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Immunoregulatory Cells And Mediators In Murine Bone Marrow

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IMMUNOREGULATORY CELLS AND MEDIATORS IN MURINE BONE MARROW

by

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ABSTRACT

This thesis defines distinct immunoregulatory mechanisms which naturally exist in murine bone marrow (BM). Physical separation of BM cells revealed two immunoregulatory cell populations: a suppressor activity present in a fraction of large, low density BM cells predominantly of myeloid and blast cell morphology; and an enhancing activity contained in a fraction of small, high density BM cells enriched for lymphocytes. Both activities are associated with the production of soluble mediators which possess analogous function to the immunoregulatory cells.

The population of cells responsible for immune suppression have been referred to as Natural Suppressor (NS) cells. NS cells are unique in that they are not antigen- or MHC-restricted, and do not require specific priming to function. They suppress a variety of immune responses, including antibody (Ab), mitogen, and mixed lymphocyte reaction (MLR) responses. NS cells were routinely found in the BM of normal as well as severe combined immune-deficient (SCID) mice, and expressed no surface markers characteristic of B, T, MØ, and NK/LAK cells. The culture of BM cells in IL-2 containing supernatants resulted in the generation of cells possessing potent NS as well as Natural Killer (NK) activity, suggesting that both NS and NK may be associated with a common family of cells.

Bone marrow cells secrete two soluble mediators which act in an analogous fashion to the suppressive and enhancing activities present in murine BM. These are bone marrow derived suppressor factor (BDSF) and bone marrow derived enhancing factor (BDEF). BDSF is contained in

a low MW (1-10 kDa) fraction of BM culture supernatant which suppresses Ab and MLR, but not mitogen-driven responses. BDSF prevents production of IL-2 in the MLR, and BDSF-suppressed MLR responses can be reconstituted by the addition of exogenous IL-2. Therefore it is proposed that absence of IL-2 production due to BDSF results in clonal anergy or non-responsiveness.

BDEF (> 10 kDa) augments Ab and MLR responses, but cannot synergize with mitogen to induce proliferation. BDEF is directly mitogenic for murine thymocytes, specifically those which do not express the receptor for the lectin peanut agglutinin (PNA^-) and most resemble mature T-cells. The ability of BDEF to induce T-cell proliferation may be intimately associated with augmentation of both humoral and cellular responses.

CHAPTER 1

NATURAL SUPPRESSOR (NS) CELLS AND OTHER IMMUNOREGULATORY MECHANISMS

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ABBREVIATIONS

Ab-	antibody
Ag-	antigen
BDSF-	bone marrow derived suppressor factor
BDEF-	bone marrow derived enhancing factor
BM-	bone marrow
BSA-	bovine serum albumin
BSS-	balanced salt solution
CFU-	colony forming unit
Con A-	concanavalin A
CSF-	colony stimulating factor
CTL-	cytotoxic T lymphocyte
DEAE-	diethylaminoethyl
DTH-	delayed type hypersensitivity
ELISA-	enzyme linked immunosorbent assay
FBS-	fetal bovine serum
Fc-	receptor for Fc portion of immunoglobulin
HLA-	human histocompatibility locus antigens
HPLC-	high pressure liquid chromatography
Ig-	immunoglobulin
IL-1 to 8-	interleukin 1 to 8
IFN-	interferon
kDa-	kilodalton
LAK-	lymphokine activated killer
LT-	lymphotoxin
MHC-	murine major histocompatibility complex
MLR-	mixed lymphocyte reaction
MØ-	macrophage
MW-	molecular weight
NC-	natural cytotoxic
NK-	natural killer
NS-	natural suppressor
Ox RBC-	ox red blood cells
PAGE-	polyacrylamide gel electrophoresis
PBS-	phosphate buffered saline
PFC-	plaque forming cell
PHA-	phytohemagglutinin
PMA-	phorbol-12-myristate-13-acetate
SRBC-	sheep red blood cells
TdT-	terminal deoxynucleotidyl transferase
TGF-	transforming growth factor
TLC-	thin layer chromatography
TLI-	total lymphoid irradiation
TNF-	tumor necrosis factor
TRF-	T-cell replacing factor

CHAPTER 1

NATURAL SUPPRESSOR (NS) CELLS AND OTHER IMMUNOREGULATORY MECHANISMS

1.1 INTRODUCTION

The immune system is comprised of a diverse network of cells derived from both lymphoid and myeloid lineages, all of which are the progeny of a common hematopoietic stem cell. The cells which participate in immune responses can be divided into two types: those which are antigen-specific, and those which are not antigen-specific. The ability to recognize specific antigen (Ag) requires the presence of an Ag-specific receptor on the cell surface. The receptors are generated by somatic rearrangement of the DNA unique to each cell, or clone, and are represented by surface immunoglobulin (Ig) on B lymphocytes, and the CD3-associated T-cell receptor (TcR) of T lymphocytes (Tonegawa, 1983; Davis and Bjorkman, 1988). According to the clonal selection theory proposed by Burnet (1959), clones bearing Ag-specific receptors are expanded during challenge with Ag leading to either a humoral or cellular immune response, or both.

In contrast to the Ag-specific functions of the B and T lymphocytes are those cells which do not require antigenic priming and lack Ag-specific receptors. These include cells which display inherent constitutive activity in the absence of clonal expansion as required by B and T cells. The cells which mediate this activity are predominantly of myeloid lineage, including those of the inflammatory response such as polymorphonuclear cells, basophils, eosinophils, and monocytes and macrophages. There is also a distinct population of cells which mediate natural immunity; the natural killer (NK), natural cytotoxic (NC), and natural suppressor (NS) cells. The origin of the NK, NC, and NS cells is at present unknown, and they have tentatively been grouped

together as a common family of cells (Maier et al., 1986). While these cells act nonspecifically by definition, they may be influenced by cytokines released as a result of an Ag-specific response (Maier et al., 1986; Holda et al., 1986), or may be transiently Ag-specific by virtue of secreted Ig binding to Fc receptors on the surface of these cells.

Coordination between Ag-specific and nonspecific cellular subtypes is believed as essential for a naturally functioning immune system. In particular, B and T cells, while responsible for immune competence, must first retain the ability to discriminate self from nonself. Breakdown of this important function leads to reactivity to self antigens and the emergence of autoimmune disease. One potential mechanism for preventing this unwanted activation of autoimmune cell networks is natural suppression, which represents a major part of the work described in this thesis. In the following sections, NS activity from diverse sources will be reviewed and compared to alternative regulatory mechanisms which also exist. The importance for these diverse types of immune regulation is apparent when one considers the adverse effects of unbridled immune reactivity, especially against self antigens.

1.2 NATURAL SUPPRESSOR CELLS

Natural suppressor (NS) activity was originally described by this laboratory (Singhal et al., 1972) as a population of cells residing in murine BM which could inhibit Ab responses of spleen cells generated in vitro against heterologous erythrocytes. Since this initial discovery, BM NS activity has also been described in rats (Noga et al., 1988), rabbits (Okumura and Kern, 1975; Soderberg, 1984), and humans (Bains et al., 1982; Mortari et al., 1986). NS activity has also been reported from tissue sources other than bone marrow. However, these sites all resemble primary hematopoietic environments which lack populations of mature lymphocytes characteristic of the peripheral immune system. The activities of NS cells have been extended to include suppression of mitogen responses and mixed lymphocyte responses (MLR) in vitro, and in all cases suppression was neither MHC-restricted nor specific for antigen.

The ability of NS cells to inhibit a wide variety of immune responses has suggested that they are involved in immunological tolerance. However, while mechanisms which contribute to the maintenance of the tolerant state have been extensively studied (such as T suppressor cells and idiotypic networks), Strober (1984) has postulated; "Cellular mechanisms that contribute to the induction rather than the maintenance of tolerance are less well understood." Interestingly, all environments which harbor NS activity are also sites where tolerance induction may occur (so-called "windows of tolerance" by Strober). This has led Maier et al. (1986) to propose their possible role as inducers of immunological tolerance to self antigens.

This notion is supported by the findings of Sadelain et al. (1989) that augmentation of NS activity in NOD mice following the injection of complete Freund's adjuvant prevents the development of autoimmune-mediated diabetes in these animals.

The following sections of this chapter will describe the activities and physical characteristics of NS cells derived from different sources.

1.2.1 BM Derived NS Activity

Bone marrow derived NS activity was first described by Singhal et al. (1972) in mice as the ability of BM cells to suppress an in vitro PFC response against heterologous erythrocyte antigens. The cells were found to be radiosensitive and suppressed the response maximally when added at culture initiation. Subsequent work demonstrated that suppression was not associated with T-cells, was found predominantly in the non-adherent fraction of BM cells, and was not mediated by intracellular inhibitory substances released upon sonication of BM cells (Drury and Singhal, 1974). Duwe and Singhal (1979a) further demonstrated that primary T-dependent and T-independent Ab responses could be suppressed, and that the addition of antigen-primed T-cells or TRF could not overcome suppression by BM cells. Lastly, the BM cells were not able to suppress secondary IgG PFC responses to SRBC.

Simultaneously several other groups had begun to investigate the nature of the suppressor elements resident in BM. Corvese et al. (1980) demonstrated that T-dependent and T-independent Ab responses were inhibited by BM cells. They found that the activity was

radiosensitive, and was present in a non-T, non-B, non-lymphocytic fraction of BM cells. Furthermore, pretreatment of mice with phenylhydrazine, which reduces the numbers of pre-B cells, did not reduce suppressor activity. Levy et al. (1981) have shown that BM cells inhibit Ab responses to SRBC in vitro, and after 24 hr preculture could suppress in vivo Ab responses and responses of spleen cells to T and B cell mitogens. The suppressor cells were found to be large cells after enrichment by velocity sedimentation. For suppression of the Ab response, treatment of BM cells with radiation or mitomycin C blocked suppressor activity. In contrast, either treatment did not affect the ability of the BM cells to suppress the mitogenic responses. The authors postulated that either 2 suppressor mechanisms were involved, or that suppression of the Ab response may require proliferating cells, while the mitogenic response does not. Of importance was the finding that the BM cells had to be precultured before suppressing the mitogenic response suggesting selection of a co-existing suppressor cell population.

Dorshkind et al. (1980) demonstrated the role of BM cells in suppressing alloantigen-induced proliferation and cytotoxic T cell generation in mixed lymphocyte cultures. In a subsequent study, Dorshkind and Rosse (1982) studied the phenotypic properties of their so-called "natural regulatory cells", and found them to be non-B, non-T, non-MØ, non-NK, Ia^- and FcR^-. While the cells needed to be viable to function, they were cortisone and radiation resistant. It was concluded that they were "immature cells of an undetermined lineage" primarily due to a paucity of phenotypic markers.

The immature nature of NS cells in the mouse had been supported

further by findings from this laboratory. Duwe and Singhal (1979b) confirmed the non-T nature of the cell showing insensitivity to treatment with anti-thymocyte antiserum. Similarly normal suppressor activity was present in the BM of nude mice. They also demonstrated that removal of MØ from BM using anti-MØ serum or carbonyl iron did not affect suppressor activity. The cells could be enriched after velocity sedimentation in fractions containing medium-to-large lymphocytes which were FcR^+. McGarry and Singhal (1982) extended these findings to demonstrate that BM suppressor activity was not sensitive to treatment with antibodies directed against Thy-1, Lyt 1.1, Lyt 2.2, I-J, Ig, or Ia. Dauphinee and Talal (1979) had previously shown that a suppressor cell population existed in BM which could be induced to express Thy-1 after exposure to thymosin or thymic peptides. However, incubation of BM cells with Con A or T-cell differentiation agents such as synthetic thymic peptide (TP5) or soluble thymic factor (STF) had no effect on BM suppressor activity, even after anti-Thy-1 treatment (McGarry and Singhal, 1982). Lastly, the BM suppressor activity was found to be sensitive to treatment with anti-H-2 and, to a lesser degree, anti-stem cell antisera, confirming the notion of others that the suppressor activity was associated with immature cells.

More recently, Sugiura et al. (1988) enriched for NS activity in murine BM as large, low density cells expressing the receptor for the lectin wheat germ agglutinin (WGA). These WGA^+ cells not only possess potent NS activity but also contain the majority of cells responsible for CFU-S in BM. They have concluded that it is stem cells which possess NS activity, provided they are cycling, since treatment of mice with 5-FU (fluorouracil) decreased NS activity in BM. This is

consistent with the hypothesis of Noga et al. (1988) that stem cell progenitors possess NS activity which can autoregulate hematopoietic events as well as block immunologically induced activation signals in the bone marrow microenvironment.

The significance of BM NS activity has also been demonstrated in vivo during transplantation. Injection of donor BM cells at the time of skin grafting from an allogeneic recipient resulted in prolonged allograft survival (Gozzo et al., 1982). BM cells which had been rendered metabolically inactive by mitomycin C treatment were not able to prolong survival of the skin grafts. Ildstad and Sachs (1984) had shown that reconstitution of irradiated mice with BM from syngeneic plus allogeneic or xenogeneic donors could lead to acceptance of allografts or xenografts, respectively. Furthermore, Sykes et al. (1988) found that mixed allogeneic chimeras could be generated by injection of T-cell depleted syngeneic plus allogeneic bone marrow. More importantly, co-injection of the syngeneic marrow also prevented GVHD associated with injection of the allogeneic BM cells. In these studies it was found that the spleens of these animals contained NS activity 8 days after transplantation which persisted for several weeks. This NS activity was mediated by a null cell phenotype and was derived from the inoculum of BM cells syngeneic to the recipient.

In rats, Noga et al. (1988) used counterflow centrifugal elutriation (CCE) to deplete lymphocytes from BM which prevented GVHD during allogeneic BM transplantation. The lymphocyte depleted marrow contained higher NS activity than unseparated BM, and further enrichment in low density Percoll fractions revealed radiosensitive NS activity comprising 0.5% of the original BM population. Incidentally,

genes of the T-cell receptor (TcR), and expressed full length mRNA transcripts for alpha, beta, and gamma chains (Hertel-Wulff et al., 1987). It was later demonstrated that the cells expressed alpha/beta TcR on their surface in association with CD3 (Strober et al., 1989), but did not express CD4 or CD8 (the classic double-negative phenotype). Antibodies directed against CD3 could stimulate the cells to proliferate in the presence of PMA and irradiated feeders. However, anti-CD3 did not inhibit their suppressive activity in culture, suggesting that CD3 was not involved in the suppressive mechanism. The lack of Ag specificity by NS clones argues against an important role for the TcR on these cells. One could speculate that TcR is expressed as a result of the culture methods employed, favoring selection of rare double-negative T-cell subsets.

An alternative means to derive long term growth of NS cells was to culture neonatal spleen cells in conditioned medium from the myelomonocytic cell line WEHI-3 which constitutively secretes IL-3 (Jadus and Parkman, 1986). Cells maintained this way were able to suppress MLR and mitogen responses without genetic restrictions. The cells were characterized as $Ly5^+$, $J11d^+$, and MHC Class I^+ while being negative for common B, T, and MØ markers. The cells were not lytic for the classical NK-sensitive target Yac-1, but did lyse the NC-sensitive target, WEHI-164. There was also a direct correlation between NS and NC activity, such that cultures which lost lytic activity also lost their NS activity. This suggests that suppression was mediated via a cytotoxic mechanism. Indeed, the relationship between NS, NK, and NC cells have prompted the proposal that these cell types are related, and Maier et al. (1986) have placed NS cells into an LGL-regulatory family

suppress a primary Ab response by lymphocytes to Ag. Soderberg (1984, 1985) has studied the role of BM NS activity in the regulation of cell proliferation within the BM compartment. He found that FcR^+ cells in BM suppressed the constitutive proliferation of the remaining FcR^- cells in the BM. The FcR^- BM cells produced a soluble growth factor which was responsible for this endogenous proliferation. Addition of FcR^+ BM cells to FcR^- cells effectively inhibited the production or release of the growth factor, suggesting the potential role of BM NS activity in regulating cell growth in bone marrow.

1.2.2 NS Activity in Neonatal Spleens

It has long been known that immunological tolerance can be easily induced in neonatal animals exposed to foreign antigen. Billingham et al. (1953) in what are now classic experiments demonstrated that intravenous injection of lymphocytes from adult mice into allogeneic neonatal recipients resulted in the establishment of permanent chimerism. More significantly, the adult chimeras were specifically tolerant of donor tissue as demonstrated by the ability of these animals to accept donor skin grafts while rejecting third party tissue. It was later demonstrated in this system that tolerance was induced for cell mediated responsiveness against Class I and Class II MHC antigens (Streilein and Gruchalla, 1981). Furthermore, tolerance at the level of humoral responses was also demonstrated by injection of nominal antigens such as heterologous blood cells and bacterial protein antigens into neonatal recipients (Smith and Bridges, 1958; Shellam and Nossal, 1968).

Consistent with the ease of tolerization in neonates were

reports that spleen cells from neonatal mice were potent inhibitors of *in vitro* immune responses (Basset *et al.*, 1977; Argyris, 1978; Okada and Strober, 1982; Oseroff *et al.*, 1984; Schwadron *et al.*, 1985; Jadus and Parkman, 1986). In most of these studies suppression of *in vitro* responses such as MLR and CTL generation was examined; however, co-injection of neonatal spleen cells with allogeneic BM cells into lethally irradiated recipients prevented GVHD and resulted in the establishment of permanent chimerism (Strober, 1984). Suppression of Ab responses to heterologous erythrocytes has also been demonstrated but may be more complex. This activity was primarily due to MØ precursors which differentiated into mature MØ during *in vitro* culture and secreted inhibitory prostaglandins (Piguet *et al.*, 1981; Snyder *et al.*, 1982). By contrast, the cells responsible for suppression of alloreactivity were not MØ and were not sensitive to the presence of indomethacin in culture (Oseroff *et al.*, 1984).

Like BM NS cells, neonatally derived NS cells lack antigen and MHC specificity (Strober, 1984). Phenotypically, these cells have also been characterized as "null cells", consistent with the immature nature of neonatal spleen such that 80% of resident mononuclear cells express neither Thy-1 nor sIg on their surface (Oseroff *et al.*, 1984). Furthermore methods aimed at depletion or selection of NS cells using antibodies against T or B cell markers did not identify NS activity. Peeler *et al.* (1983) have also demonstrated the non-macrophage nature of NS activity in neonatal spleens. During maturation of the animal, however, increased expression of cell surface markers for B and T cells correlates with waning of NS activity. For example, 30 days after birth less than 20% of mononuclear cells in the spleen bear the null

phenotype, at which time NS activity is also not detectable (Oseroff et al., 1984). Thus, the ability to induce tolerance in neonates may be directly related to the activities of splenic NS activity, as proposed by Strober (1984), while tolerance in adults can only be induced after manipulation of antigen, such as deaggregation of gamma globulins as demonstrated by Dresser (1962).

1.2.3 Induction of NS Activity

The following sections will deal with means of inducing NS activity in vivo, usually associated with regenerating hematopoietic tissue.

1.2.3.1 Total Lymphoid Irradiation (TLI)

TLI, as the name implies, consists of fractionated doses of irradiation (200 rads) to lymphoid tissues, specifically lymph nodes, spleen, and thymus. By shielding bone marrow, lungs, and other non-lymphoid tissue, the cumulative dose of irradiation can be as high as 4000 rads without causing mortality (Strober, 1984). Interestingly, after TLI, the spleen is repopulated with cells derived from hematopoietic tissue thereby resembling the spleens of neonatal mice, which contain low numbers of cells expressing Thy-1 or sIg (Oseroff et al., 1984). Similar to neonates, animals treated with TLI are quite tolerant to foreign antigen. For example, Slavin et al. (1977,1978) demonstrated that allogeneic BM injected into TLI-treated mice resulted in permanent chimerism in the absence of GVHD. These chimeras accept marrow-donor strain skin grafts and normally reject grafts from third-party donors. Tolerance can also be induced to the heterologous serum proteins BSA or BGG after TLI (Zan-Bar, 1978). The ability to induce

tolerance wanes with time as lymphoid cell development progresses; nevertheless the ability to induce tolerance to serum proteins can last as long as 100 days (Zan-Bar, 1978).

The spleens of TLI-treated mice contain high levels of NS activity shortly after treatment as assessed by 1) their ability to inhibit proliferation of responder cells and the generation of CTL from mixed leukocyte cultures *in vitro* (Okada and Strober, 1982) and 2) inhibition of GVHD *in vivo* (King *et al.*, 1981). These experiments showed that suppression was Ag- and MHC-unrestricted, and, as in neonatal spleen, that NS activity disappeared 30 days after treatment. NS cells could not be enriched after treatment with antibodies against T-cell, B-cell, or MØ surface markers (Oseroff *et al.*, 1984). NS cells also lacked NK activity. Furthermore, suppression was not mediated by a promonocyte since *in vitro* culture did not result in differentiation into mature MØ. Parerthetically, suppression was not sensitive to the presence of indomethacin in culture (Oseroff *et al.*, 1984).

TLI has been used to treat Hodgkin's disease, rheumatoid arthritis, and in organ and BM transplantation (Kaplan, 1980; Strober *et al.*, 1983; Slavin, 1987). In mice, TLI prolongs skin allograft survival, prevents GVHD, and has been used to prevent autoimmunity in New Zealand mice (Slavin *et al.*, 1976; King *et al.*, 1981; Kotzin and Strober, 1979). Thus, TLI allows for the emergence of NS activity providing the host with a microenvironment suitable for tolerance induction. This underscores the important role that NS cells may play in immune homeostasis.

1.2.3.2 ^{89}Sr-Treatment of Mice

^{89}Sr is a bone seeking isotope which destroys bone marrow cells when injected into mice. After BM destruction, the spleen emerges as the primary site of hematopoiesis, populated by stem cells and immature cells, thereby resembling bone marrow. Resident within the spleens of ^{89}Sr-treated mice is NS activity similar to that observed in bone marrow. Merluzzi et al. (1978) had originally shown that these splenic suppressors were non-T, non-B, non-MØ, radiosensitive cells which could inhibit primary and secondary Ab responses in vitro to T-dependent and T-independent Ags. Levy et al. (1981) further demonstrated that the cells could be positively selected by velocity sedimentation, in fractions containing large cells which could subsequently suppress Ab responses and mitogen activation. Irradiation (1000 R) or mitomycin C treatment of cells resulted in abrogation of suppression of Ab but not mitogen responses. This suggested either multiple suppressor populations, or differing sensitivity of the same cell in different assay systems. Importantly these authors concluded that the presence of suppressor activity in the BM and ^{89}Sr-spleens inhibited lymphoid function thereby allowing myelopoiesis to proceed more efficiently.

1.2.3.3 Cyclophosphamide-induced NS Activity

Treatment of mice with a high dose of cyclophosphamide (200 mg/kg body weight) results in splenic cellular depletion followed by lymphoid regeneration. Concomitantly there is the appearance of non-specific suppressor cells in the spleen 5 days to 13 days after treatment (McIntosh et al., 1979). Segre et al. (1985) showed that these cells suppressed both primary and secondary antibody responses in

vitro, and could be isolated as low density cells on discontinuous Percoll gradients. The cells were classified as null cells as shown by their insensitivity to Ab treatments against T, B, MØ, or NK cell markers. Attempts to induce T-cell differentiation in the presence of thymosin followed by treatment of the cells with anti-Thy 1.2 Ab did not remove suppressor activity, suggesting that the cells were not pre-T cells.

The same laboratory studied the role of these cells in immunological tolerance by attempting to prevent the onset of autoimmune manifestations in NZB mice by injection of spleen cells from cyclophosphamide-treated syngeneic mice (Greeley et al., 1985). They demonstrated prolonged suppression of both Coombs and natural thymocytotoxic antibodies; however, anti-DNA autoantibodies and total IgM and IgG levels were not affected. Renal histology did not differ from controls, showing marked glomerulonephritis. Survival times of treated animals were not improved compared with controls. These results suggest that NS cells evoked by cyclophosphamide treatment may be able to induce immunological tolerance. The fact that not all autoimmune manifestations were inhibited may reflect the limitations of this particular study. Perhaps more effective ways of inducing NS activity in autoimmune-prone animals could potentially result in greater prevention of the diseased state.

1.2.3.4 NS Activity Associated with Graft-vs-Host Disease

Claman and co-workers (reviewed by Holda et al., 1985) have used a model of chronic GVH reactions against minor histocompatibility antigens. In their model, donor cells (B10.D2) are identical at the

MHC and Mls loci and not reactive in an MLR with the recipient (Balb/c). Unlike most models of GVHD which employ parent into F1 spleen cell injections, the minor model of Claman requires prior sub-lethal irradiation (600 R) of the recipient before injection of donor cells. However, the GVH reaction which follows results in the typical wasting syndrome, including hair loss, skin lesions, collagen deposition, and dermal mononuclear cell infiltrate.

Spleen cells from GVH mice taken 10 days after injection display the classic immunodeficiency associated with GVH reactions, showing inability to respond to the mitogens Con A or LPS. Accompanying this immune deficiency is the ability of the same spleen cells to suppress the MLR and mitogen responses of normal spleen cells, in an MHC-unrestricted fashion (Maier et al., 1985a). Attempts to remove or enrich for the suppressor cells using isolation techniques for T-cells, B-cells, or MΦ, were unsuccessful. Thus the cells were of the classic null cell phenotype characteristic of NS cells. Percoll fractionation of GVH spleen cells showed that NS activity was enriched in low density bands, and also revealed the presence of mitogen reactive lymphocytes in the small, high density band. This suggests that functional lymphocytes are present during GVH immune deficiency, but are suppressed by NS cells in the spleen (Holda et al., 1985).

Lastly, it was found that both donor T-cells and non-T-cells (NS) are required in the GVH inoculum to create immune deficiency and immunosuppressive activity. Holda et al. (1985) postulated that T-cell signals in the form of lymphokines may activate NS activity in the donor inoculum. This was supported by evidence that supernatants from Con A stimulated rat spleen cells could enhance NS activity of both BM

and GVH spleens. Further dissection looking for components responsible for activating NS cells indicated that while IFN-gamma and IL-2 were both required, IFN-gamma was much more effective (Holda et al., 1986). Using BM cells it has recently been shown that IL-3, IL-4, and IL-6 can all augment NS activity. This can be inhibited by the addition of anti-IFN-gamma Ab to the culture system. Therefore lymphokines released during early events in an immune response may be one mechanism by which NS cells become activated and subsequently mediate suppressive function.

1.2.3.5 Cloning of Cells with NS Activity

The cloning of NS cells followed an initial report by Oseroff et al. (1984) that NS activity from TLI-spleens could be maintained in vitro for 6-8 weeks in medium containing Con A stimulated rat spleen cell supernatants (rat Cas). The cells which survived in culture resembled the starting population in their ability to suppress MLR cultures and lack of phenotypic markers as determined by FACS analysis. Following the initial study, the same group reported the cloning of NS cells from both TLI-spleens (Hertel-Wulff et al., 1984) and neonatal spleens (Schwadron et al., 1985) by long term culture in rat Cas. In both cases, the NS cell lines were much more effective at suppressing MLR cultures than the cell populations from which they were derived. While the cloned cells possessed no NK activity they did resemble NK cell clones in expression of Thy-1 and asialo GM1 (ASGM1) but not Lyt-1, Lyt-2, Ig, MAC-1, Fc, and Ia surface markers. Further analysis of these lines indicated that they were members of the T-cell lineage, since molecular probing revealed that NS clones rearranged beta-chain

genes of the T-cell receptor (TcR), and expressed full length mRNA transcripts for alpha, beta, and gamma chains (Hertel-Wulff et al., 1987). It was later demonstrated that the cells expressed alpha/beta TcR on their surface in association with CD3 (Strober et al., 1989), but did not express CD4 or CD8 (the classic double-negative phenotype). Antibodies directed against CD3 could stimulate the cells to proliferate in the presence of PMA and irradiated feeders. However, anti-CD3 did not inhibit their suppressive activity in culture, suggesting that CD3 was not involved in the suppressive mechanism. The lack of Ag specificity by NS clones argues against an important role for the TcR on these cells. One could speculate that TcR is expressed as a result of the culture methods employed, favoring selection of rare double-negative T-cell subsets.

An alternative means to derive long term growth of NS cells was to culture neonatal spleen cells in conditioned medium from the myelomonocytic cell line WEHI-3 which constitutively secretes IL-3 (Jadus and Parkman, 1986). Cells maintained this way were able to suppress MLR and mitogen responses without genetic restrictions. The cells were characterized as $Ly5^+$, $J11d^+$, and MHC Class I^+ while being negative for common B, T, and MØ markers. The cells were not lytic for the classical NK-sensitive target Yac-1, but did lyse the NC-sensitive target, WEHI-164. There was also a direct correlation between NS and NC activity, such that cultures which lost lytic activity also lost their NS activity. This suggests that suppression was mediated via a cytotoxic mechanism. Indeed, the relationship between NS, NK, and NC cells have prompted the proposal that these cell types are related, and Maier et al. (1986) have placed NS cells into an LGL-regulatory family

including NC and NK cells.

1.2.3.6 NS Cells - Epilogue

It is clear from the above discussion that NS cells are typically found in areas of hematopoiesis or where induced tissue injury has led to hematopoietic or lymphoid regeneration. Typically, in each instance, they do not express surface markers for any specific lineage and have been classified as null cells. Morphologically, they resemble large granular lymphocytes and are typically enriched in low density cell fractions. They are not restricted with respect to antigen or MHC and appear to be able to block events associated with immunological activation.

Some minor discrepancies exist such as 1) the ability to suppress primary or secondary responses or both, and, 2) sensitivity to either irradiation or mitomycin C treatment. However, this may reflect the diversity of the NS cell population derived under different experimental conditions which may impart selection for one NS cell type over another. Similarly the readout systems used may influence the characteristics of the particular NS populations described. Clearly, the development of monoclonal antibodies against specific but as yet uncharacterized markers on NS cells is necessary for further study of these cells.

1.3 OTHER TYPES OF SUPPRESSOR CELLS

Suppressor activity has been described for diverse cell types in many different readout systems. These include suppression of immune responses by either Ag-specific or Ag-nonspecific mechanisms, both genetically restricted or non-restricted. As well, many of these cells release soluble mediators which effect suppression. The evolution of such a diversity of suppressor mechanisms (including NS activity) points to their importance in limiting unwanted lymphoid activation.

1.3.1 NK Cells

The first reports of immune regulation by NK cells were by Nabel et al. (1981,1982). They reported that cloned NK cell lines could lyse LPS-activated B-cells, and could inhibit Ig synthesis when added to culture or when co-injected into irradiated mice during adoptive transfer of Ab-forming cells and antigen. This was followed by the classic work of Abruzzo and Rowley (1983) demonstrating that NK cells could terminate ongoing primary antibody responses in vivo and in vitro by acting on accessory cells which have interacted with antigen. They suggested that induction of NK activity results in termination of the Ab response usually 4-6 days after immunization with antigen. They further demonstrated that antigenic competition, during which immunization by one antigen results in a suppressed response to a second antigen, could be explained by induction of NK activity (Abruzzo and Rowley, 1986).

Inhibition of Ab production has also been shown in humans. Brieva et al. (1984) demonstrated that NK cells could directly block Ab

production by autologous lymphoblastoid B-cells which appeared 5-7 days after immunization with tetanus toxoid. Arai et al. (1983) demonstrated suppression of IgM and IgG secretion by human PBLs stimulated with PWM, and that the potential mechanism was via an effect on T_H cells but not directly on B-cells.

The regulatory ability of NK cells is not solely restricted to Ab formation. Gilbertson et al. (1986) have shown that NK cells can inhibit CTL generation in an MLR by either eliminating or suppressing dendritic cells necessary for antigenic stimulation. D'Amore and Golub (1985) have shown that NK cells generated in an MLR have the ability to regulate NK cytolytic activity. NK cells have also been shown to lyse targets in fetal thymus and BM (Hansson et al., 1981), and subsequent work demonstrated their effectiveness at regulating hematopoiesis (O'Brien et al., 1983; Holmberg et al., 1984). Azuma and Kaplan (1988) had shown that BM cells activated by IL-2 containing supernatants in culture possessed NK/LAK activity and were potent mediators of NS and veto activities. They have also shown that these cultured NK/LAK cells inhibit BM graft rejection and lethal GVHD associated with adoptive transfer of semi-allogeneic and allogeneic BM into irradiated recipients (Azuma et al., 1989). These results are pertinent to the complications associated with human BM transplantation and suggest a potential therapeutic value of NK cells.

The mechanisms by which NK cells impart regulatory activity are uncertain. It has been suggested that inhibition is associated with the cytolytic activity of the NK cells (James and Ritchie, 1984). Targan et al. (1985) have demonstrated that release of the NK cytotoxic factor (NKCF) is responsible for NK mediated suppression of the Ab

response. There is also evidence that NK cells can produce interferon-gamma which may mediate inhibition by NK cells (Arai et al., 1983; Handa et al., 1983).

Defects in NK activity may be associated with immune dysfunction. For example, NK activity is functionally impaired in human SLE and rheumatoid arthritis (Karsh et al., 1981; Katz et al., 1982). More recently, Pan et al. (1986) have demonstrated impaired NK function in mice expressing the lpr gene which is associated with increased autologous responses to self antigens. The implication is that autoreactivity may be controlled by NK cells, and defective NK function may in part be related to the occurrence of autoimmune disease.

1.3.2 Veto Cells

Veto cells were originally described by Miller (1980) as a population of cells present in nude spleen which could inhibit the generation of CTL in a mixed lymphocyte culture. He also found that veto activity was present in neonatal spleen, BM, and thymus. They were called "veto cells" due to their ability to veto T cell activation leading to CTL; however, they were only active when the veto cell population was syngeneic to the stimulator population in an MLC. This led to the proposal that the veto cell functioned to prevent the generation of autoreactive CTL within an organism.

Muraoka and Miller (1980) demonstrated that BM cells and T cell colonies derived from BM could suppress the generation of CTL in vitro. They found that the veto cell in BM was Thy-1$^-$ and radiosensitive, while the veto cell in T cell colonies was Thy-1$^+$ and radioresistant.

They concluded that the Thy-1$^-$ veto cell in BM was the precursor to the Thy-1$^+$ veto cell which developed in the T colony system.

Veto activity is not restricted to BM cells or their progeny after T cell colony formation. Veto activity also appears to be a normal function of cloned CTL (reviewed by Fink et al., 1988). Apparently any CTL can mediate veto activity independently of their own ability to recognize a ligand via the T cell receptor. Thus veto activity is dictated not by the veto cell itself, but by the T cell which recognizes it, presumably via recognition of MHC antigens on the surface of the veto cell. While the mechanism of inactivation is unknown, there is conclusive evidence that they are not cytolytic for the cell which they inactivate, and they do not function simply by exhausting lymphokines in culture (Fink et al., 1988). The function of veto cells appears to be mainly directed towards Class I MHC restricted CTL, although it has been suggested that T helper cells may be susceptible to inactivation by a veto cell which expresses Class II antigens.

1.3.3 Macrophages

Macrophages have previously been shown to suppress a variety of immune responses, such as Ab production (Brown et al., 1980), mitogen activation (Allen and Moore, 1979), and generation of cytotoxic T cells (Klimpel and Henney, 1978). In each of these cases, suppression was due to activation with bacillus Calmette-Guerin (BCG) resulting in the emergence of suppressor MØ in the spleen. Bennett et al. (1978) have proposed that suppressor activity induced by BCG was the result of the migration of NS cells from the bone marrow to the spleen.

More recently, Kato et al. (1985) have shown that after stimulation with BCG, NS-type cells migrate from the BM to the peritoneal cavity, and that these cells could suppress DTH responses. This migration required metabolically viable cells in the BM since pretreatment of mice with methotrexate prior to BCG inoculation prevented the appearance of suppressor MØ in the peritoneum. They have described the suppressor activity in BM as NS, although unlike classic NS these cells phagocytized carbonyl iron particles and were adherent to Sephadex G-10, characteristic of MØ. This is consistent with the findings of Piguet et al. (1981) that monocyte precursors exist in neonatal spleen which had the ability to suppress Ab responses, thus rendering the comparison with NS cells.

Suppressor MØ may also be induced by Corynebacterium parvum (Kirchner et al., 1975) and have been implicated as the cells responsible for immunosuppression in tumor bearing hosts (Herberman et al., 1979; Parhar and Lala, 1985). The mechanism of action of suppressor MØ either involves direct contact with the responding population or the release of soluble mediators (Allen et al., 1978). These include prostaglandins of the E series, polyamine oxidases, and cyclic AMP. Recent work by Lala et al. (1986) has shown that treatment of tumor bearing mice with the prostaglandin synthetase inhibitor indomethacin results in clearance of the tumor burden due to increased immune function.

1.3.4 B-Lymphocytes

The ability of B cells to act as suppressors was initially described by Katz et al. (1974) in the regulation of DTH responses.

Later work demonstrated that B cells could non-specifically inhibit primary and secondary antibody responses of spleen cell cultures to T dependent Ags, provided that they were first activated with LPS (Gilbert and Hoffmann, 1983). Petrov and Khaitov (1977) had also demonstrated *in vivo* that injection of syngeneic BM, lymph node or spleen into mice immunized with SRBC could inhibit anti-SRBC PFC formation, and the cells responsible were characterized as B cells. Matsumoto and Shibata (1985) have shown that BM cells cultured in the presence of Con A can suppress LPS generated polyclonal Ab responses of spleen cell cultures. The suppressor cells were found to be B cells, and the authors postulated that in the presence of Con A immature B cell progenitors developed into sIg positive suppressor cells. Consistent with this Hoskin *et al.* (1983) and Gronvik *et al.* (1987) have described NS-type activity associated with cells in the spleen of pregnant mice. These non-specific suppressor cells could be enriched on Percoll gradients at a density characteristic of resting B-cells, and could be purified using the lectin soybean agglutinin which is typically used to purify B lymphocytes (Reisner *et al.*, 1976). They present evidence which suggests that these cells may be involved in preventing immune reactivity against paternal antigens of the fetus during pregnancy.

B cells can also act as antigen specific regulators as a result of Ig production. In 1974, Jerne proposed that antibodies could regulate each other via specific idiotypic interactions, the so-called "network theory" of immune regulation. According to this theory, an antibody is immunogenic by virtue of its Ag-binding region, or idiotype. This idiotypic determinant induces an antibody response

leading to the generation of anti-idiotypic Ab. Consequently a network arises such that new antibodies are generated against each new idiotypic determinant. It is believed that these antibodies interact with one another to form a regulatory web, which is disturbed by the introduction of antigen. An imbalance of idiotype to anti-idiotype results in an immune response to antigen (Paul and Bona, 1982).

A second type of Ag-specific suppression has been described by Sinclair and Panoskaltsis (1987) as end-product feedback. In this model, B-cells are inactivated by crosslinking of antigen by surface Ig and constitutively secreted IgG bound to the Fc receptor. This negative signal can be abrogated by interleukin-4 (O'Garra et al., 1987) or more specifically by either heterologous antibody directed against the Fc portion of IgG, or rheumatoid factors (Panoskaltsis and Sinclair, 1989). While both the sIg and suppressive IgG may be directed against the same or different epitopes on an antigen, the interaction may also represent an idiotype-anti-idiotype interaction (Sinclair and Panoskaltsis, 1987). This hypothesis is in agreement with the findings of Borel (1980) and Scott et al. (1979) that hapten coupled to intact IgG could induce hapten-specific tolerance in vivo.

1.3.5 T-Lymphocytes

There is a large body of literature describing the characteristics and function of T-suppressor cells which can only be briefly reviewed here. Suppressor T lymphocytes, originally described by Gershon and Kondo (1970), have long been recognized as Ag-specific, MHC-restricted cells which regulate primarily helper T cell function. T-suppressor cells are induced by immunization with antigen resulting

in the interaction of several T-cell subsets which are intermediates leading to a final effector stage (reviewed by Green et al., 1983; Dorf and Benacerraf, 1984). Green has described these intermediates as follows. The initial cell in the pathway is called T_S1, the suppressor-inducer, which is $Ly1^+$, $Qa1^+$, and $I\text{-}J^+$. This cell interacts with an intermediate called T_S2, or suppressor-transducer, which is $Ly1,2^+$, $I\text{-}J^+$, and $Qa1^+$. This cell stimulates the last cell in the series, called T_S3 or suppressor-effector, which is $Ly2^+$, $I\text{-}J^-$, and $Qa1^-$ and in cooperation with a naive $Ly1^+$, $I\text{-}J^+$ cell inactivates the helper T cell specific for the same Ag. These interactions are not only Ag-restricted but also MHC-restricted (at the I-J locus) and V_H restricted. The same cells mediate their suppressive effects via soluble factors which are Ag, MHC, and V_H restricted at certain stages (Green et al., 1983; Flood et al., 1986).

It has also been shown that suppressor T cells could interact with effector T-cells or B-cells via recognition of idiotype on the surface of those cells, an extension of Jerne's network hypothesis (Sercarz et al., 1989). Sercarz has also demonstrated that antigen is made up of distinct epitopes (or determinants) specific for stimulating either T_H, T_S, or B cells (Turkin and Sercarz, 1977; Krzych et al., 1985). This led to the demonstration that removal of T_S-inducing determinants from antigen results in a response by genetically non-responsive mice, suggesting an in vivo role of T_S in maintaining anergy to a particular antigen (Yowell et al., 1979).

1.4 SCOPE OF THE THESIS

This thesis will describe two distinct immunoregulatory activities inherent in murine bone marrow. The first, which has been extensively reviewed here, is natural suppressor (NS) activity. Chapter 2 will examine further the characteristics of NS cells in BM and examine the potential relationship between NS and NK activities. Chapter 3 describes a suppressor factor derived from BM which mimics BM NS activity. The activities associated with this factor, called bone marrow derived suppressor factor (BDSF), reveal a potent suppressive mechanism for controlling immunological activation. The studies in this chapter were designed to determine the potential mechanism by which BDSF acts. Data is presented to suggest that BDSF may inhibit IL-2 driven proliferation of T-cells. Finally, Chapter 4 describes the activities of bone marrow derived enhancing factor, or BDEF. This chapter also provides partial characterization of this molecule and distinguishes it from several well characterized cytokines. The data presented also correspond with previous notions that BDEF may be associated with T cell differentiation or activation. In conclusion, these activities may represent mechanisms present in bone marrow which regulate growth and activation events in BM necessary for proper lymphoid development.

CHAPTER 2

BONE MARROW DERIVED NATURAL SUPPRESSOR CELLS: CHARACTERISTICS AND BIOLOGICAL ACTIVITIES

2.1 INTRODUCTION

This laboratory was the first to demonstrate that BM was a rich source of suppressor cells for Ab synthesis in both mice (Singhal et al., 1972; Duwe and Singhal, 1979) and humans (Bains et al., 1982; Mortari et al., 1986). Since these cells were non-specific in their action and did not require specific priming, they were referred to as "Natural Suppressor" (NS) cells, similar to the previous designations of "Natural Killer" (NK) and "Natural Cytotoxic" (NC) cells. BM derived NS cells have also been shown to suppress mitogen activation (Maier et al., 1985) and mixed lymphocyte responses (Dorshkind et al., 1980; Sugiura et al., 1988). In vivo, BM NS cells have been shown to prevent GVHD during the generation of mixed allogeneic chimeras (Sykes et al., 1988) and to inhibit hematopoiesis (Sadelain et al., 1989).

The presence of NS cells in the BM may account for the inability of BM to mount a primary Ab response while being a major site of IgG production during secondary Ab responses (Benner et al., 1981). This is supported by the findings of Duwe and Singhal (1979) that BM NS cells inhibit primary Ab responses while not affecting secondary Ab responses. However, to some degree lack of primary responsiveness may be due to a lack of all functional cell types necessary for a response to antigenic challenge (Benner et al., 1974; DeGast and Platts-Mills, 1979). Interestingly, if NS cells are removed from BM, the remaining cells have the capacity to make Ab to a primary antigenic challenge provided that accessory cells are added (Gorczynski et al., 1977; Ryser and Dutton, 1977; Mortari et al., 1986).

While functional activity associated with NS cells has been

widely described, they have been elusive with respect to phenotype and lineage. For practical purposes NS cells have been categorized as null cells which are non-adherent, resemble large granular lymphocytes, and express Fc-gamma receptors on their surface (Maier et al., 1986). They have been potentially characterized as immature myeloid cells (Corvese et al., 1980), early stem cell progenitors (Noga et al., 1988; Sugiura et al., 1988), and members of the NK lineage (Mortari et al., 1986). The designation of NS cells as members of an LGL-regulatory family, including NK and NC cells, has been put forward by Maier et al. (1986). The regulatory abilities of both NK and NC cells have been well documented and are reviewed in sections 1.2.3.5 and 1.3.1.

The major focus of this chapter was to determine the physical characteristics of cells in murine BM which mediate NS activity. This included physical separation based on size and density, as well as depletion of cells by Ab-mediated cytotoxicity using antibodies specific for both mature NK cells and NK precursors. Attempts were also made to generate cultured lines of NS cells from BM as Schwadron et al. (1985) had achieved using neonatal spleen and spleens of TLI-treated mice as a source of NS activity. The intended goal was to characterize NS cells in fresh BM or from continuously growing cell lines and evaluate any potential relationships which existed between NS and NK cells. Ultimately, the characterization of NS cell function and phenotype may lead to the development of regimes to either boost or depress endogenous NS activity in the appropriate clinical situations.

2.2 MATERIALS AND METHODS

2.2.1 Mice

Balb/c, DBA/2, CBA, and C57Bl/6 mice were originally obtained from Charles River Breeding Laboratories (St. Constant, Que.) and were bred and maintained in our own animal care facility. SCID and C.B-17 mice were kindly provided by Dr. R.A. Phillips (Ontario Cancer Institute). In all experiments, the mice used were between 8-12 weeks of age.

2.2.2 Media for Tissue Culture

Cells were routinely washed in a balanced salt solution (BSS) which was prepared in the following way: Solution A consisted of 2.0 g dextrose, 0.12 g KH_2PO_4, 0.716 g Na_2HPO_4-$7H_2O$, and 20 mg phenol red, all in one litre of double distilled water. Solution B consisted of 0.372 g $CaCl_2$-$2H_2O$, 0.8 g KCl, 16 g NaCl, 0.4 g $MgCl_2$-$6H_2O$ and 0.4 g Mg_2SO_4-$7H_2O$ all in one litre of double distilled water. Solutions A and B were mixed together, pH adjusted, filter sterilized, and stored at 4^oC.

Medium for the generation of antibody responses *in vitro* consisted of Eagle's MEM (Gibco, Burlington, Ont.) supplemented with 1% L-glutamine (200 mM), 1% non-essential amino acids (100 X), 1% sodium pyruvate (100 mM), 3% sodium bicarbonate (7.5% solution), 0.5% gentamicin, and 10% fetal bovine serum (all Gibco).

Nutritional cocktail required for daily feeding in the generation of antibody responses consisted of 140 ml of Eagles MEM supplemented with 20 ml essential amino acids (50X), 10 ml non-essential amino acids (100X), 10 ml L-glutamine, and 2.0 g dextrose.

The pH of the mixture was adjusted to 5.0 with 1N NaOH, then 22.6 ml of 8.8% $NaHCO_3$ was added and the solution was filter sterilized and stored in 8 ml aliquots at -20°C. Before use in tissue culture, each 8 ml aliquot was supplemented with 4 ml FBS.

For Con A responses, RPMI-1640 medium (Gibco) was supplemented with 3% sodium bicarbonate, 10% FBS, and 1% penicillin/streptomycin (Gibco). In the MLR, the medium used was the same, with exception of FBS, which was used at a concentration of 5%. All media were adjusted to pH 7.0 to 7.2.

2.2.3 Antibody Synthesis In Vitro

Tissue culture conditions were a modification of those described by Mishell and Dutton (1967). For the generation of plaque forming cells (PFC), 1 X 10^7 spleen cells were cultured in a total volume of 1 ml medium in 24-well Nunc plates (Gibco). To generate a response against SRBC (Woodlyn Laboratories, Guelph, Ont.), 30 ul of a 3% suspension of SRBC was added to each culture. Cultures were maintained at 37°C in a humidified chamber containing 10% CO_2 plus air. The cultures were rocked on a rocker platform (Bellco, Vineland, N.J.) and fed daily with 90 ul of a nutritional cocktail mixture. After 5 days, individual cultures were harvested and assayed for SRBC-specific IgM PFC according to the double slide method described by Cunningham and Szenberg (1968).

2.2.4 Con A Responses

Spleen cells were cultured at a density of 5 X 10^5 cells per well in a total volume of 250 ul in 96-well flat-bottomed plates (Nunc). Con A (Calbiochem-Behring, La Jolla, Ca.) was added to the cell suspension prior to plating, to achieve a final concentration of

0.5 ug/ml. Cultures were incubated for 72 hr and pulsed for the final 6 hr of culture with 0.5 uCi of [methyl-^{3}H] thymidine (New England Nuclear Corp., Boston, MA.), after which time plates were harvested onto glass fiber filter paper using a Titertek cell harvester (Flow Laboratories, Mississauga, Ont.). [^{3}H] thymidine incorporation was determined by liquid scintillation counting.

2.2.5 Mixed Lymphocyte Responses

MLRs were generated by culturing 2 X 10^5 responder spleen cells with 2 X 10^5 stimulator spleen cells in a total volume of 200 ul in 96-well round-bottomed plates (Nunc). Alternatively, 5 X 10^5 responder cells were cultured with 5 X 10^5 stimulator cells in 96-well flat-bottomed plates (Nunc). Stimulators were prepared by incubation of 10^6 cells/ml in 50 ug/ml of Mitomycin C (Sigma Chemical Co., St. Louis, MO) for 30 min at 37^{o}C. The cells were then washed 3 times in BSS before use in culture. Cultures were incubated for 96 hr, which included pulsing with 0.5 uCi of [^{3}H] thymidine for the final 4-6 hr of culture. Plates were harvested as above, and radioactivity was assessed by liquid scintillation counting.

2.2.6 Antibodies

The following monoclonal antibodies were purchased as indicated: rat anti-mouse L3T4 (Becton-Dickinson, Mississauga, Ont.); rat anti-mouse MAC1 (Sera-Lab, Sussex, England); rabbit anti-mouse asialo GM1 (Wako Chemicals, Dallas, Tx.); and rat anti-mouse IL2 receptor (Boehringer Mannheim, Dorval, Que.). The alloantisera against the surface markers Qa2/3 and Qa1/TL were the kind gift of Dr. Shoji Kimura (Memorial Sloan-Kettering Cancer Center). The monoclonal antibody against the surface marker NK 1.1 (rat anti-mouse) was the

kind gift of Dr. Gloria Koo (Merck Sharp & Dohme Research Laboratories).

2.2.7 Antibody Treatment of BM Cells

BM cells were prepared by flushing tibias and femurs with sterile BSS using a syringe and a 26-gauge needle, and a single cell suspension was obtained by repeated aspiration of BM tissue fragments with syringe and needle. The cells were washed 3 times prior to further use. BM cells (10^7/ml) were incubated at 4°C for 45-60 min with RPMI alone or appropriately diluted antisera. The dilutions used were as follows: NK 1.1 (1/100), ASGM-1 (1/50), MAC-1 (1/50), TAC (0.1 ml), L3T4 (0.2 ml), Qa2,3 (1/320), and Qa1,TL (1/160). As a second step, the cells were incubated with a goat anti-rat IgG Ab (Bio/Can Scientific, Mississauga, Ont.) where appropriate. Following incubation, the cells were washed once, resuspended in the same volume of RPMI containing a 1/10 dilution of Low-Tox Rabbit Complement (Cedarlane Laboratories, Hornby, Ont.), and further incubated at 37°C for 45-60 min. After incubation, the cells were washed 2 times, resuspended in medium, and counted prior to use so that equal numbers of treated cells could be added to specific assays.

2.2.8 Elutriation of BM Cells

BM cells were prepared as described above. Additionally, BM cells were treated with DNAse and 2,4-N-dispase (Boehringer Mannheim) to decrease agglutination of cells. After the final wash, the cells were passed through a wire mesh to remove any particulate tissue that still existed.

The elutriation system consisted of a J2-21 M centrifuge equipped with the JE-6 elutriation rotor and separation chamber (Beckman

Instruments, Toronto, Ont.). The initial rotor speed was adjusted to 4200 rpm, and buffer containing 1% FBS was pumped through at a rate of 26 ml/min. Before injection of cells, air bubbles were removed from the system. After injection of the BM cell suspension, the initial population eluted was RBCs, which were discarded. The rotor speed was subsequently lowered (by 100 rpm increments) and the number and size of cells being collected was monitored by a Coulter Counter and Channelyzer (Coulter Electronics Inc., Hialeah, Fla.). The BM cells were collected as 100 ml fractions and pooled according to size based on the individual Coulter profiles. Typically, 2-5 X 10^8 BM cells were separated at one time, and after each run viable cell recovery was > 70%.

2.2.9 Indomethacin Treatments

Indomethacin (Sigma) was suspended in ethanol and stored as a 10^{-2} M stock solution. For use in tissue culture the stock solution of indomethacin was appropriately diluted in tissue culture medium. In all experiments, ethanol diluted the same way was used as a control.

2.2.10 BM Reconstitution of Lethally Irradiated Mice

Balb/c mice were lethally irradiated with 850R emitted from a ^{137}Cs source in a Gammacell 20 Irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada). Irradiated mice were reconstituted by tail-vein injection of Balb/c BM cells within 6 hrs of treatment. Reconstituted mice were maintained on water supplemented with antibiotics, but otherwise were fed unsterilized food and maintained in normal animal housing conditions.

2.2.11 Short Term Culture of Bone Marrow Cells

BM cells from Balb/c or C57Bl/6 mice were cultured in 24-well

plates at a density of 2 X 10^6 cells/well in RPMI plus 10% FBS, and supplemented with 25% v/v of supernatants from rat spleen cells which had been stimulated with 2.5 ug/ml of Con A for 48 hrs (referred to as rat Cas). Con A was removed from rat Cas by absorbing two times with 0.2 g/100 ml Sephadex G-25 (Pharmacia) for 15 min at room temperature (Oseroff et. al., 1984). Rat Cas was found to be supportive of growth of the IL-2 dependent cell line CTLL-2 (ATCC, Rockville, Md.). BM cells were cultured in rat Cas supplemented for up to 6 days. Continuing cultures were fed every 2 days by removing one-half of the medium and replacing it with fresh medium.

2.2.12 Long Term Culture of Bone Marrow Cells

Whole BM cells from Balb/c mice were cultured in 1 ml volumes in 24-well plates at a density of 2-5 X 10^6 cells/well along with equal numbers of irradiated (2500 R) syngeneic spleen cells which served as a feeder layer. The cells were cultured in RPMI plus 10% FBS, and were supplemented with 25% v/v of rat Cas.

The cultures were fed every 2-3 days by removing 1/2 of the medium and replacing it with fresh medium containing the same concentration of rat supernatant. Cultures were replenished with new feeder layers every 7-14 days. Growth was negligible after 2-3 weeks of culture, after which time a dominant cell type arose which was homogeneous in appearance. Wells showing positive growth were allowed to expand to 3/4 confluence, were harvested, and replated into fresh 24-well plates with growth medium and feeder cells. Growth of the replated cells required feeding every 2-3 days as above. The maximum lifespan of the cells in culture was limited to 2-3 months after the initial plating.

2.2.13 NK Assay

Prior to the assay, YAC-1 lymphoma cells (ATCC) growing in exponential phase were harvested and incubated at 37°C with 100 uCi of ^{51}Cr (ICN Canada, Montreal, Que.) in a volume of 1 ml at a density of 1 X 10^6 cells/ml. After 45-60 min of incubation, the cells were washed and kept in RPMI plus 10% FBS in the incubator until the time of plating.

For the assay, spleen, BM, and long term cultured BM cells (LTBMC) were prepared and suspended in RPMI plus 10% FBS. The responder cells were plated in 96-well round-bottomed plates at densities of 1 X 10^6, 5 X 10^5, and 2.5 X 10^5 cells/well along with 1 X 10^4 labelled YAC-1 cells in a total volume of 200 ul to give responder:target ratios of 100:1, 50:1, and 25:1 respectively. Plates were incubated for 12 hr to maximize killing, then centrifuged for 5 min at 1000 rpm and 100 ul of supernatant was harvested from each well of the plate for gamma counting. Total release was determined by culturing YAC-1 targets in the presence of Triton X-100 (Sigma). Spontaneous release was derived from YAC-1 cells cultured alone in medium.

2.2.14 Cytospin and Staining of Cells

Cytospins of cells were prepared using a Shandon Elliott Cytospin (Fisher). Approximately 1 X 10^6 cells in a volume of 200 ul and 50 ul of FBS were added to a cytospin holding cup secured to a glass microscope slide. The cells were centrifuged at 700 rpm for 5 min, forcing them onto the microscope slide. The slides were air dried and then fixed with methanol for 5 min. Slides were stained with Jenner-Giemsa stain for morphological examination of cells. Briefly, fixed slides were rinsed with Giemsa buffer, which consisted of 50 ml

of 0.2 M KH_2PO_4 mixed with 23.7 ml of 0.2 N NaOH. Slides were then flooded with a 1:2 dilution (in Giemsa buffer) of Jenner's stain (BDH Chemicals, Toronto, Ont.) for 3 min. The slides were then rinsed with Giemsa buffer and flooded with a 1:8 dilution (in Giemsa buffer) of Giemsa stain (BDH) for 10 min. Slides were then rinsed with Giemsa buffer, blotted dry using bibulous paper, and saved for microscopic examination.

2.2.15 Statistics

All data were analyzed according to the one- or two-tailed Student's t-test where applicable. P values greater than .05 were considered to be not significant. All experiments were performed in quadruplicate unless indicated otherwise.

2.3 RESULTS

2.3.1 Suppression by BM Derived NS Cells

The addition of BM cells to cultures of spleen cells participating in PFC, MLR, or Con A responses resulted in a dose dependent suppression of each response (Fig. 2.1). Suppression was maximal in the PFC response (Fig. 2.1 A) at a spleen cell:BM ratio of 2:1, and in the MLR and Con A responses suppression was maximal at a ratio of 1:1 (Fig. 2.1 B,C).

In all of the responses in Figure 2.1 the BM cells added to culture were syngeneic to the spleen cells used as the responder population. However, suppression was not found to be MHC restricted between the responding and NS cells. In a representative experiment, addition of BM cells syngeneic (Balb/c) or allogeneic (CBA) to the responding population (Balb/c) in an MLR resulted in identical levels of suppression (Fig. 2.2). To demonstrate that suppression was due to suppressor cells in the BM, and not nonspecific events caused by overcrowding, either BM cells, spleen cells, or thymocytes were added to an MLR at equal doses. The results in Table 2.1 clearly demonstrate that only BM cells could suppress proliferation in the MLR.

2.3.2 Kinetics of Suppression by BM NS Cells

BM cells were added to an MLR at various time intervals to determine whether suppression was restricted to a specific stage of the response. The addition of 2×10^5 BM cells, which represents a maximal suppressive dose, at days 0 and 1 of culture resulted in suppression of the response (Fig. 2.3 A). However, addition of BM cells at day 2 of culture had no effect, suggesting that suppression was restricted to

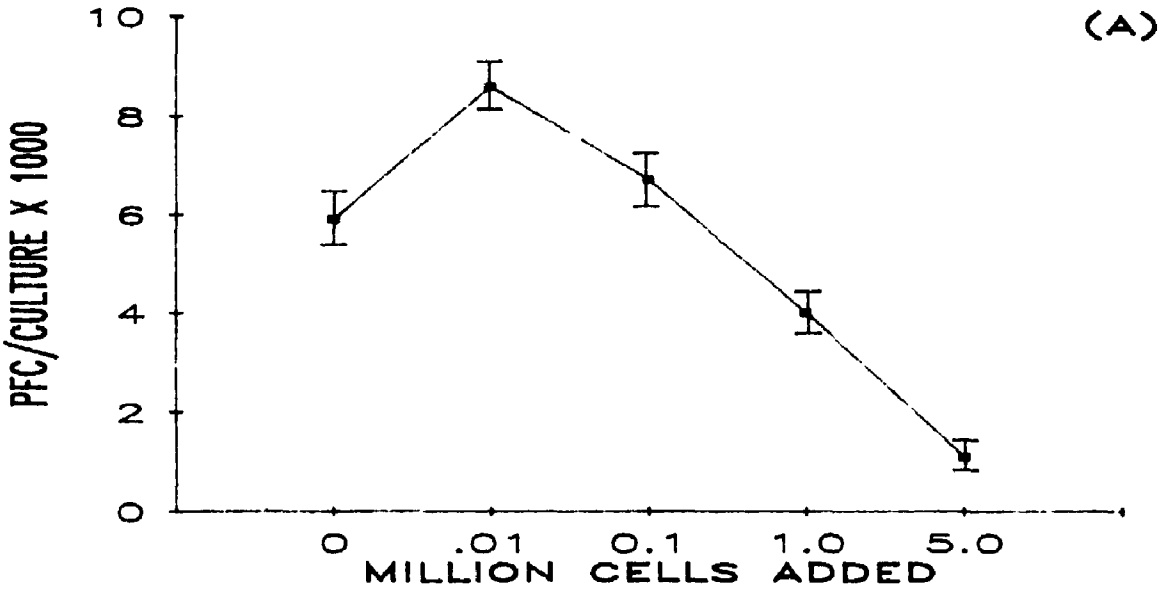
FIGURE 2.1

Suppression of PFC, MLR, and Con A Responses by BM Cells

(A) Increasing doses of DBA/2J BM cells were added to DBA/2J spleen cells + SRBC. The background response of unstimulated cells was 313 ± 124 PFC/culture.

(B) Increasing doses of C57Bl/6 BM cells were added to a C57Bl/6 vs. Balb/c MLR culture. The background response of responding cells was 4,904 ± 1,446 CPM.

(C) Increasing doses of C57Bl/6 BM cells were added to C57Bl/6 spleen cells cultured with 0.5 ug/ml of Con A. The background response of unstimulated cells was 2,438 ± 443 CPM. In all 3 panels vertical bars represent standard deviation.


(A)
PFC/CULTURE X 1000
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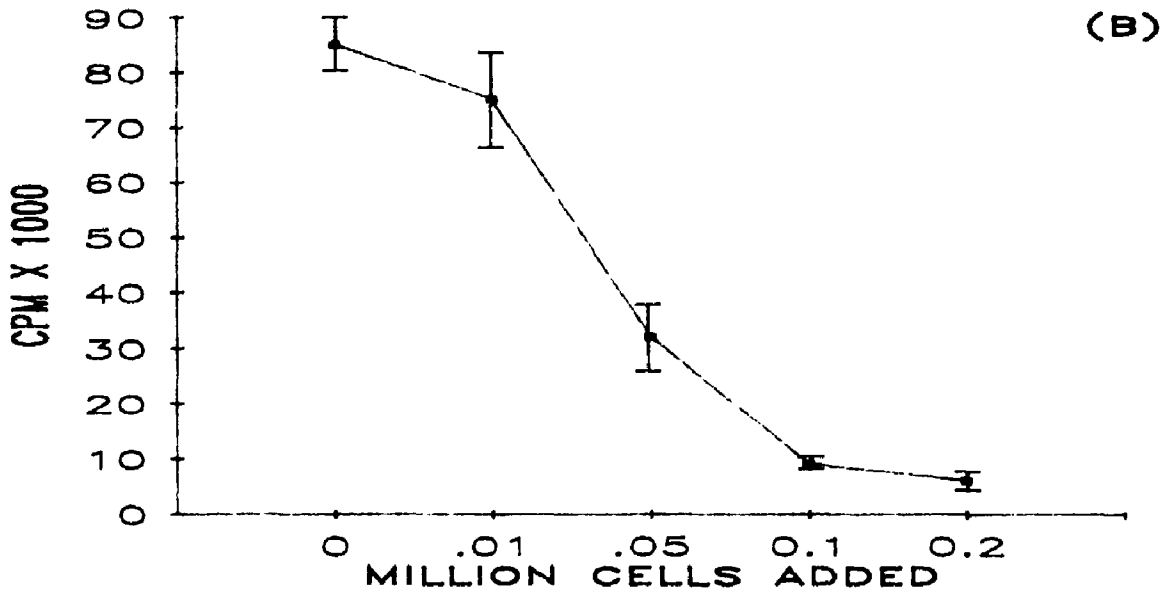
8
6
4
2
0
0
.01
0.1
1.0
5.0
MILLION CELLS ADDED


(B)
CPM X 1000
90
80
70
60
50
40
30
20
10
0
0
.01
.05
0.1
0.2
MILLION CELLS ADDED

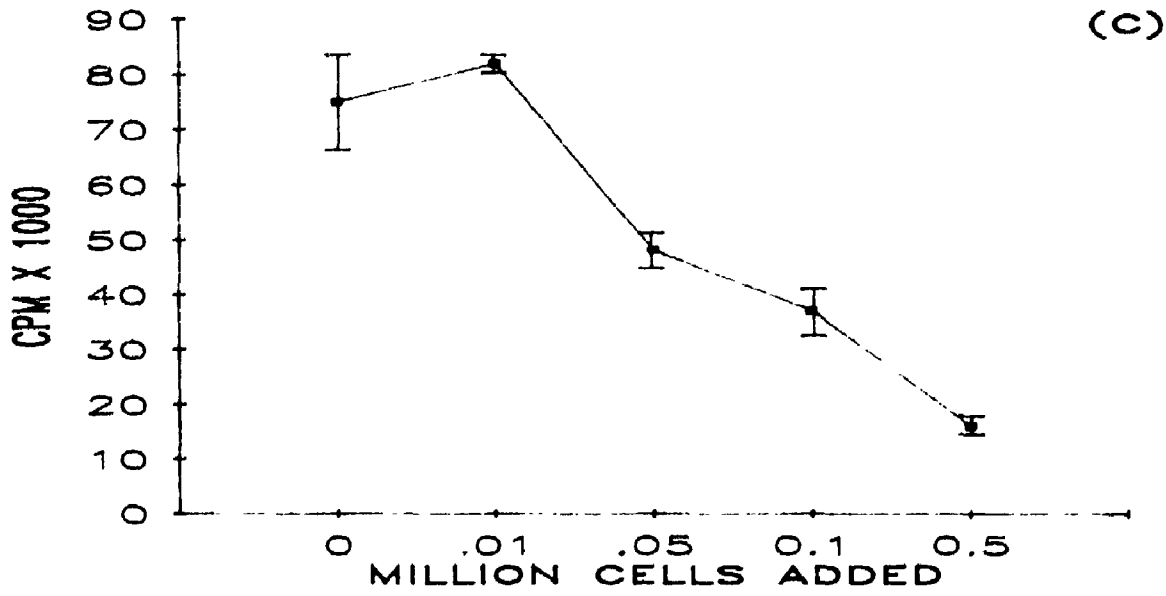

(C)
CPM X 1000
90
80
70
60
50
40
30
20
10
0
0
.01
.05
0.1
0.5
MILLION CELLS ADDED

FIGURE 2.2

Suppression by BM Cells in the MLR is not MHC Restricted

Increasing doses of either Balb/c or CBA BM were added to a Balb/c vs. C57Bl/6 MLR culture. The background response of Balb/c vs. Balb/c was 14,500 ± 2,502 CPM. Vertical bars represent standard deviation.

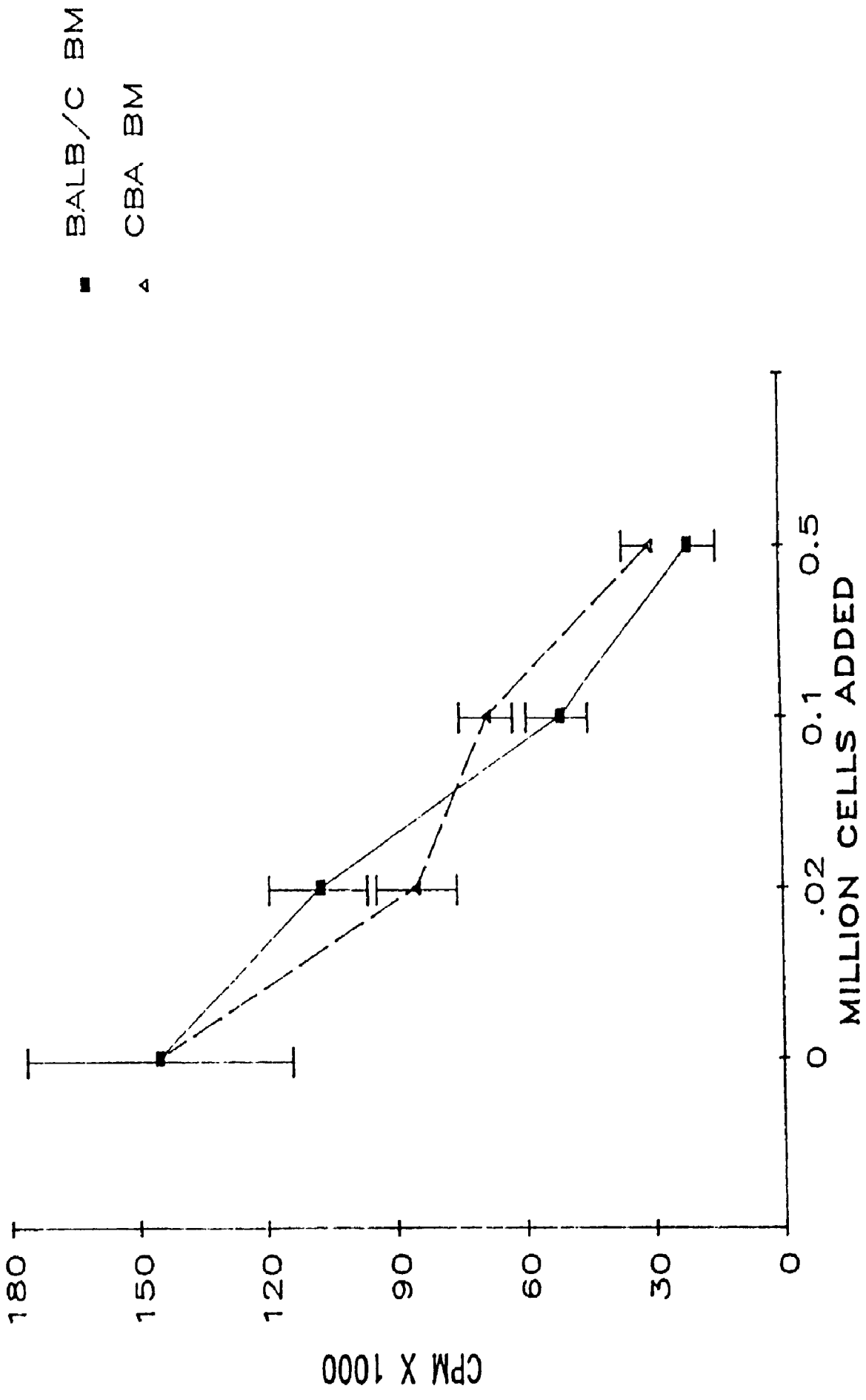
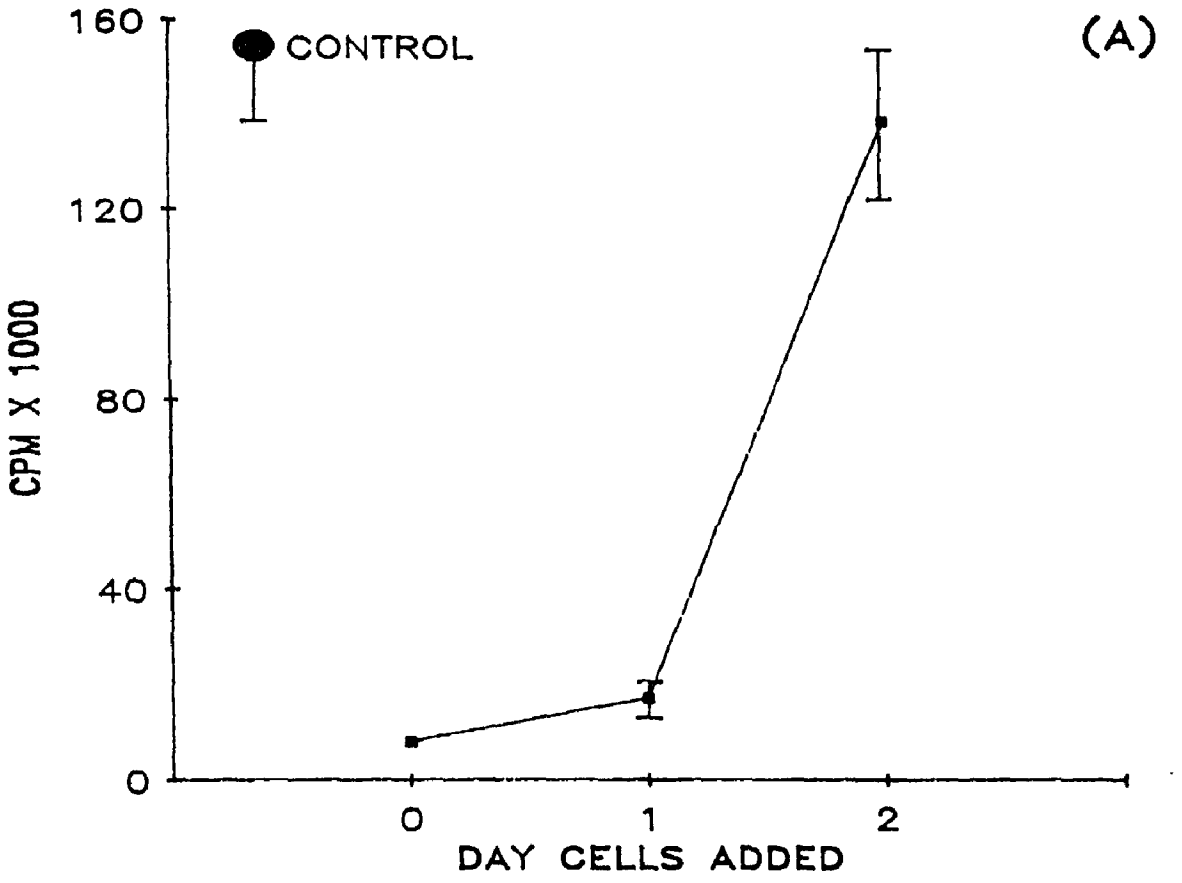

BALB/C BM
CBA BM
CPM X 1000
180
150
120
90
60
30
0
0
.02
0.1
0.5
MILLION CELLS ADDED

FIGURE 2.3

The Kinetics of Suppression by BM in the MLR

(A) 2 X 10^5 Balb/c BM cells were added to a Balb/c vs. C57Bl/6 MLR culture at days 0, 1, and 2 of culture. ^{3}H-TdR uptake was monitored 4 days after culture initiation. The background response of cultures stimulated with autologous cells was 12,933 ± 2,825 CPM.

(B) MLR cultures consisting of C57Bl/6 vs. Balb/c spleen cells were set up in the presence or absence of 2 X 10^5 C57Bl/6 BM cells, and ^{3}H-TdR uptake was monitored on days 3, 4, and 5 after culture initiation. The background responses of cultures stimulated with autologous cells was 2,950 ± 473, 2,656 ± 353, and 2,310 ± 296 CPM on days 3, 4, and 5, respectively. In both panels vertical bars represent standard deviation.


(A)
CONTROL
160
120
80
40
0
CPM X 1000
0
1
2
DAY CELLS ADDED

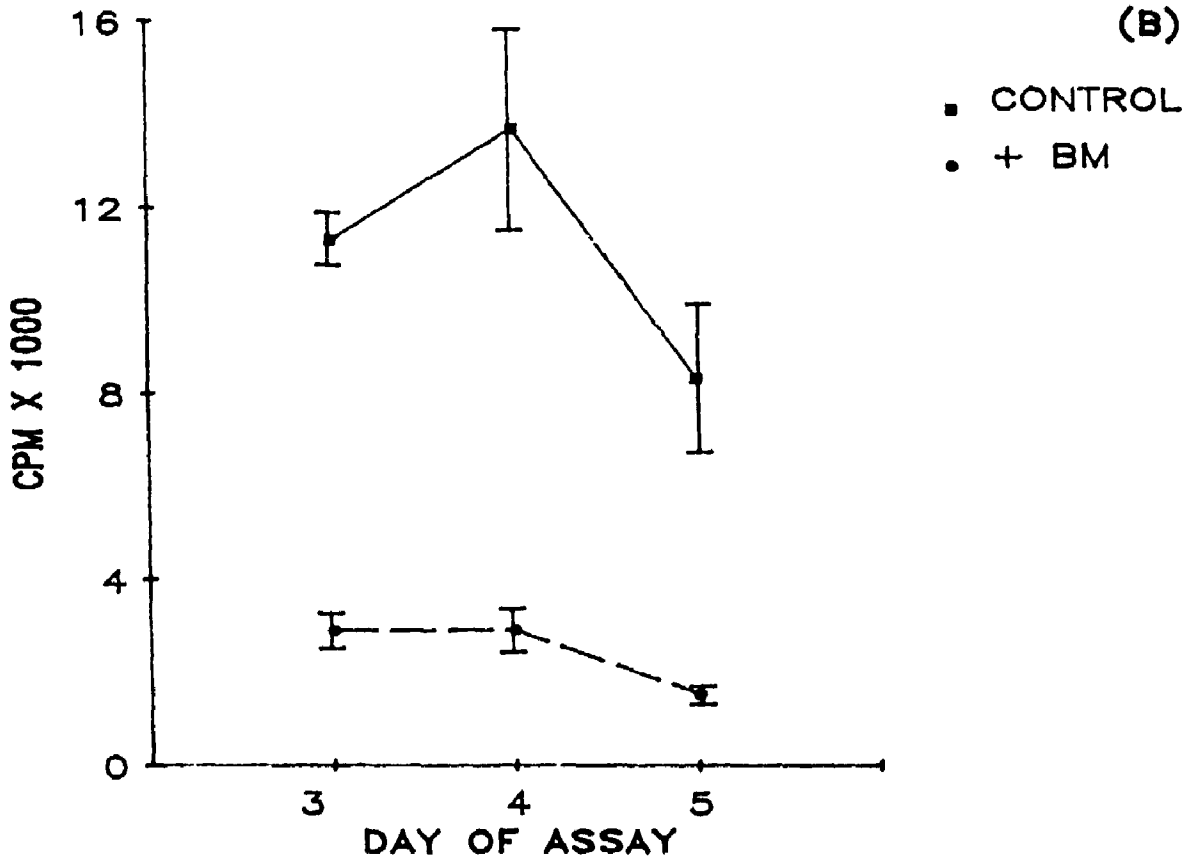

(B)
CONTROL
+ BM
16
12
8
4
0
CPM X 1000
3
4
5
DAY OF ASSAY

TABLE 2.1

Suppression of the MLR by BM, but not Spleen or Thymus

Cells Added	Number of Cells Added	CPM ± S.D.	P Value[a]
--	--	75,205 ± 10,950	--
BM	2×10^5	26,060 ± 5,816	<.0005
	1×10^5	34,070 ± 5,534	<.0005
	5×10^4	47,582 ± 3,228	<.005
Spleen	2×10^5	81,879 ± 8,977	N.S.
	1×10^5	92,802 ± 22,583	N.S.
	5×10^4	74,314 ± 20,413	N.S.
Thymus	2×10^5	60,362 ± 11,693	N.S.
	1×10^5	73,311 ± 6,155	N.S.
	5×10^4	71,529 ± 18,387	N.S.

[a] The MLR consisted of C57Bl/6 spleen cells cultured with mitomycin C treated Balb/c spleen cells. BM cells, spleen cells, or thymocytes from C57Bl/6 mice were added at culture initiation at the doses indicated. The background response of unstimulated cultures was 9,537 ± 2,561 CPM.

b P value compared to control with no cells added. N.S. refers to not significant.

the activation stage.

To determine whether the addition of BM cells simply shifted the kinetics of the response, MLR cultures to which 2 X 10^5 BM cells were added at day 0, were assayed on either days 3, 4, or 5. Suppression was strong and was approximately equal relative to the control at each time point (Fig. 2.3 B).

2.3.3 Suppression by BM NS Cells is not Inhibited by Indomethacin

Indomethacin, a prostaglandin synthetase inhibitor, was added to PFC and MLR cultures containing BM cells to determine whether suppression was mediated via the synthesis and release of suppressive prostaglandins. As shown in Figure 2.4, the addition of either 10^{-5} or 10^{-6} M indomethacin to PFC (A) or MLR (B) cultures did not abrogate suppression due to BM NS cells. Suppression closely parallelled that in control cultures treated with equivalent dilutions of ethanol (the solvent for indomethacin).

2.3.4 SCID Mice Possess Normal Levels of BM NS Activity

SCID mice are characterized by a lack of mature, functional lymphocytes due to the inability to functionally rearrange genes encoding antigen-specific cell surface receptors (Schuler et al., 1986). In spite of this, SCID mice retain a normal myeloid component and possess normal NK function (Dorshkind et al., 1985). NS activity was compared between BM of SCID mice and their normal littermate C.B-17, and normal NS function was present in both mice. The response of SCID spleen cells cultured with SRBC was 188 $\pm$ 54 PFC/culture, confirming the immune deficiency of SCID mice.

2.3.5 NS Activity in Spleens of Lethally Irradiated, BM Reconstituted Mice

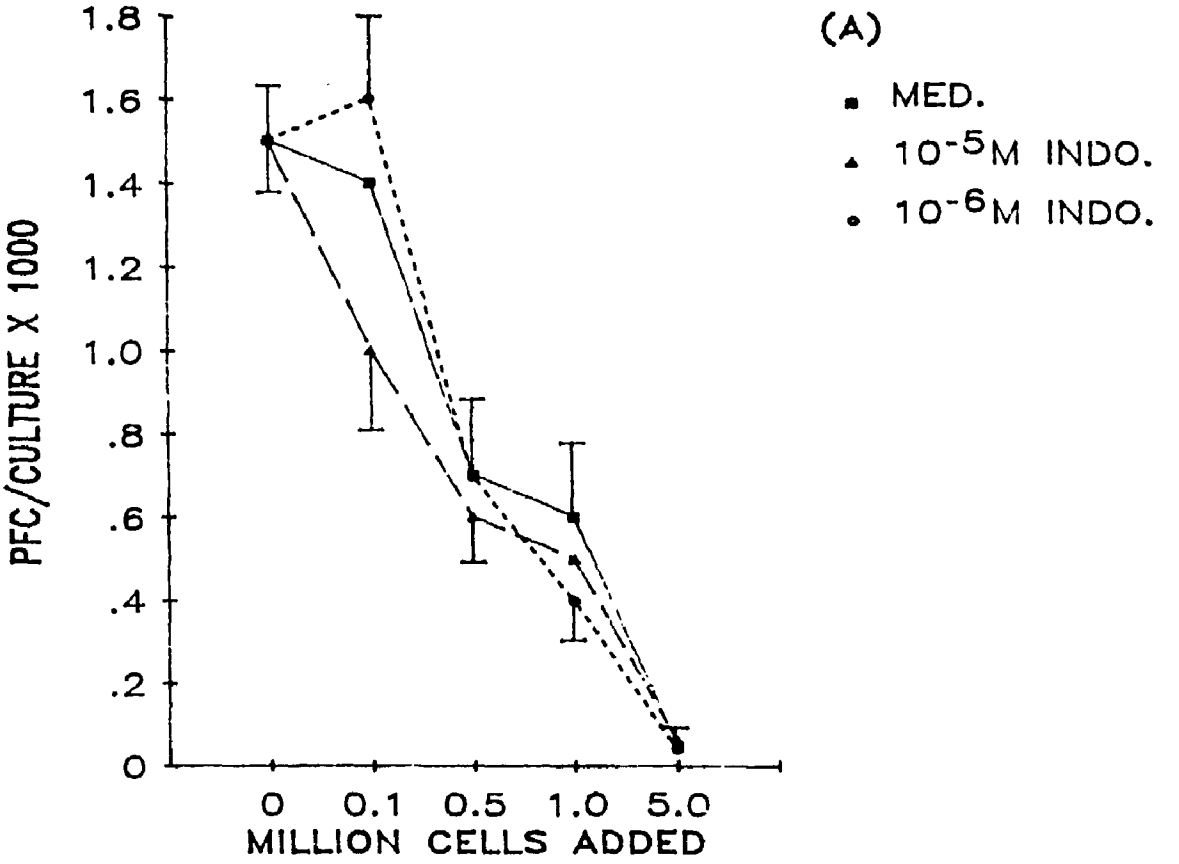
The spleens of lethally irradiated mice reconstituted with

FIGURE 2.4

Suppression by BM is not Inhibited by Indomethacin

(A) Increasing doses of Balb/c BM cells were added to Mishell-Dutton cultures of Balb/c spleen cells + SRBC with or without indomethacin. As a control, cultures were treated with medium containing a 1/1000 dilution of ethanol. The control response in the presence of 10^{-5} M indomethacin was 983 ± 193 PFC/culture, and for 10^{-6} M indomethacin was 1,363 ± 139 PFC/culture. The background response of unstimulated cells was 96 ± 43 PFC/culture.

(B) Increasing doses of Balb/c BM cells were added to a Balb/c vs. C57 MLR culture with or without indomethacin. Control cultures were treated with medium containing a 1/1000 dilution of ethanol. The control response in the presence of 10^{-5} M indomethacin was 223,925 ± 71,889 CPM, and for 10^{-6} M indomethacin was 238,369 ± 27,971 CPM. The background response was 12,106 ± 4,860 CPM. In both panels vertical bars represent standard deviation.


(A)
MED.
10-5M INDO.
10-6M INDO.
PFC/CULTURE X 1000
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
0 0.1 0.5 1.0 5.0
MILLION CELLS ADDED

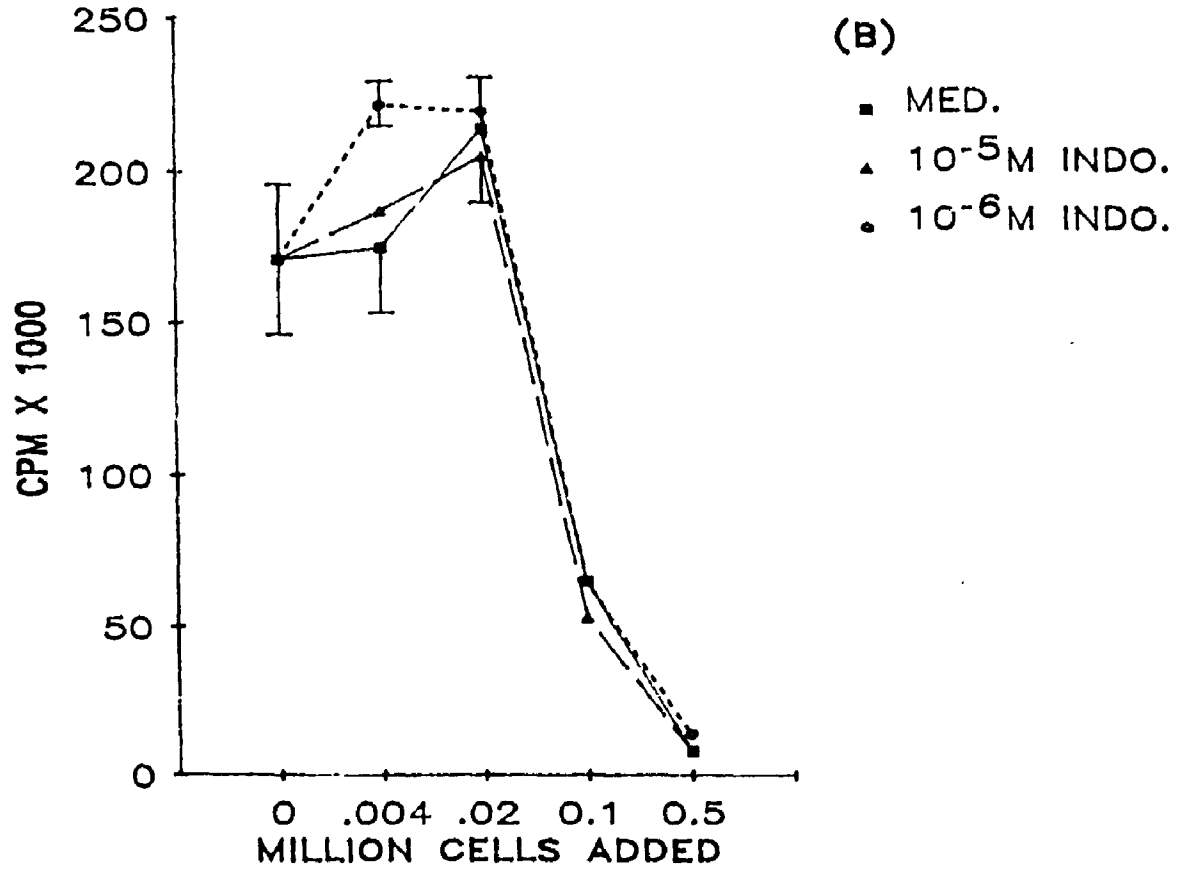
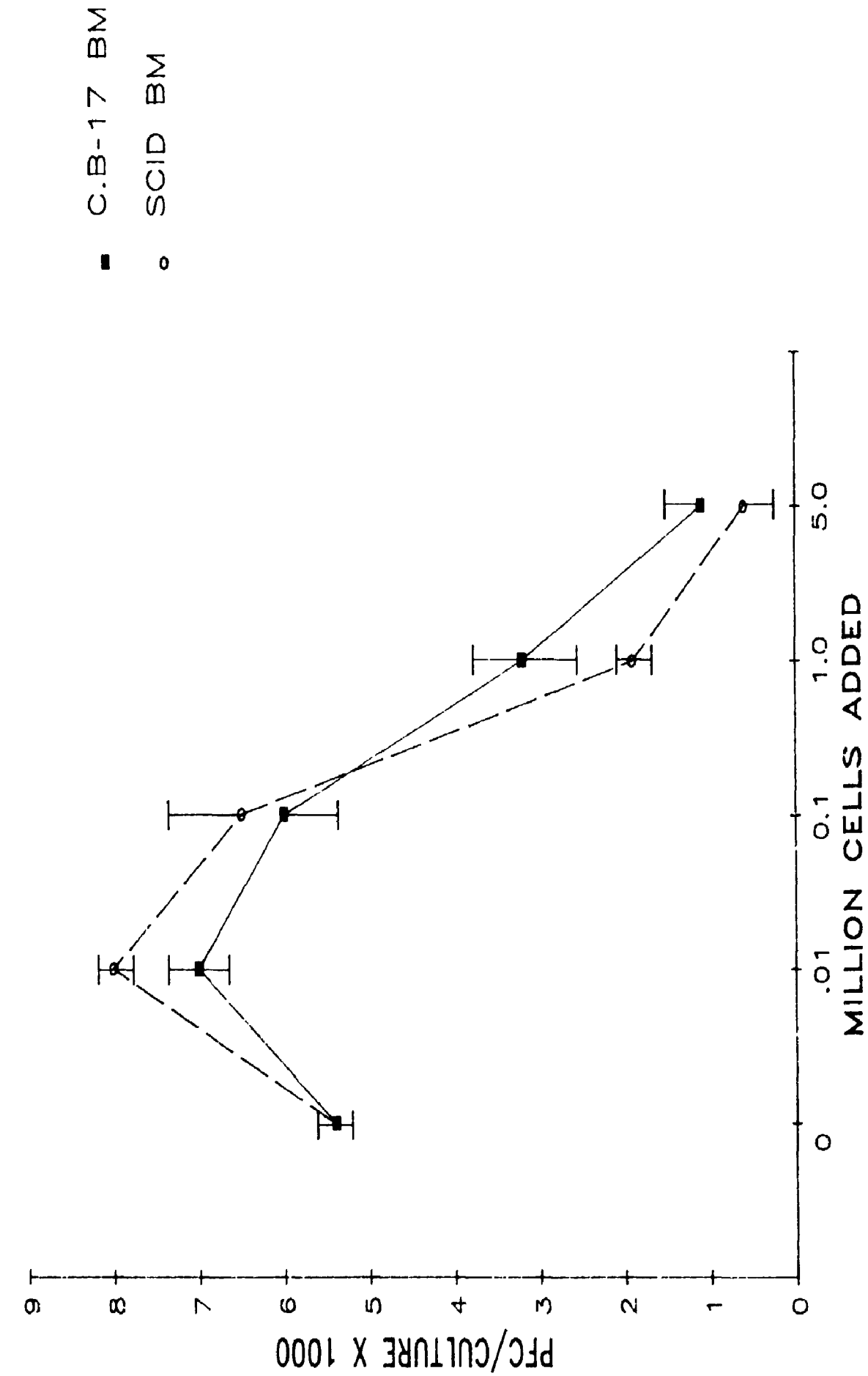

(B)
MED.
10-5M INDO.
10-6M INDO.
CPM X 1000
250
200
150
100
50
0
0 .004 .02 0.1 0.5
MILLION CELLS ADDED

FIGURE 2.5

Suppression of the PFC response to SRBC by SCID and C.B-17 BM

1×10^7 C.B-17 spleen cells were cultured with varying doses of either C.B-17 (■, solid line) or SCID (○, broken line) BM cells + SRBC. The background response of C.B-17 spleen cells in the absence of SRBC was 718 ± 278 PFC/culture. Vertical bars represent standard deviation.


C.B-17 BM
SCID BM
PFC/CULTURE X 1000
0
1
2
3
4
5
6
7
8
9
0
.01
0.1
1.0
5.0
MILLION CELLS ADDED

syngeneic BM contain hematopoietic progenitor cells (CFU-S) and cells of the myeloid lineage 10 days after reconstitution, thus resembling normal adult BM (Till and McCulloch, 1961). Balb/c mice were lethally irradiated with 850R and reconstituted with increasing doses of BM cells. Ten days after reconstitution spleens were removed from each group of three mice, pooled, and tested for NS activity in a PFC response. Figure 2.6 demonstrates that spleens from all three groups contained NS activity. which appeared to be greater as higher numbers of BM cells were used to reconstitute the mice. As a control a group of three lethally irradiated mice were injected with BSS only and at day 10 the number of cells recovered was too low for assay in the PFC response (approximately 1 X 10^6 cells/spleen). In comparison mice reconstituted with 10^5 BM cells yielded 20 X 10^6 cells/spleen, and those reconstituted with 10^6 and 10^7 BM cells yielded 35 X 10^6 cells/spleen.

2.3.6 Separation of Immunoregulatory Cell Populations in Murine BM by Elutriation

To enrich for NS activity BM cells were fractionated according to size and density by counterflow centrifugal elutriation. BM cells were divided into 4 fractions shown in Figure 2.7. Fraction 1 (F1) contained small, high density cells which were predominantly of lymphocytic morphology as determined by Jenner-Giemsa staining of cytospin preparations. Fractions 2 and 3 (F2 and F3) contained progressively larger, lower density cells of monocytic/myeloid lineage. Fraction 4 (F4) was comprised of the largest, lowest density cells, containing myeloid cells, megakaryocytes, and blast cells. The percent recovery of BM cells in each fraction was as follows: F1, 21% ; F2, 12% ; F3, 47% ; F4, 20%.

FIGURE 2.6

Suppression of the PFC Response by Spleen Cells From Lethally Irradiated, BM Reconstituted Mice

Balb/c mice were lethally irradiated (850 R) and injected IV with either 10^5, 10^6, or 10^7 syngeneic BM cells. Spleens were harvested from groups of 3 mice 10 days after BM reconstitution. Single cell suspensions of spleen cells from the respective groups were added to Mishell-Dutton cultures of Balb/c spleen cells in the presence of SRBC. The positive control is represented by the closed square. The background response of unstimulated cells was 50 ± 35 PFC/culture. Vertical bars represent standard deviation.

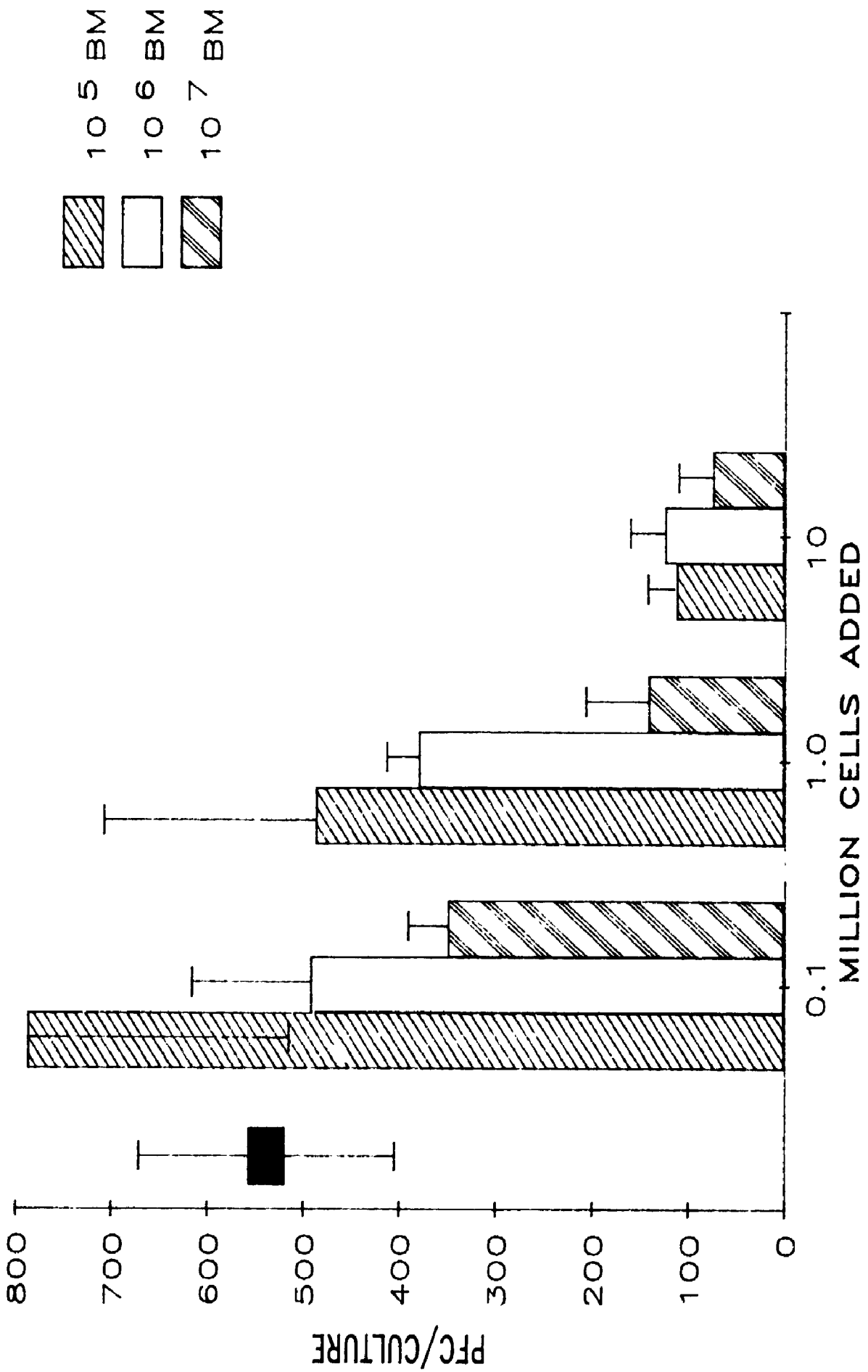
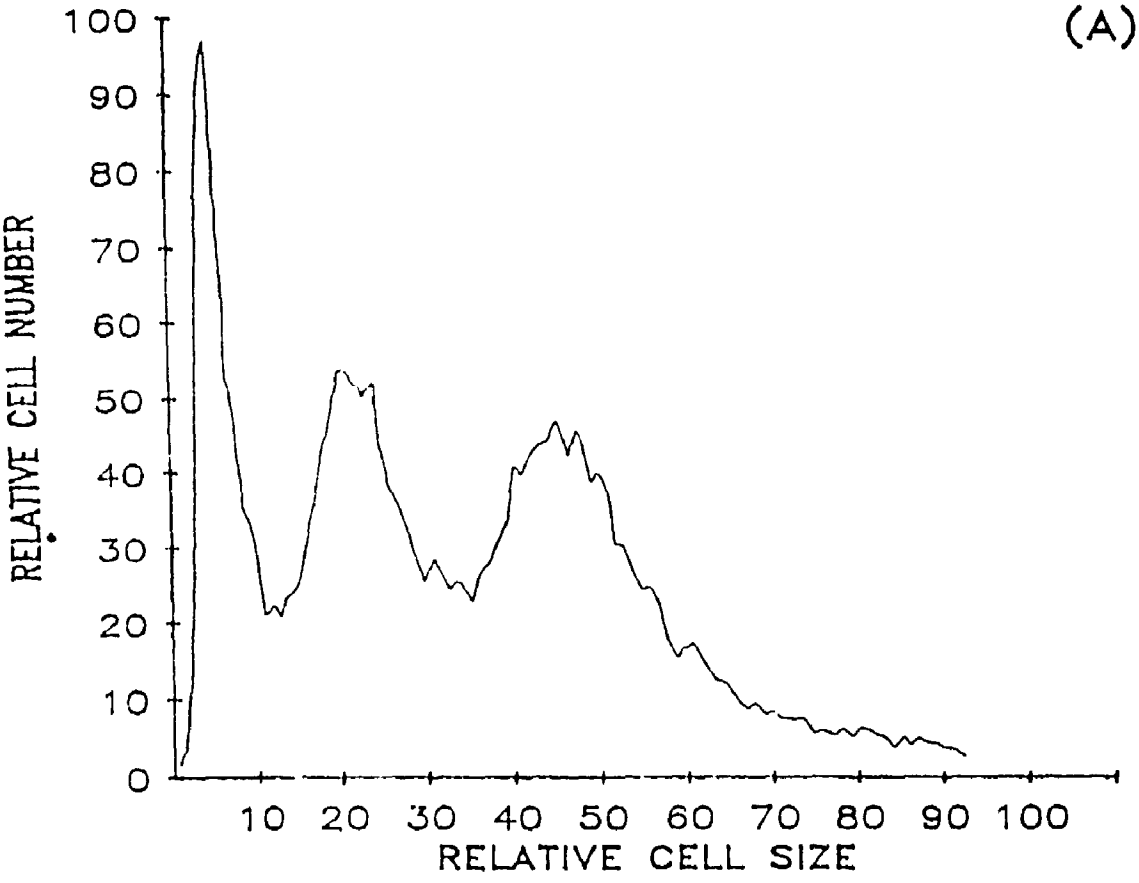

10 5 BM
10 6 BM
10 7 BM
PFC/CULTURE
800
700
600
500
400
300
200
100
0
0.1
1.0
10
MILLION CELLS ADDED

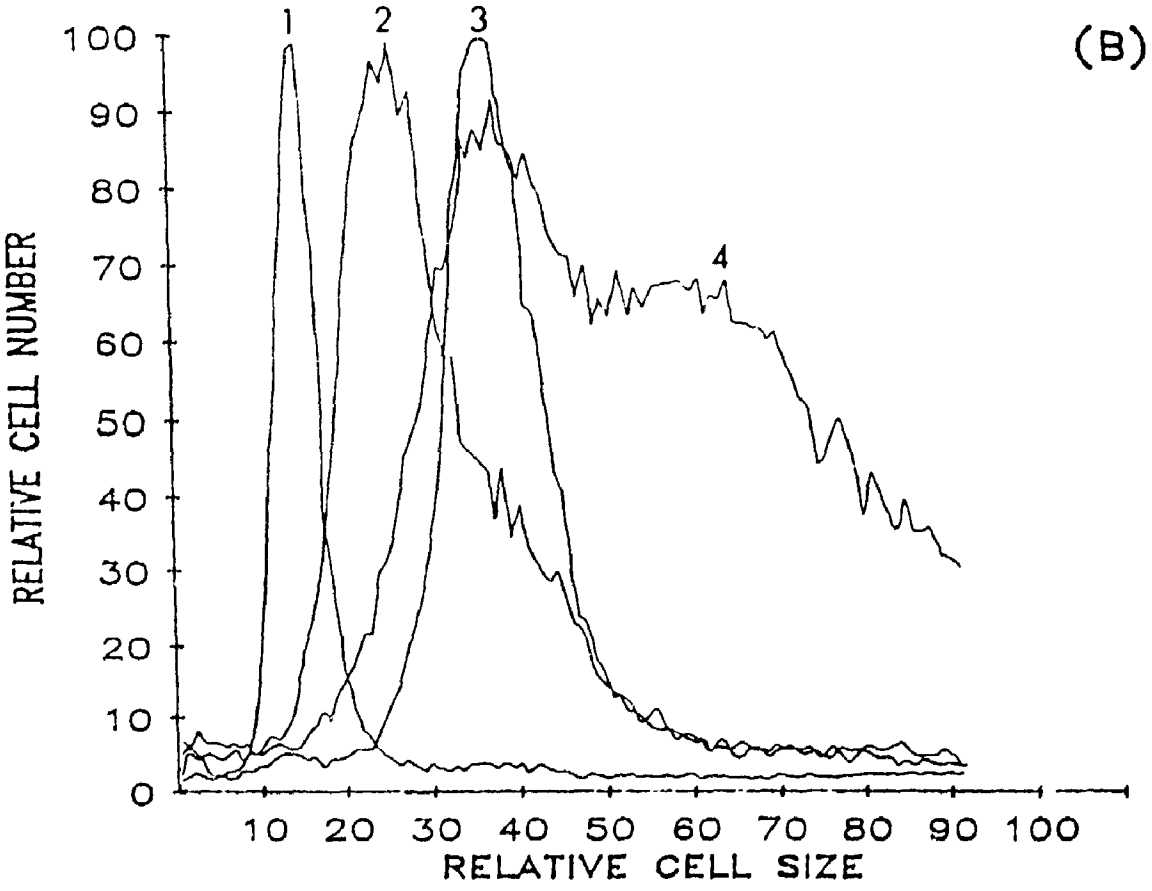
FIGURE 2.7

Cell Size Profile of Unfractionated and Elutriated BM Cells

(A) Profile of unfractionated Balb/c BM cells sized on a Coulter Channelizer prior to elutriation.

(B) Elutriated Balb/c BM cells were sized and pooled into four fractions according to their relative sizing. The numbers 1-4 represent the profile of each individual fraction.


(A)
RELATIVE CELL NUMBER
100
90
80
70
60
50
40
30
20
10
0
10 20 30 40 50 60 70 80 90 100
RELATIVE CELL SIZE


(B)
1
2
3
4
RELATIVE CELL NUMBER
100
90
80
70
60
50
40
30
20
10
0
10 20 30 40 50 60 70 80 90 100
RELATIVE CELL SIZE

The individual fractions displayed distinct immunoregulatory activity when added to cultures of spleen cells challenged with SRBC (Table 2.2). F1 enhanced the PFC response to SRBC, while F2 cells showed marginal activity (19%) and F3 cells displayed increased suppressor activity (38%). The majority of suppressor activity was present in F4 (80%) suggesting that NS cells reside in the population of large, low density cells in BM. On a cell per cell basis suppression was enriched in F4 compared to the normal level of NS activity observed with whole BM. It therefore appears that BM derived NS activity resides in the F4 BM cell population. The proliferative potential of elutriated BM cells as assessed by ^{3}H-TdR uptake shows that F4 cells proliferated at the highest rate (Table 2.3)

In a subsequent elutriation fractions of BM cells added to MLR cultures possessed identical activity to that seen in the PFC response. As shown in Figure 2.8, BM was divided into 3 fractions, and again F1 represents the smallest, high density cells and F3 the largest, low density cells. The percent recovery after elutriation was as follows: F1, 12% ; F2, 83% ; F3, 5%. F1 cells displayed enhancing activity when increasing doses of cells were added to culture, while F2 displayed suppressor activity equivalent to unfractionated BM at every dose. Strikingly, F3 cells were highly suppressive (perhaps reflecting greater enrichment), even when only 5 X 10^4 cells were added to the culture, as compared to whole BM added at the same dose.

In the generation of the MLR, there are no reports in the literature using BM as a source of stimulator cells. Table 2.4 demonstrates that compared to spleen, BM cells are poor stimulators regardless of the strain of mouse used as a source of spleen and BM.

TABLE 2.2

The Effect of Enriched Mouse Bone Marrow Fractions on the In Vitro PFC Response to SRBC

Cells Added[a] to Culture	PFC ± S.D./ Culture	% Suppression[b]	P VALUE[c]
None	6,875 ± 925	—	—
Whole BM	2,251 ± 530	67	<.001
Fraction 1	9,438 ± 1,085	enh.	<.005
Fraction 2	5,563 ± 835	19	<.05
Fraction 3	4,250 ± 730	38	<.005
Fraction 4	1,417 ± 221	80	<.001

[a] 1 X 10^6 whole or fractionated Balb/c BM cells were added per culture of 10^7 Balb/c spleen cells + SRBC. (The background response of spleen cells cultured in the absence of SRBC was 1,188 ± 385 PFC/culture).

[b] % suppression was calculated relative to cultures containing spleen cells + SRBC alone.

$$\% \text{ SUPPRESSION} = 1 - \frac{\text{SPLENIC PFC + BM}}{\text{SPLENIC PFC}} \times 100$$

[c] P values were calculated relative to the control response of spleen cells + SRBC alone. P values >.05 were not considered significant.

TABLE 2.3

Proliferative Rate of Elutriated BM Cells

Cells	^{3}H-TdR Uptake[a] (CPM ± SD)
Whole BM	4,933 ± 933
Fraction 1	263 ± 110
Fraction 2	422 ± 82
Fraction 3	2,680 ± 349
Fraction 4	23,374 ± 4,404

[a] 5 X 10^{5} Balb/c BM cells were cultured in 96-well flat-bottomed plates for 24 hr followed by a 24 hr pulse with 0.5 uCi of ^{3}H-TdR. CPM values were obtained by liquid scintillation counting.

FIGURE 2.8

The Effect of Elutriated BM Fractions in the MLR

Enriched fractions of elutriated C57Bl/6 BM cells were added to a C57Bl/6 vs. Balb/c MLR culture. Unfractionated C57Bl/6 BM cells were added at equal doses as a control. The positive control is represented by the closed circle. The background response of cultures stimulated with autologous cells was 8,370 ± 1,672 CPM. Vertical bars represent standard deviation.

TABLE 2.6

Suppression of Anti-SRBC Responses by Ab Treated

BM Derived NS Cells

Treatment	Ratio of Responder:BM Cells[a]	% Suppression[b]
EXP. 1		
C′ Treated	2:1	97
	10:1	75
	50:1	56
Anti-NK 1.1 + C′	2:1	98
	10:1	82
	50:1	53
EXP. 2		
C′ Treated	2:1	83
	10:1	26
	50:1	12
Anti-ASGM1 + C′	2:1	90
	10:1	51
	50:1	26
Anti-MAC1 + C′	2:1	89
	10:1	48
	50:1	--
EXP. 3		
C′ Treated	2:1	77
	10:1	56
	50:1	22
Anti-L3T4 + C′	2:1	83
	10:1	67
	50:1	11
Anti-IL2R + C′	2:1	81
	10:1	61
	50:1	22

[a] 10^7 C57Bl/6 spleen cells + SRBC were cultured with varying doses of C57Bl/6 BM cells.

[b] % suppression was calculated relative to the control values of C57Bl/6 spleen cells + SRBC alone. These values were 13,363 ± 2,076, 731 ± 167, and 1,313 ± 127 PFC/culture for experiments 1,2, and 3, respectively.

TABLE 2.4

BM Cells are Poor Stimulators in the MLR[a]

Stimulator[b] Cells	CPM ± S.D.	Stimulation[c] Index
C57Bl/6 Spleen	2,918 ± 507	—
C57Bl/6 BM	1,824 ± 303	—
Balb/c Spleen	77,559 ± 9,678	26.6
Balb/c BM	9,472 ± 1,976	3.2
C3H Spleen	79,602 ± 13,829	27.2
C3H BM	7,047 ± 1,880	2.4
DBA Spleen	123,675 ± 13,914	42.4
DBA BM	5,958 ± 1,071	2.0

[a] C57Bl/6 spleen cells were used as the responder population.

[b] Stimulator cells were inactivated by treatment with Mitomycin C as described in Materials and Methods (Section 2.2.5).

[c] Stimulation index refers to the experimental value divided by the control value, which is represented by C57Bl/6 spleen cells.

TABLE 2.5

Stimulation in the MLR by Elutriated BM Fractions[a]

Stimulator[b] Cells	CPM ± S.D.	Stimulation[c] Index
Balb/c Spleen	7,250 ± 1,622	—
C57Bl/6 Spleen	89,808 ± 12,698	12.3
Unf. C57Bl/6 BM	13,486 ± 2,842	1.9
F1 C57Bl/6 BM	45,194 ± 4,670	6.2
F2 C57Bl/6 BM	9,446 ± 1,375	1.3
F3 C57Bl/6 BM	942 ± 173	—

[a] Balb/c spleen cells were used as the responder population.

[b] Stimulator cells were inactivated by treatment with Mitomycin C as described in Materials and Methods (Section 2.2.5).

[c] Stimulation index refers to the experimental value divided by the control value, which is represented by Balb/c spleen cells.

When the elutriated BM cell fractions used in Figure 2.8 were tested for their ability to serve as stimulators in an MLR, F1 was found to contain cells capable of stimulation, albeit to a lesser extent than that of spleen cells (Table 2.5). Stimulation by F2 was the same as the syngeneic control, while F3 cells were totally ineffective at stimulating any response at all, suggesting either a paucity of MHC Class II antigens or the strong suppressive influence of F3.

2.3.7 BM Derived NS Cells Lack Phenotypic Markers Characteristic of Veto or NK/LAK Cells

While the lineage of the cells which mediate murine NS activity is presently unknown, several groups have shown that they are not of B-cell, T-cell, or MØ origin (Duwe and Singhal, 1979; McGarry and Singhal, 1982). However it has been shown that mature NK/LAK cells do possess NS activity in addition to cytotoxic capabilities (Azuma and Kaplan, 1988). Tables 2.6, 2.7, and 2.8 demonstrate that depletion of specific cells from BM did not remove NS activity as tested on PFC, MLR, or Con A responses, respectively. Antibodies directed against markers found on NK/LAK cells such as NK 1.1, ASGM1, IL-2 receptor (p55) and MAC1 were not able to deplete NS activity from BM after complement-mediated lysis. Also, antibodies directed against the Class I Ags Qa2/3 and Qa1/TL did not deplete BM of NS activity after complement-mediated lysis. Furthermore, antibody against the Th cell marker L3T4 also did not abrogate BM derived NS activity (Table 2.6).

2.3.8 Suppressor Activity of BM Cells Cultured in IL-2 Containing Medium

The lymphokine IL-2 has been shown to induce the formation of NK cells from precursors in BM (Koo *et al.*, 1984), and to augment BM NS activity (Holda *et al.*, 1986). To determine whether NS cells in BM

TABLE 2.6

Suppression of Anti-SRBC Responses by Ab Treated BM Derived NS Cells

Treatment	Ratio of Responder:BM Cells[a]	% Suppression[b]
EXP. 1		
C′ Treated	2:1	97
	10:1	75
	50:1	56
Anti-NK 1.1 + C′	2:1	98
	10:1	82
	50:1	53
EXP. 2		
C′ Treated	2:1	83
	10:1	26
	50:1	12
Anti-ASGM1 + C′	2:1	90
	10:1	51
	50:1	26
Anti-MAC1 + C′	2:1	89
	10:1	48
	50:1	—
EXP. 3		
C′ Treated	2:1	77
	10:1	56
	50:1	22
Anti-L3T4 + C′	2:1	83
	10:1	67
	50:1	11
Anti-IL2R + C′	2:1	81
	10:1	61
	50:1	22

[a] 10^7 C57Bl/6 spleen cells + SRBC were cultured with varying doses of C57Bl/6 BM cells.

[b] % suppression was calculated relative to the control values of C57Bl/6 spleen cells + SRBC alone. These values were 13,363 ± 2,076, 731 ± 167, and 1,313 ± 127 PFC/culture for experiments 1,2, and 3, respectively.

TABLE 2.7

Suppression of the MLR by Ab Treated

BM Derived NS Cells

Treatment	Ratio of Responder:BM Cells[a]	% Suppression[b]
EXP. 1		
C′ Treated	4:1	91
	20:1	44
	40:1	18
Anti-NK 1.1 + C′	4:1	93
	20:1	71
	40:1	34
Anti-ASGM1 + C′	4:1	93
	20:1	49
	40:1	22
Anti-MAC1 + C′	4:1	95
	20:1	46
	40:1	36
EXP. 2		
C′ Treated	4:1	62
	20:1	34
	40:1	11
Anti-Qa2,3 + C′	4:1	60
	20:1	18
	40:1	8
Anti-Qa1,TL + C′	4:1	80
	20:1	22
	40:1	20

[a] 2 X 10^5 C57Bl/6 spleen cells + 2 X 10^5 mitomycin treated Balb/c stimulator cells were cultured with varying doses of C57Bl/6 BM cells.

[b] % suppression was calculated relative to control cultures in the absence of BM cells. These values were 140,215 ± 23,267 and 84,743 ± 4,987 CPM in experiments 1 and 2, respectively.

TABLE 2.8

Suppression of Con A Responses by Ab Treated

BM Derived NS Cells

Treatment	Ratio of Responder:BM Cells[a]	% Suppression[b]
EXP. 1		
C′ Treated	1:1	73
	5:1	59
	10:1	22
Anti-NK 1.1 + C′	1:1	81
	5:1	66
	10:1	4
Anti-ASGM1 + C′	1:1	74
	5:1	68
	10:1	17
EXP. 2		
C′ Treated	1:1	60
	5:1	60
	10:1	26
Anti-MAC1 + C′	1:1	68
	5:1	48
	10:1	38
EXP. 3		
C′ Treated	1:1	79
	5:1	51
	10:1	36
Anti-Qa2,3 + C′	1:1	89
	5:1	58
	10:1	53
Anti-Qa1,TL + C′	1:1	80
	5:1	32
	10:1	22

[a] 5×10^5 C57Bl/6 spleen cells + Con A were cultured with varying doses of C57Bl/6 BM cells.

[b] % suppression was calculated relative to the control of C57Bl/6 spleen cells + Con A alone. These values were 23,855 ± 6,011, 57,572 ± 737, and 74,790 ± 8,715 for experiments 1,2, and 3, respectively.

could be expanded in culture, BM cells were plated in the presence of rat Cas, an IL-2 containing supernatant, for a period of up to 6 days. Cultures were assayed at days 2, 4, and 6 for NK activity and at days 2 and 4 for NS activity in the PFC response. In Figure 2.9 A, the cultured BM cells displayed strong NK activity which was maximal from day 4 cultures. The same cells were assayed for NS activity (Fig. 2.9 B), which at the highest dose was significantly greater using day 4 BM as opposed to day 0 or 2 BM. Cell recovery was 42% at day 2, 13% at day 4, and 12% at day 6. C57Bl/6 BM cells cultured for 4 days in complete medium containing human IL-2 were harvested and the kinetics of suppression was determined (Fig. 2.10). The results indicate that cultured BM cells could suppress the MLR when added at either day 0 or day 2. By contrast, fresh BM cells could only suppress the response when added at culture initiation.

2.3.9 NS Activity of Long Term Bone Marrow Cultures (LTBMC)

BM cells from Balb/c mice were cultured in the presence of rat Cas over a long period of time in an attempt to obtain cell lines possessing NS activity (see Material and Methods Section 2.2.12). The cultures went through a crisis period during the first 4 weeks, but after this time a population of large, round, non-adherent cells began to grow at a relatively slow rate. Growth continued until approximately 12 weeks after culture initiation, at which time the cells could no longer be stimulated to grow and died in culture, even after repeated attempts to split the cells into new plates with fresh feeder layers and medium.

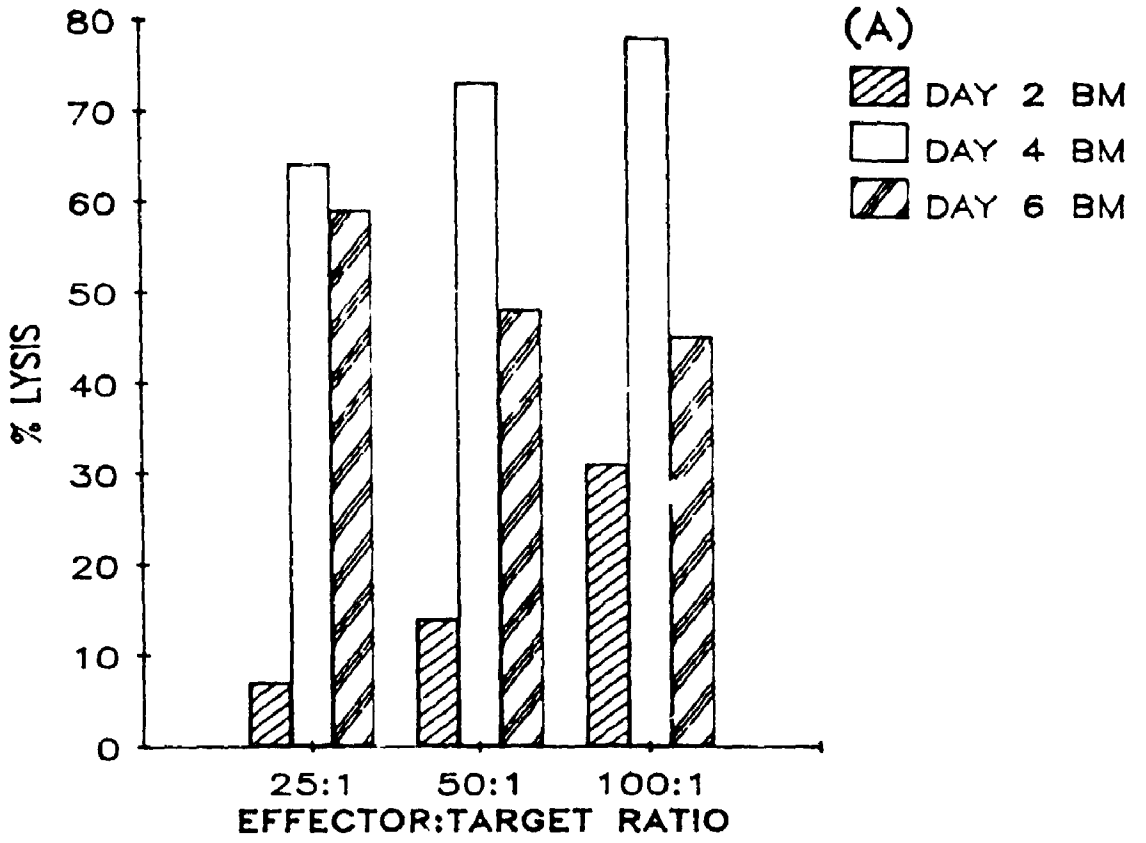
LTBMC were harvested after 12 weeks of culture and were compared with fresh syngeneic BM cells for the ability to suppress both

FIGURE 2.9

BM Cells Cultured in Rat Cas Display NK and NS Activities

(A) C57Bl/6 BM cells cultured in 25% rat Cas were harvested at days 2, 4, and 6 and assayed for NK activity. All values are the means of quadruplicate cultures. Standard deviations were less than 5% of the means in all cases.

(B) C57Bl/6 BM cells cultured in 25% rat Cas were harvested at days 2 and 4 and assayed for NS activity in the PFC assay. Fresh C57Bl/6 BM cells were added as a positive control for NS activity. The positive control of C57Bl/6 spleen cells is represented by the closed circle. The background response of unstimulated spleen cells was 134 ± 30 PFC/culture. Vertical bars represent standard deviation.


(A)
DAY 2 BM
DAY 4 BM
DAY 6 BM
% LYSIS
80
70
60
50
40
30
20
10
0
25:1
50:1
100:1
EFFECTOR:TARGET RATIO

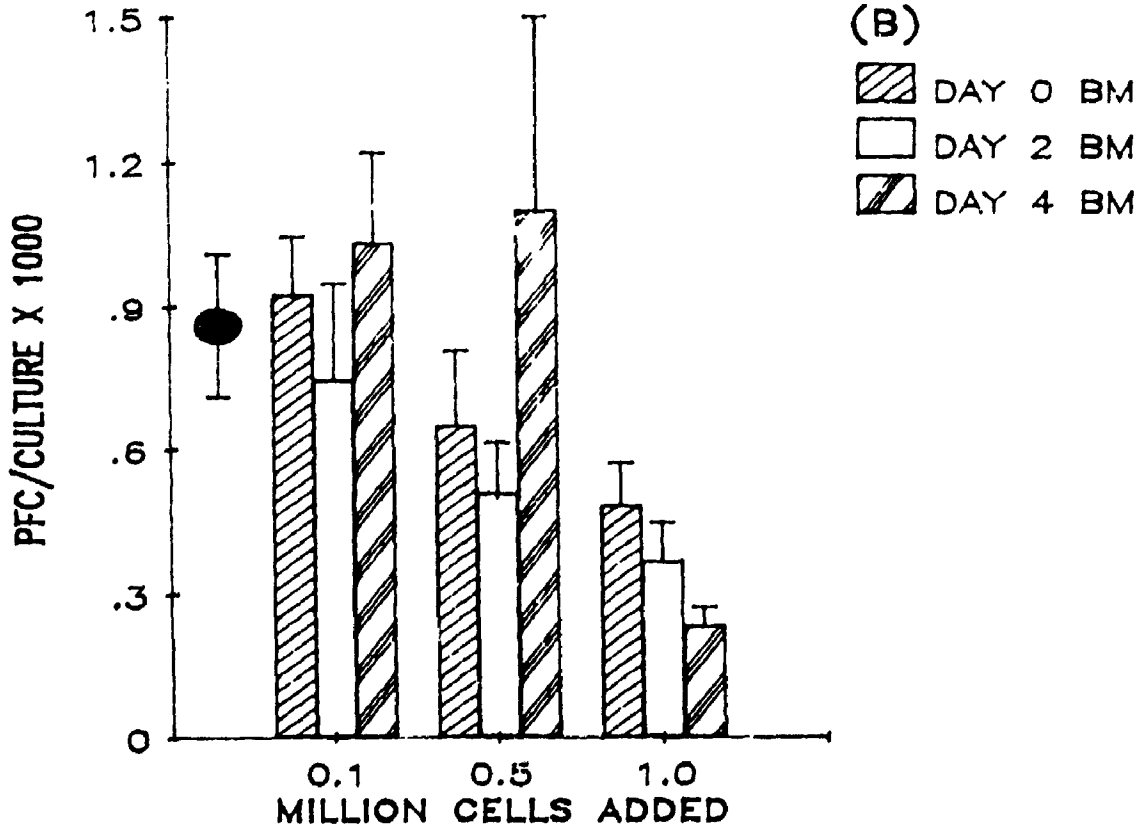
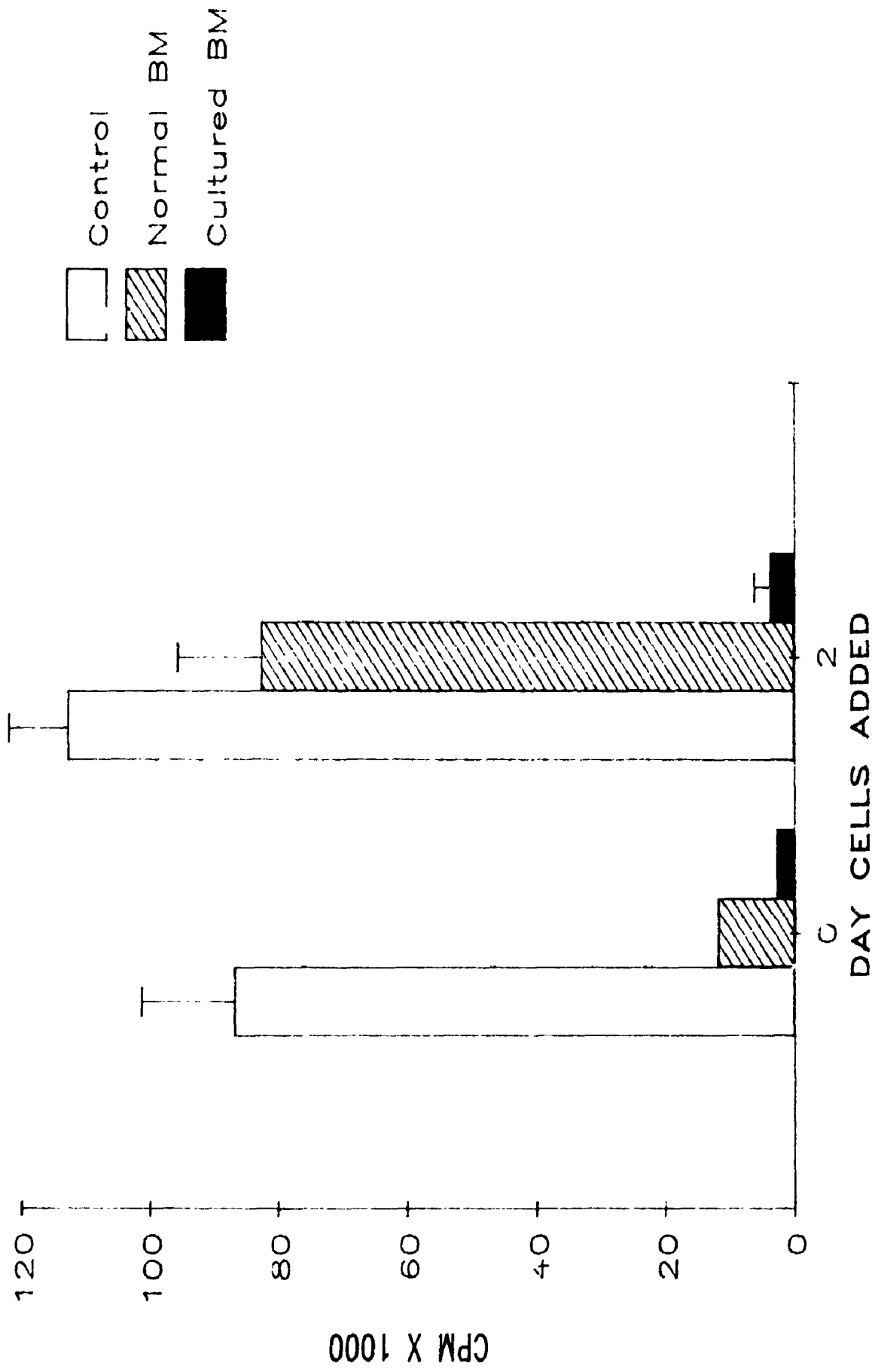

(B)
DAY 0 BM
DAY 2 BM
DAY 4 BM
PFC/CULTURE X 1000
1.5
1.2
.9
.6
.3
0
0.1
0.5
1.0
MILLION CELLS ADDED

FIGURE 2.10

Kinetics of Suppression in the MLR by cultured BM Cells

C57Bl/6 BM cells were cultured for 4 days in complete medium containing human IL-2 (1/100 dilution in culture). Cells were harvested and compared with fresh BM for suppression in a C57Bl/6 vs Balb/c MLR. The cells were added to separate MLR cultures at either day 0 or day 2. The background response of the day 0 MLR was 5,575 ± 2,572 CPM, and for the day 2 MLR was 8,332 ± 3,298 CPM. Vertical bars represent standard deviation.


Control
Normal BM
Cultured BM
CPM X 1000
120
100
80
60
40
20
0
0
2
DAY CELLS ADDED

the PFC response (Fig. 2.11 A) and the MLR (Fig. 2.11 B). As shown in both panels of Figure 2.11, LTBMC suppressed the PFC response and MLR with much greater potency than fresh BM when equal numbers of each type of cells were added to culture. All BM cultures generated in such a way possessed strong NS activity.

In addition the LTBMC were also assayed for cytotoxicity against the NK sensitive cell line YAC-1. As seen in Table 2.9, LTBMC possessed strong NK activity at all three effector:target ratios tested. By contrast, NK activity of fresh spleen was greatest at the E:T ratio of 100:1, and diminished at 50:1 and 25:1. However, the NK activity of LTBMC at an E:T ratio of 25:1 was strikingly higher than that of fresh spleen at 100:1 (58% vs 44% lysis, respectively). By comparison, fresh BM cells were not effective mediators of NK activity even at an E:T ratio of 100:1 (3% lysis).

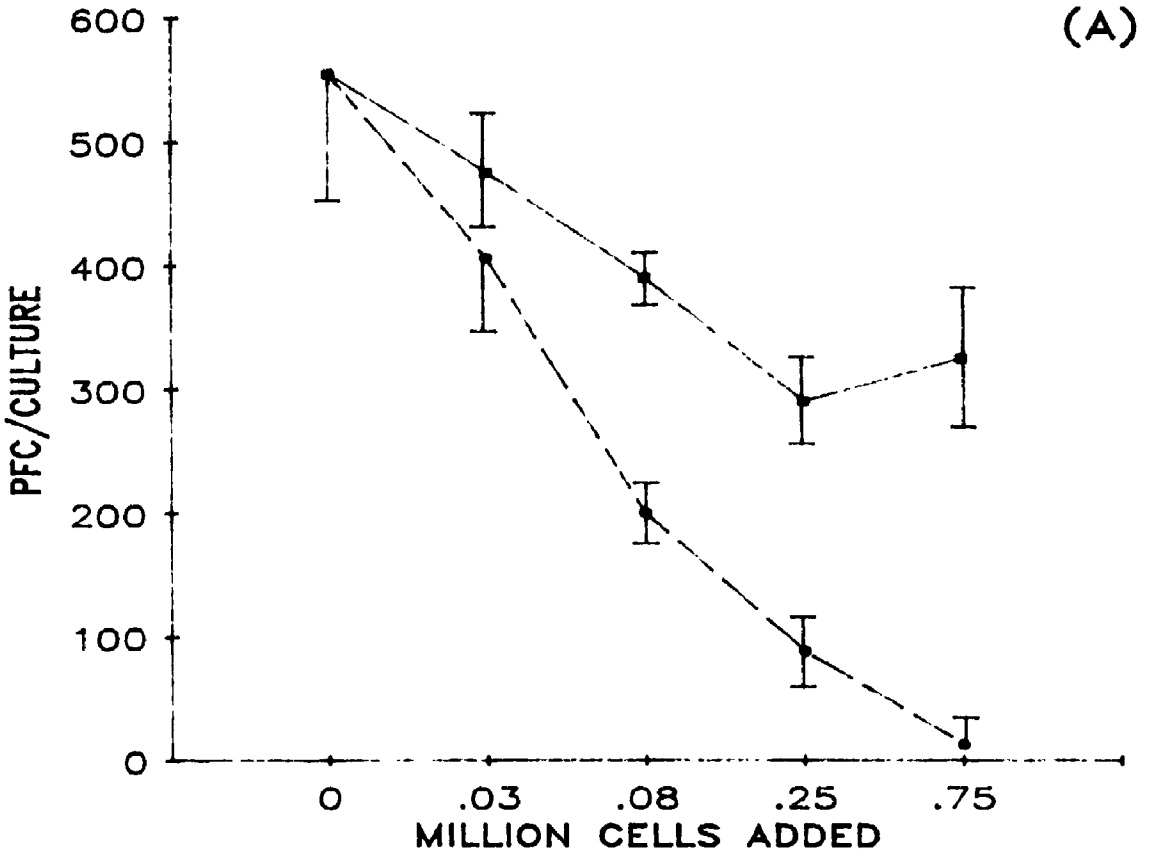
To determine whether LTBMC constitutively secreted a soluble suppressor factor, supernatants from cultures of actively growing LTBMC were harvested and subjected to sequential Amicon ultrafiltration to yield 3 MW fractions: >50 kDa, 10-50 kDa, and <10 kDa in size. All three fractions were tested in a PFC assay for suppressor activity. As a comparison, separate cultures were treated with Bone Marrow Derived Suppressor Factor (BDSF), which is constitutively produced from BM cells cultured in serum-free medium and suppresses the PFC and MLR responses similar to BM NS cells (see Chapter 3). Figure 2.12 demonstrates that no suppressor activity was present in any of the fractions tested, in comparison to BDSF. In fact, the high molecular weight fractions significantly enhanced the PFC response in a dose-dependent manner.

FIGURE 2.11

Suppression of the PFC Response and MLR by BM and LTBMC

(A) 1×10^7 Balb/c spleen cells were cultured with SRBC and varying doses of either Balb/c BM (■, solid line) or LTBMC (●, broken line) generated from Balb/c BM. The background response of Balb/c spleen cells in the absence of SRBC was 21 ± 29 PFC/culture.

(B) 2×10^5 Balb/c spleen cells were cultured with 2×10^5 mitomycin C treated C57 spleen cells with varying doses of either Balb/c BM (■, solid line) or LTBMC (●, broken line) from Balb/c BM. The background proliferation of unstimulated Balb/c responders was 14,364 ± 3,255 CPM. In both panels vertical bars represent standard deviation.


(A)
600
500
400
300
200
100
0
PFC/CULTURE
0
.03
.08
.25
.75
MILLION CELLS ADDED

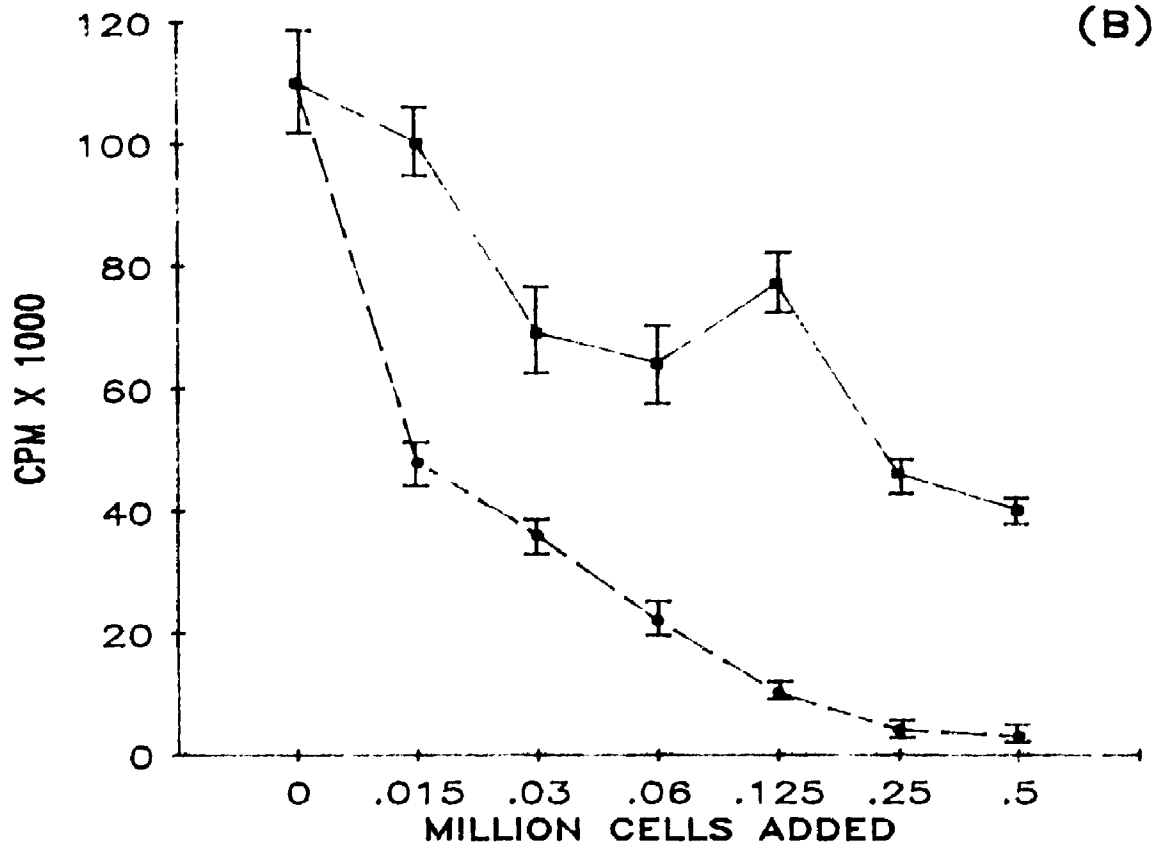

(B)
120
100
80
60
40
20
0
CPM X 1000
0
.015
.03
.06
.125
.25
.5
MILLION CELLS ADDED

TABLE 2.9

NK Activity of Spleen, Bone Marrow, and LTBMC

Responder[a] Cells	Responder:Target Ratio	% Lysis[b]
Spleen	100:1	44
	50:1	19
	25:1	17
Bone Marrow	100:1	3
	50:1	0
	25:1	0
LTBMC	100:1	59
	50:1	62
	25:1	58

[a] All cells used in the assay were of Balb/c origin. The details of the NK assay are outlined in Materials and Methods section 2.2.13.

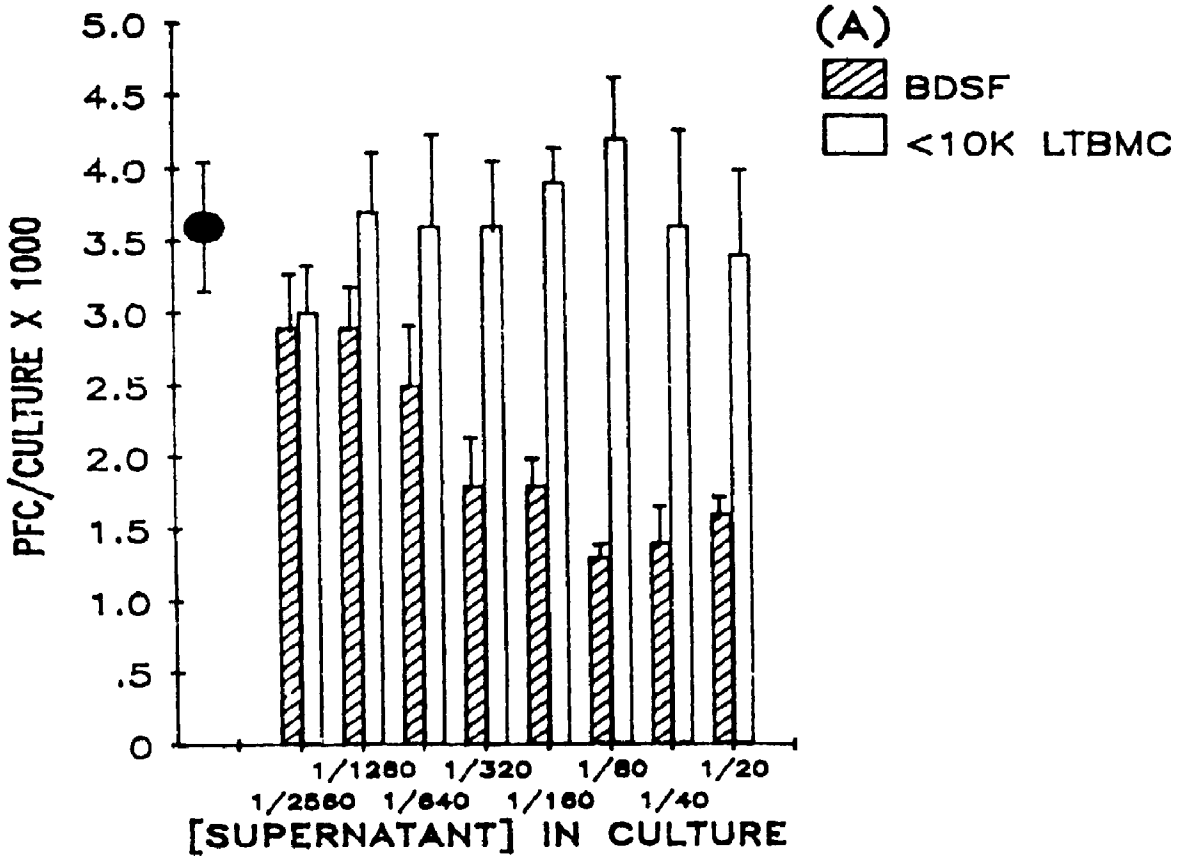
[b] % lysis was calculated as follows:

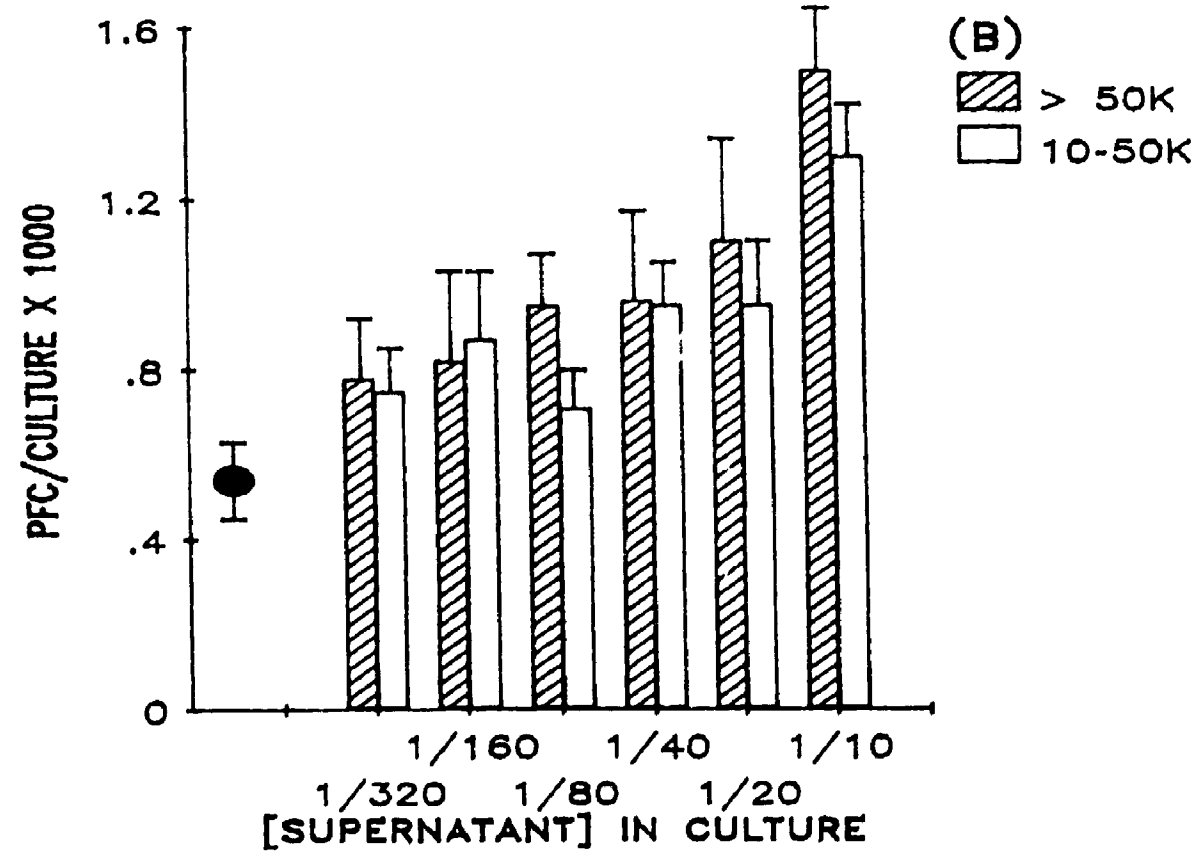
$$\% \text{ Lysis} = \frac{\text{Experimental} - \text{Spontaneous Release}}{\text{Total Release} - \text{Spontaneous Release}} \times 100$$

FIGURE 2.12

LTBMC do not Constitutively Secrete a Soluble Suppressive Factor

Supernatants from actively growing LTBMC were harvested and fractionated by Amicon ultrafiltration. Concentrated fractions, consisting of MW < 10kD, 10-50kD, and > 50kD, were tested for suppressive activity in a PFC assay. In (A), BDSF, prepared from 48 hr cultures of Balb/c BM cells in serum-free medium, was added as a positive control for suppression. In both (A) and (B), the positive control of Balb/c spleen cells is represented by the closed circle. The background responses of unstimulated cells was 38 ± 35 PFC/culture for (A), and 75 ± 27 PFC/culture for (B). In both panels vertical bars represent standard deviation.


(A)
BDSF
<10K LTBMC
PFC/CULTURE X 1000
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0
1/1280
1/320
1/80
1/20
1/2560
1/640
1/160
1/40
[SUPERNATANT] IN CULTURE


(B)
> 50K
10-50K
PFC/CULTURE X 1000
1.6
1.2
.8
.4
0
1/160
1/40
1/10
1/320
1/80
1/20
[SUPERNATANT] IN CULTURE

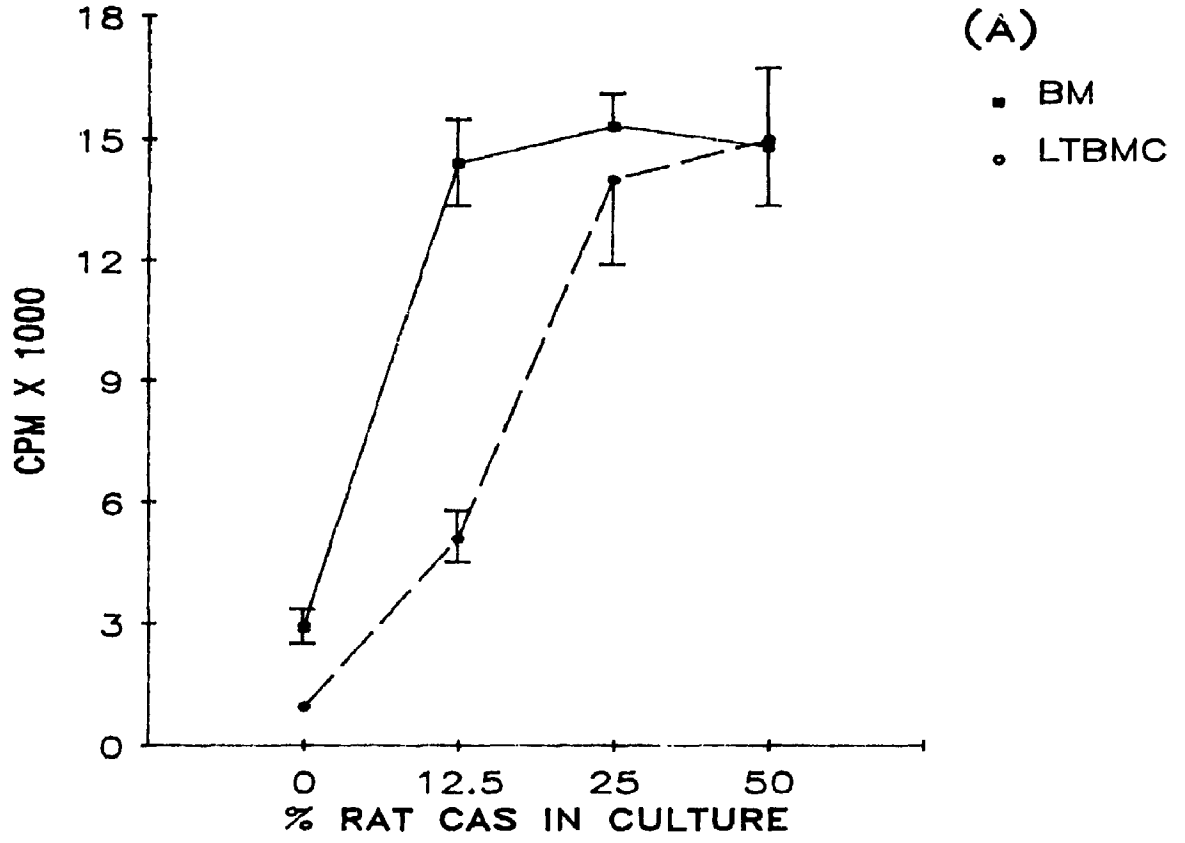
To determine whether the LTBMC may have been suppressor T-cells which expanded in culture due to contaminating amounts of Con A, LTBMC were stimulated with either rat Cas or Con A in a proliferation assay. The results (Fig. 2.13) demonstrate that while the cells proliferated in the presence of rat Cas, there was no proliferation in the presence of doses of Con A known to be mitogenic for splenic lymphocytes. Therefore it is unlikely that LTBMC contain suppressor T-cells which could potentially arise in the presence of Con A and IL-2.

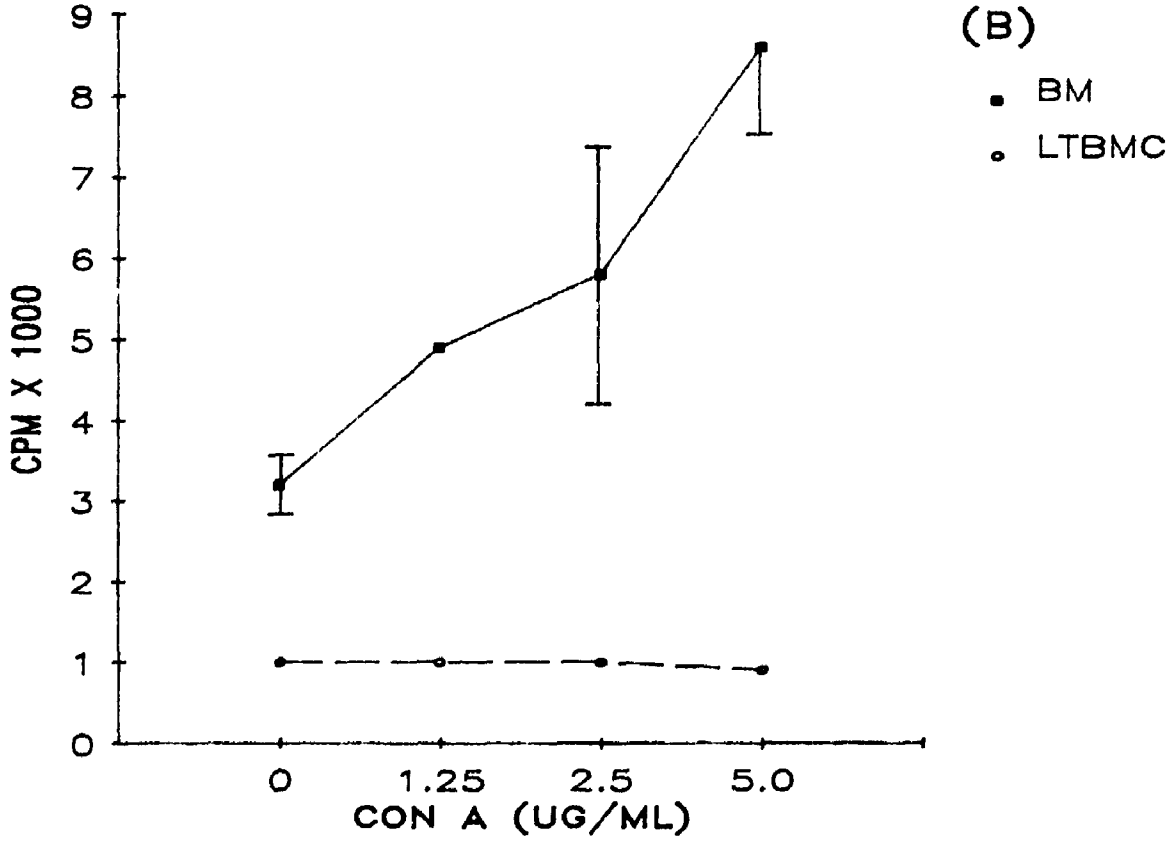
FIGURE 2.13

LTBMC Proliferate in the Presence of Rat Cas, but not Con A

(A) 5 X 10^5 Balb/c BM or Balb/c LTBMC cells were cultured with increasing doses of rat Cas in 96-well flat-bottomed plates. After 48 hr cultures were pulsed with ^{3}H-TdR, and harvested 24 hr later to determine ^{3}H-TdR uptake.

(B) The culture conditions were the same as in (A), substituting Con A for rat Cas. In both panels vertical bars represent standard deviation.


(A)
BM
LTBMC
CPM X 1000
18
15
12
9
6
3
0
0
12.5
25
50
% RAT CAS IN CULTURE


(B)
BM
LTBMC
CPM X 1000
9
8
7
6
5
4
3
2
1
0
0
1.25
2.5
5.0
CON A (UG/ML)

2.4 DISCUSSION

Natural suppression describes the ability of an LGL-like population of null cells to suppress immune responses without requiring specific priming or MHC restriction. This laboratory had previously described this activity derived from both murine (Singhal et al., 1972; Duwe and Singhal, 1979) and human (Bains et al., 1982; Mortari et al., 1986) BM cells. The results in this study support these observations, and confirm the findings of others that BM derived NS cells suppress mitogen activation (Holda et al., 1986) and mixed lymphocyte responses (Dorshkind et al., 1980; Sugiura et al., 1988).

It was previously shown that suppression of primary IgM Ab responses by BM cells operated at the inductive phase of the response (Duwe and Singhal, 1979; Bains et al., 1982). The results here support this finding in the MLR, and furthermore demonstrate that suppression was not the result of a shift in the kinetics of the peak response. The results also support the hypothesis that NS cells block events associated with the activation of antigen-reactive cells, as Duwe and Singhal (1979) and McGarry and Singhal (1982) had previously demonstrated.

A unique feature of BM NS cells is their ability to function without a requirement for antigenic specificity or MHC restriction. However, while unstimulated responses were unaffected by BM NS cells (Duwe and Singhal, 1979), antigen stimulation was necessary to direct the suppressive signal. The finding that suppression in the MLR was not MHC restricted is supported by similar results from others in both the MLR (Dorshkind and Rosse, 1982) and Ab response (McGarry and

Singhal, 1982; Bains et al., 1982). The lack of MHC restriction also distinguishes BM NS cells from the Veto cells first described by Miller (1980) and Muraoka and Miller (1980). Veto activity is associated with Thy-1$^-$ BM derived cells which have the potential to differentiate into Thy-1$^+$ cells under conditions which promote T-cell growth (Muraoka and Miller, 1980). Both the Thy-1$^-$ and Thy-1$^+$ cells have been shown to suppress proliferation and CTL generation in mixed lymphocyte cultures. Miller (1980) postulated that veto cells may initially exist as precursors in BM which seed to the thymus and inactivate self-reactive CTL. However, veto activity was MHC restricted in that suppression was dependent upon MHC matching between the BM veto cells and the stimulator cells used in the MLC. As shown here in the MLR, BM NS activity was not MHC restricted since BM NS cells syngeneic to the responder or allogeneic to both responder and stimulator suppressed the MLR with equal potency. The findings of Miller (1980) imply the presence of a suppressor population in BM distinct from NS activity.

The mechanism of suppression by BM NS cells has been the focus of study by many labs, and this laboratory has postulated that NS activity is mediated by a soluble lipid mediator termed Bone Marrow Derived Suppressor Factor (BDSF) which is discussed in detail in Chapter 3. Choi et al. (1988) have shown that murine NS activity could be partially inhibited by the prostaglandin synthetase inhibitor indomethacin. However, in the present study suppression of both the PFC and MLR responses by BM were unaffected by the incorporation of indomethacin into the cultures. Suppression associated with BM derived MØ was shown to be mediated via the release of inhibitory prostaglandins (Bennett and Mitchell, 1980). Piguet et al. (1981) also

demonstrated that neonatal spleen contained a population of monocytic cells which could suppress Ab responses to SRBC in culture, and this activity was inhibited by indomethacin. However, Oseroff et al. (1984) have shown that NS cells from neonatal spleen or spleens from TLI-treated animals were not affected by the presence of indomethacin when suppressing the MLR. Clearly, the results here demonstrated that BM NS activity on either MLR or Ab responses acted via a prostaglandin-independent mechanism.

NS activity, as indicated in the paragraph above, is not solely associated with BM. Other sites of NS activity include the spleens of neonatal mice (Jadus and Parkman, 1986; Oseroff et al., 1984), the spleens of mice undergoing lymphoid repopulation induced by TLI (reviewed by Strober, 1984), the spleens of mice subjected to cyclophosphamide (Segre et al., 1985) and strontium-89 treatments (Levy et al., 1981), the spleens of CFA treated mice (Sadelain et al., 1989), and the spleens of mice undergoing chronic GVHD (Maier et al., 1985). All of these sites represent areas of active hematopoiesis, and NS activity cannot be found in secondary lymphoid organs containing predominantly mature lymphocytes (Strober, 1984; Bains et al., 1982). The results here also indicate that NS activity was not associated with mature lymphocytes, since normal levels of NS activity are present in SCID BM when compared with their immunocompetent littermate C.B-17. However, this does not rule out the possibility that lymphoid progenitor cells, which have not yet undergone gene rearrangements encoding the antigen-receptor, may contain NS activity. Sugiura et al. (1988) have shown that BM NS activity is associated with a population of cells expressing receptors for the lectin wheat germ agglutinin, and

coincidentally the same population is enriched in cells responsible for CFU-S formation. Till and McCulloch (1961) demonstrated that repopulation of irradiated mice with syngeneic BM cells resulted in splenic colony formation in 2 waves, in which colonies examined at day 7 were derived from committed erythrocyte progenitors, while colonies examined at days 10-12 were mainly myeloid in morphology and containing cells which could generate CFU-S upon transfer to a secondary recipient. When NS activity was examined here in lethally irradiated mice which had been reconstituted with syngeneic BM, it was shown that day 10 spleens could suppress a PFC response. A similar result has recently been reported by Sykes et al. (1990). The presence of NS activity in spleens of BM reconstituted mice is consistent with the potential designation of NS cells as immature members of the myeloid lineage (Corvese et al., 1980). Even more interesting is the hypothesis of Noga et al. (1988) that NS cells may be stem cell progenitors possessing both immunoregulatory and autoregulatory functions. However, Sykes et al. (1990) have shown that depletion of Sca-1^+ pluripotent stem cells from BM does not affect NS activity. Still, this does not rule out the possibility that Sca-1^- stem cells exist which possess NS function.

The separation of NS activity in murine BM has been achieved in 2 ways: i) Adherence to the lectin wheat germ agglutinin (Sugiura et al., 1988) and ii) Separation based on size (Gorczynski and McRae, 1977; Duwe and Singhal, 1979; Dorshkind and Rosse, 1982; Holda et al., 1986; Sugiura et al., 1988). More recently, Mortari and Singhal (1988) had shown that NS activity could be enriched in human BM by counterflow centrifugal elutriation (CCE). In this study, CCE was used to enrich

NS activity from murine BM. The results demonstrated that two immunoregulatory activities were present. One was an immunoenhancing activity present in a fraction of small, high density cells of lymphocytic morphology (discussed in Chapter 4). The other was NS activity, residing in a fraction of large, low density cells of mixed myeloid and blast cell morphology. On a cell per cell basis NS activity was greatly enriched from whole BM, especially in the MLR. Baccarini et al. (1986) showed that Percoll fractions of low density are also effective at enriching for NK cells from spleens of adult animals. However, NK activity was absent in sites of active hematopoiesis, such as described by Mortari et al. (1986) in human BM, Oseroff et al. (1984) in murine neonatal spleen, and Yung et al. (1985) in murine long term (Dexter) BM cultures. The fraction enriched in NS activity also had the greatest proliferative rate of BM cells, which agreed with previous findings demonstrating that BM NS activity was sensitive to treatments which inhibited cellular proliferation (Singhal et al., 1972; Fuchs et al., 1978; Corvese et al., 1980; Bains et al., 1982; and Sugiura et al., 1988).

Muraoka and Miller (1980) demonstrated that BM cells were a poor source of stimulators in an MLR. This finding was confirmed here in that stimulation due to BM cells was poor compared to spleen, regardless of the strain combinations used. However, when elutriated BM fractions were tested as stimulators in an MLR, significant stimulation was observed against the lymphocyte-rich fraction. This was in contrast to the fraction which contained NS activity, in which stimulation was absent, and the level of proliferation was well below background. Therefore, while BM contains cells which can induce a

mixed lymphocyte response, none may be seen due to the overriding suppressive activity of NS cells. This is especially relevant to clinical situations involving BM transplantation, where NS cells may be important in preventing graft-vs-host reactions (Strober, 1984) and host-vs-graft reactions against MHC antigens of the bone marrow inoculum. During allogeneic BM transplantation, a dichotomy exists such that elimination of lymphocytic cells (especially T cells) will prevent GVHD (Noga et al., 1988; Parkman, 1989); however removal of donor T cells resulted in reduced stem cell capacity of marrow, suggesting that T cells aid in regenerating hematopoietic tissue (Thomas, 1985; Martin et al., 1985). Therefore devising means of stimulating NS activity during BM transplantation may prevent undesirable complications associated with immune activation. To this end Sykes et al. (1988) have shown that co-injection of T-cell depleted BM cells syngeneic to the host facilitated allogeneic BM chimerism.

Although NS cells have not been placed into a specific cell lineage, it has been suggested that they may belong to an LGL-regulatory family whose members mediate NK and NC effector functions (Maier et al., 1986). Like NS cells, NK and NC cells do not require antigenic priming and are not MHC restricted in their action. Azuma and Kaplan (1988) have shown that NS and Veto activity can be mediated by BM cells cultured in Con A-conditioned medium. These cultured cells are cytolytic for the NK-sensitive target YAC-1, and clones of cells which were noncytolytic were apparently defective in their ability to prevent allogeneic BM chimerism (Azuma et al., 1989). The cells resembled mature NK/LAK cells expressing the surface markers Qa2, Qa5, Thy-1, ASGM1, and NK1.1. The data in this study demonstrated that

complement-dependent lysis of fresh BM using antibodies against the surface antigens Qa2/3, Qa1/TL, ASGM1, NK1.1, MAC-1, IL-2R, and L3T4 did not deplete BM of endogenous NS activity. Although Qa2 has been shown to be a potential marker for precursors of NK/LAK cells as generated by long term BM culture (Azuma and Kaplan, 1988), antibody directed against Qa2 had no effect on endogenous BM NS activity. The lack of effect of antibody against Qa1/TL surface antigens distinguishes BM NS cells from nonspecific suppressor cells of T-dependent Ab responses found in the spleens of TLI-treated mice (Strober, 1984). Antibody directed against the T-helper cell marker L3T4 did not abrogate BM derived NS activity suggesting also that suppression was not mediated by potential $CD4^+$ Class II restricted T-cells as proposed by Lanzavecchia (1989).

The culture of BM cells for 3-7 days in CAS-supplemented medium resulted in the generation of cell populations possessing potent NS and NK/LAK activity. This was in agreement with similar studies by Azuma and Kaplan (1988) that NK/LAK cells mediated NS and Veto activities. Two groups demonstrated that the generation of NK cells from BM in short-term culture was mainly dependent upon IL-2 and to some extent IFNs (Koo and Manyak, 1986; Migliorati et al., 1988). Holda et al. (1986) had also shown that NS activity from BM and from the spleens of mice undergoing chronic GVHD could be augmented by IL-2 and especially IFN-gamma. Thus, one could speculate that activation of NS and NK cells may occur via common mechanisms. Interestingly, kinetic studies here indicated that while fresh BM only suppressed the MLR at culture initiation (day 0), BM cells cultured in human IL-2 for 4 days suppressed the MLR when added at day 0 or day 2 of the response. Since

the MLR is essentially an IL-2 driven response (Ilonen and Karttunen, 1984), it is possible that BM NS cells added at culture initiation may differentiate to NK/LAK type cells in the MLR culture. Whether this represents a mechanism by which BM NS cells, or perhaps an alternate population, suppresses immune responses remains to be seen.

Strober's lab had used essentially the same technique to generate long term cultures and clones of NS cells (lacking NK activity) from spleens of neonatal mice and spleens of TLI-treated mice (Oseroff et al., 1984; Schwadron et al., 1985; Strober et al., 1989). Interestingly, these cells were $CD3^+$ $CD4^-$ $CD8^-$, and expressed alpha/beta TcR on their surface. In an attempt to derive long-term NS clones, BM cells were cultured in rat Cas as Strober had used in his derivation of NS clones. The long-term BM cultures (or LTBMC) possessed potent immunosuppressive activity in both PFC and MLR assays when comparing them with fresh BM. However unlike the NS cell lines generated by Strober, the LTBMC demonstrated strong NK activity as compared to syngeneic spleen cells and BM. The ability of NK cells generated in LTBMC to suppress the PFC and MLR responses was not surprising in view of the large body of evidence implicating the immunoregulatory function of NK cells (see Section 1.3.1 for a review of the regulatory properties of NK cells). In several attempts to generate NS activity in LTBMC, culture of BM cells in rat Cas consistently resulted in the generation of cultures possessing NK activity. The fact that cell survival was limited to a maximum of 12 weeks in culture may have represented a technical problem, despite all attempts to maintain cultures for a longer time period and to clone cells by limiting dilution.

The consistent generation of NK cells possessing potent NS activity prompted a potential lineage relationship between NS and NK cells. Clearly the LTBMC were not T-cells as demonstrated by their inability to proliferate in the presence of Con A. Mortari et al. (1986) had shown that human BM NS cells were non-lytic, HNK-1^+ (CD57), OKM1^+, OKT3^- and SSEA-1^+ which fit into the maturation scheme of NK cells as described by Abo et al. (1984). HNK-1^+ OKM-1^+ cells which could suppress T cell proliferation and B cell Ig production in humans had been described by Tilden et al. (1983). A potential link between NK and NS activities was made by McGarry (1981) who demonstrated that beige mice, which lack NK activity due to a stem cell defect (Roder and Duwe, 1979), also had reduced BM NS activity. Levy et al. (1981) had suggested that the presence of NS cells in ^{89}Sr-treated mice is consistent with the possibility that these cells represent early precursors to NK cells. Furthermore Pan et al. (1986) reported that decreased NK activity in lupus-prone mice expressing the lpr gene correlated with increased autoantibody responses in these animals, suggesting a natural immunoregulatory function for NK cells.

While NS and NK cells may be related in lineage and function, the mechanism of suppression by NS and NK appears to be different. This lab has reported that BM produces a soluble lipid mediator of low molecular weight called BDSF (see Chapter 3) which mediates BM NS activity. NK cells are proposed to act via a cytotoxic effect on the responding cells, potentially via the NK cytotoxic factor, NKCF (Targan et al., 1985). NK cells also produce IFN-gamma (Heron et al., 1976; Kashahara et al., 1983) which has been shown to have anti-proliferative effects and is a potent inhibitor of hematopoiesis (Broxmeyer et al.,

1983; Broxmeyer et al., 1985; Raefsky et al., 1985). In this study, supernatants from actively growing cultures of LTBMC were fractionated by Amicon ultrafiltration and were found to contain no suppressive moiety capable of mediating suppressor activity, suggesting a requirement for cell-cell contact between the responder and potential NK suppressor cells from long-term cultures.

Janeway (1989) has proposed that NK cells may represent the primitive precursor of the CTL, such that acquisition of clonally distributed receptors on NK cells may have resulted in the ability to distinguish self from non-self. This is supported by the similarity of cytolytic effector mechanisms which they possess. However, unlike present day CTL, NK cell recognition of its target is not restricted, suggesting that NK cells possess non-clonally distributed recognitive mechanisms. Since NS cells are not restricted in a clonal fashion, one may speculate that NS, in an analogous fashion to NK, may represent a primordial suppressor mechanism. It is possible that NS cells have co-evolved with other non-specific effector populations, and retain the ability to function in situations where Ag-specific regulatory mechanisms are inoperative.

CHAPTER 3

IMMUNOLOGICAL SUPPRESSION BY BONE MARROW DERIVED SUPPRESSOR FACTOR, BDSF

3.1 INTRODUCTION

The production of soluble mediators by cells of the immune system is well recognized as a mechanism by which positive and negative regulatory signals can be delivered to the appropriate target. Initial work in this laboratory by Duwe and Singhal (1978) demonstrated the production of two soluble factors released by bone marrow cells. This thesis deals with one of those factors, termed bone marrow derived suppressor factor, or BDSF. The studies outlined in this chapter describe further the activities associated with BDSF and define a potential mechanism of action for this mediator. The activities and biochemical nature of BDSF indicate that it is a unique molecule which is naturally produced in the BM. However, consistent with the inherent complexity of the immune system, several other distinct regulatory molecules have also been described which inhibit growth and activation associated with immune function. The unique nature of many of these agents is that they are antigen nonspecific and genetically unrestricted. The rest of this section will be devoted to a review of previous work on BDSF, and several related immunosuppressive molecules.

3.1.1 Bone Marrow Derived Suppressor Factor (BDSF)

Duwe and Singhal (1978) initially demonstrated that suppression of the antibody response in vitro by murine BM cells could occur across cell impermeable membranes, suggesting the elaboration of a soluble mediator of suppression. The fractionation of bone marrow cell conditioned medium revealed the presence of suppressive activity in a preparation of 1-10 kDa in size. They called this material bone marrow

suppressor factor, or B-SF. To avoid confusion with B-cell stimulatory factor (BSF, now IL-4), the designation was changed to BDSF (Mortari and Singhal, 1988), and will be referred to as such for the rest of this thesis. The production of BDSF was observed in several different strains of mice, and suppression was not MHC restricted. BDSF was produced not only by whole BM but also the velocity sedimentation enriched suppressor cell fraction in BM as described by Duwe and Singhal (1979a).

Duwe and Singhal (1978) demonstrated that BDSF suppressed in vitro and in vivo Ab responses to SRBC. BDSF had no effect on mitogen-induced proliferation of spleen cells, but was itself mitogenic for both spleen and BM cells. The suppressive activity of BDSF was maximal when added at culture initiation as opposed to 48 hr later. McGarry et al. (1982) demonstrated that BDSF must be present at least during the first 8-12 hr of culture, suggesting early activation events to be the target of suppression.

The physical properties of BDSF were also examined by McGarry et al. (1982) and revealed a more restricted MW range of 1-3.5 kDa as determined by sequential dialysis. Treatment of BDSF preparations with trypsin, neuraminidase, and RNAse all had no effect on suppressive activity. It was eventually determined that BDSF was a glycolipid, for several reasons. Firstly, BDSF was heat stable, even to boiling. Boiled preparations of BDSF formed a precipitate, which when redissolved in medium was suppressive. Secondly, either the boiled BDSF precipitate or lyophilized BDSF were both soluble in chloroform:methanol (1:1) and were strongly carbohydrate positive as determined by the Mollisch test and staining of TLC plated material

with alpha-naphthol. Thirdly, scrapings of C:M dissolved BDSF run on TLC plates could be eluted and were shown to be suppressive in culture. Although BDSF contained no sialic acid, as determined by resorcinol/HCl staining, its physical properties most closely resembled those of gangliosides, which contain sialic acid.

Human bone marrow cells have also been shown to produce BDSF, which is similarly obtained in a MW fraction of between 1-10 kDa after ultrafiltration. Bains et al. (1986) examined the role of human BDSF in regulating T-lymphoid colony formation. BDSF suppressed T-colony formation of both tonsillar and BM derived precursors in the presence of T-cell conditioned medium. BDSF inhibited colony formation by both E_R^- and E_R^+ inocula, and maximal suppressive activity occurred prior to mitogenic activation by T-cell conditioned medium.

Mortari and Singhal (1988) identified the cells which produced BDSF from human BM as large, low density cells which expressed the surface marker HNK-1. They demonstrated that human BDSF could suppress both human in vitro Ab responses and Con A-induced proliferation; the target for suppression in these systems was resident in the E_R^- population of responding cells. Like murine BDSF, human factor acted early during induction of the response and did not affect cell viability. Physically, human BDSF was found to be less than 1.5 kDa MW and was purified in the lipid fraction of Amicon prepared BM supernatants. Production of the factor was insensitive to indomethacin treatment, indicating that it was unlikely to be one of the prostaglandins.

Weingust et al. (1989) examined the potential of human BDSF in regulating both normal Ab and auto-Ab responses. They showed that BDSF

suppressed total IgM responses of peripheral blood and tonsillar lymphocytes stimulated with the polyclonal activators EBV and PWM. Human BDSF was also able to block production of rheumatoid factor (RF) from normal and rheumatoid PBL stimulated with the same mitogens. These results suggest a potential role for BDSF in preventing inadvertent activation of autoantibody-producing cells in the BM compartment.

3.1.2 Gangliosides

Gangliosides are sialic-acid containing glycosphingolipids which are commonly found associated with either the plasma membrane or with lipoproteins in serum (Marcus, 1984). Gangliosides were first reported to have immunoregulatory function by Miller and Esselman (1975) who demonstrated that murine brain gangliosides could inhibit the anti-SRBC response of spleen cells in vitro. The regulatory role of gangliosides has been extended to include suppression of mitogen-responses (Lengle et al., 1979; Whisler and Yates, 1980; Ladisch et al., 1983; and Marcus et al., 1987), antigen-induced proliferation (Ladisch et al., 1983; Ladisch et al., 1984), alloantigen-induced proliferation (Whisler and Yates, 1980; Ladisch et al., 1983), and NK-mediated cytotoxicity (Bergelson et al., 1989).

Although Miller and Esselman (1975) had initially demonstrated that the target of suppression in the Ab response was a B-cell, recent evidence from several groups has also implicated T-cells, MØ, and NK cells as potential targets. Similarly, the same group reported that gangliosides acted both early and late; however, much evidence has accumulated to suggest that early events in activation are

preferentially inhibited. For example, it was demonstrated that gangliosides could inhibit RNA and protein synthesis, DNA synthesis, and Ca^{++} flux following stimulation with Con A or the Ca^{++} ionophore A23817 (Marcus, 1984). Further work demonstrated more definitively that treatment with gangliosides resulted in accumulation of cells in the G_0/G_1 phase of the cell cycle (Marcus et al., 1987). This suggested that gangliosides were inhibiting transition from G_0/G_1 to the S phase of the cell cycle.

The ability of gangliosides to inhibit cell cycle progression can be linked to observations that gangliosides inhibit the proliferative signal mediated by IL-2. The interaction of antigen- or mitogen-activated T cells with IL-2 allows for transition through G_1 and into the S phase of the cell cycle (Bettens et al., 1984). Merritt et al. (1984) demonstrated that bovine brain gangliosides inhibited proliferation of the IL-2 driven murine cell line CT-6, and that this effect was reversible if gangliosides were removed. Robb (1986) confirmed these results by demonstrating that gangliosides inhibited IL-2 dependent growth of the murine cell line HT-2 and of 5 day old human PHA lymphoblasts, which could be reversed by the addition of exogenous IL-2. He also demonstrated by binding studies that gangliosides prevented the interaction of IL-2 with the IL-2 receptor by binding directly to IL-2. There was no apparent affect on the IL-2R of the responding cells. Marcus et al. (1987) similarly demonstrated that during the Con A response gangliosides inhibited interaction of IL-2 with IL-2R while not affecting expression of IL-2R on responding cells.

Along these lines Offner and Vandenbark (1985) demonstrated

that gangliosides could inhibit the *in vivo* transfer of EAE in rats by an encephalitogenic T-cell line which reacts with guinea pig MBP. Furthermore, IL-2 driven proliferation of this line was inhibited *in vitro*, as was binding of an anti-CD4 monoclonal antibody to the cells. Further work by the same group (Offner *et al.*, 1987) demonstrated that gangliosides prevented binding of anti-CD4 reagents to murine, rat, and human T-cells, while not affecting other T-cell markers. This was due to a rapid modulation of CD4 from the cell surface, which regenerated over a period of 24 hr when cells were washed free of gangliosides. They speculated that gangliosides interfered with activation via the CD4 molecule. This type of inhibition could potentially affect CD4-dependent signal transduction mediated via the CD4 associated tyrosine kinase, **lck**, a member of the **src** oncogene family (Vega *et al.*, 1990).

3.1.3 Fatty Acids and Their Metabolites

The ability of fatty acids to regulate immune responses is typically mediated by metabolic end products, namely the prostaglandins and leukotrienes. Both of these are derived from 20-carbon atom fatty acids, primarily arachidonic acid. The derivation of arachidonic acid requires the liberation from membrane phospholipids after interaction with membrane phospholipases A_2 and C (Meade and Mertin, 1978; Rola-Pleszczynski, 1985). Depending on the cell type and source of stimulation, arachidonic acid undergoes oxidation and cyclization to yield either prostaglandins or leukotrienes. This requires the action of the enzymes cyclooxygenase or lipoxygenase, respectively (Rola-Pleszczynski, 1985).

While much attention has focussed on the immunoregulatory properties of the arachidonic acid metabolites, fatty acids themselves can also regulate immune responses. Both linoleic acid (precursor of arachidonic acid) and more so arachidonic acid inhibited antigen- and mitogen-induced lymphocyte proliferation, while the saturated fatty acids palmitic, stearic, and oleic acid had little effect (Meade and Mertin, 1978). These findings were extended in vivo to prolongation of allograft survival, and to tumor growth, where high-fat diets rich in unsaturated fats predisposed rats to DMBA (dimethylbenzanthrene) induced mammary tumors (reviewed by Meade and Mertin, 1978). More recently, Santoli and Zurier (1989) demonstrated that the PGE precursors arachidonic and dihomogamma linolenic acid could inhibit IL-2 production by PHA-stimulated human PBL. Similar findings were noted using the Jurkat cell line. Stearic, oleic, and palmitic acids had no effect, consistent with the findings of Meade and Mertin (1978). Furthermore, the effect was not dependent upon conversion to PGE, since activity of the fatty acids was not affected by the presence of indomethacin. Thus, simple changes in the fatty acid profile of human lymphocytes may influence their function.

Prostaglandins have been implicated as potent immunosuppressive agents in many systems. Meade and Mertin (1978) have reviewed the activities of prostaglandins, specifically of the E series, in suppressing mitogen-induced T-cell proliferation, generation of CTL, in vivo graft rejection, and Ab production by B-cells. The major producers of prostaglandins are MØ, and generation of prostaglandins may represent one mechanism by which MØ exhibit immunosuppressive activity. PGE_2 has also been shown to prevent MØ activation and

inhibit phagocytosis of L. monocytogenes (Abbot and Myers, 1986; Hutchison and Myers, 1987), and to inhibit IL-1 production by monocytes via an increase in intracellular cyclic AMP levels (Knudsen et al., 1986).

Leukotrienes are the product of the conversion of arachidonic acid by the enzymes 5- and 15-lipoxygenase (Samuelsson et al., 1987). They are produced by polymorphonuclear cells, MØ, basophilic leukemia cells, and BM derived mast cells, but not lymphocytes (Rola-Pleszczynski, 1985). Their effects include inhibition of mitogen-induced cell proliferation, Ig synthesis, MLR, generation of suppressor T cells, and inhibition of NK activity (reviewed by Rola-Pleszczynski, 1985; Samuelsson et al., 1987). The exact mechanism of action of leukotrienes is unknown. Leukotrienes can give rise to increased intracellular cyclic nucleotides, in particular, cyclic GMP, and in this way are analogous to PGE_2.

3.1.4 Suppressor Factors Associated with Tumors

There is much evidence to suggest that the general level of immunosuppression associated with a tumor burden functions to dampen host immune surveillance and allow for tumor development. In many cases this suppression is mediated by soluble factors elaborated by tumor cells themselves or by the host. One such mechanism is shedding of gangliosides from the membranes of tumor cells. For example, Ladisch et al. (1983) demonstrated that the YAC-1 lymphoma produced high quantities of free gangliosides when cultured in vitro or as ascites in vivo, and these were strongly inhibitory. Similarly, Stallcup et al. (1984) have demonstrated that plasma membranes prepared

from normal and malignant cells could inhibit generation of CTL *in vitro* as well as LPS-induced B-cell proliferation. This suppression was nonspecific and may be involved in growth regulation of normal cells through contact inhibition. Immunosuppressive factors have also been described in serum of cancer patients, and one such factor is alpha-1-acid glycoprotein or immunosuppressive acidic protein (IAP). This protein, which is present in high amounts in serum of cancer patients but only in low amounts in normals, suppresses PHA- and alloantigen-induced proliferative responses (Bennett and Schmid, 1980). Sawada *et al.* (1984) have proposed that increased IAP levels may be a relevant clinical indicator for the development of gynecologic cancer.

One of the common ways to study immunosuppressive factors associated with tumors is to examine ascites fluid during tumor passage in rodents *in vivo*. For example, Wile *et al.* (1984) have found that the ascites fluid of rats injected IP with the Walker 256 carcinoma contained substances which inhibited PHA-induced proliferation of spleen cells. Biochemical analysis of the active ingredients revealed 2 prostaglandins, PGE_2 and PGF_2-alpha. Interestingly, cultures of tumor cells produced no suppressor activity, while cultures of thymus, spleen, or liver cells produced significant suppressor activity. Thus, production of the immunosuppressive agent was by the host and not the tumor. In contrast, *in vivo* passage of the mastocytoma P815 in mice revealed a suppressive activity in the ascites fluid (Cornelius and Normann, 1988). The P815 ascites was shown to suppress Ab, MLR, and mitogen responses. The active moiety was < 10 kDa, could be extracted in chloroform-methanol, and was not sensitive to proteolytic enzymes. The solubility of this molecule in aqueous medium was believed to be

due to its association with a high molecular weight carrier. Unlike the rat model above, there were insufficient quantities of PGE_2 to account for the suppression, and suppressive activity could be obtained by culturing P815 cells in serum-free medium.

There are many reports of *in vitro* derivation of suppressive factors which are elaborated from tumor cells or tissue from tumor-bearing animals. Jessup *et al.* (1985) described production of a suppressive factor after culture of methylcholanthrene-induced or spontaneous fibrosarcomas of C3H/He mice. This factor, called tumor-derived suppressor factor (TDSF), inhibited DTH responses to dinitrochlorobenzene (DNCB) by activating either MØ or $CD8^+$ T-cell suppressors. It was determined that TDSF was an RNA-protein complex. Similarly Pope (1985) described production of a suppressive factor from the spleens of mice bearing the M-1 fibrosarcoma. This suppressor factor could activate $Thy\text{-}1^+$, $CD4^+8^+$ suppressor cells from unprimed precursors, and in turn these suppressor cells secreted a soluble factor which inhibited Ab synthesis. The addition of conditioned medium containing T-cell growth factors could reverse suppression, suggesting potential interference at this level. Almawi *et al.* (1987) generated an IL-3 dependent cell line (M1-A5) from the M-1 fibrosarcoma bearing mice which produced the initial suppressor-inducing factor (SIF). The release of the factor was dependent upon prostaglandins, but effector function was not. The SIF preparations from cultured M1-A5 cells actually contained 2 moieties, an SIF-alpha (70 kDa) and SIF-beta (5.5 kDa), and both suppressed *in vitro* and *in vivo* Ab responses to SRBC in a genetically non-restricted fashion.

Lastly, Santoli *et al.* (1986) described a factor produced by

unstimulated cultured human T-cell leukemic lines, called T-leukemia derived suppressor lymphokine (TLSL). This factor could inhibit a variety of functions, including proliferation of leukemia and lymphoma lines, mitogen- or alloantigen-induced T-cell proliferation, and lymphoid and myeloid hematopoietic colony formation. This factor was not produced by monocytic, myeloid, or B-leukemia/lymphoid cell lines. The inhibition of proliferation appeared to be due to an arrest in the G_0/G_1 phase of the cell cycle.

3.1.5 Alpha Fetoprotein

Alpha fetoprotein (AFP) is a major component of fetal and newborn sera as well as maternal serum during the second half of pregnancy (Abramsky et al., 1982). The natural inability of the mother to reject the fetus may be associated with the potent immunosuppressive properties of AFP. Murgita and Tomasi (1975a) and Murgita and Wigzell (1976) originally demonstrated that AFP could inhibit both T-dependent and T-independent Ab responses in vitro. AFP also suppressed both mitogen- and alloantigen-induced proliferation of lymphocytes (Murgita and Tomasi, 1975b) and both spontaneous and induced NK activity (Cohen et al., 1986). Hooper and Murgita (1981) reported that AFP could block proliferation in an autologous MLR, and suggested that one potential role of AFP may be control of autoresponses during early development.

The potential mechanism of action of AFP in inducing nonresponsiveness was by the generation of suppressor T-cells, as described by Murgita et al. (1977). Peck et al. (1982) further contributed to this by examining the effects of AFP during an MLR. They found that AFP acted early in the response; however there was no

direct effect on responding T-cells. Monocyte-enriched cells pretreated with AFP could stimulate T suppressor cells to inhibit generation of CTL *in vitro*, and did so by elaborating a soluble factor which specifically activated Ts which suppressed the primary MLC.

Abramsky *et al.* (1982) were able to inhibit induction of EAE in guinea pigs by daily treatment with AFP after a disease-inducing challenge with bovine CNS homogenate or MBP in CFA. AFP was also able to inhibit cell mediated immune responses and binding of anti-MBP Ab to MBP *in vitro*. The ability of AFP to block induction of EAE was related to the clinical remission of human autoimmune disease during pregnancy, and subsequent relapse postpartum when AFP levels have returned to normal (see Abramsky *et al.*, 1982). Thus AFP may have potential clinical relevance in treating autoimmune disease. Its main endogenous function however may be associated with the lack of immune responsiveness in the neonate.

3.1.6 Interferon (IFN)

It was demonstrated several years ago that interferons were potent inhibitors of immune function. Gisler *et al.* (1974) and Johnson *et al.* (1975) had demonstrated that IFN could suppress primary Ab responses to T-dependent and T-independent antigens. Interferon can also inhibit mitogen- and alloantigen-induced proliferation. (Lindahl-Magnusson *et al.*, 1972; Heron *et al.*, 1976; and Weinstein *et al.*, 1977).

The ability of interferon to inhibit hematopoietic cell development has also been well documented. Broxmeyer *et al.* (1985) have shown that IFN-gamma, IFN-alpha, and IFN-beta all acted

individually to inhibit CFU-GM, BFU-E, and CFU-GEMM colony formation from human BM *in vitro*. IFN-gamma and IFN-alpha could also act synergistically, especially when culturing cells at low O_2 tension. The results are relevant to the clinical situation where IFN-gamma has been implicated in hematopoietic hypoproliferative disorders, such as aplastic anemia (Zoumbos *et al.*, 1985), where abnormal production of IFN has been described in these patients.

Raefsky *et al.* (1985) also demonstrated the ability of rIFN-gamma and rIFN-alpha to inhibit human myeloid colony formation (CFU-C) and erythroid colony formation (CFU-E). The effects of IFN in this system could be overcome by addition of human placenta conditioned medium, which is a good source of colony stimulating factors. The direct target of IFN appeared to be the progenitor cells. In cases where synergy between IFNs occurred the role of auxiliary cells was important. More recently, Gajewski and Fitch (1990) have demonstrated that not only does IFN-gamma inhibit proliferation of BM cells, but also of IL-3 dependent cell lines.

The role of IFN in immunosuppression has also been implicated *in vivo* during GVHD (Klimpel *et al.*, 1990). Production of IFN-gamma and IFN-beta by spleen cells of mice undergoing chronic GVHD resulted in suppression of *in vitro* mitogen responsiveness. If mice were injected with Ab to IFN-gamma, there was immune recovery and no lymphoid hypoplasia associated with GVHD. The recovery of immune function in these animals may also be related to inhibition of natural suppressor (NS) activity and IFN-beta normally associated with chronic GVHD as previously described (Cleveland *et al.*, 1988).

3.1.7 Transforming Growth Factor-beta (TGF-beta)

TGF-beta is one of a family of growth factors which can induce non-malignant cells to become transformed and to grow in an anchorage-independent fashion (Sporn et al., 1986). In addition, TGF-beta also possesses potent immunosuppressive activity for a large variety of functions. Kerhl et.al. (1986a) demonstrated that TGF-beta inhibited IL-2 dependent proliferation of human T-cell blasts prepared by pre-incubation with Con A. Other features of this effect included down-regulation of IL-2R and transferrin receptor on the activated T-cell blasts. Interestingly, activated T cells can synthesize and secrete TGF-beta, and this is associated with increased TGF-beta receptor expression on these cells. This suggests that normally, TGF-beta may act as a physiological regulator of T-cell growth.

Kerhl et al. (1986b) also reported that B-cells have high affinity receptors for TGF-beta which increase in number after B-cell activation. They showed that TGF-beta could inhibit IL-2 induced B-cell proliferation and IL-2 or BCDF induced Ig-secretion. Similar to T-cells, activated B-cells also produced significant quantities of TGF-beta, suggesting that TGF-beta may be an autonomous regulator of B-cell function. Consistent with this Lee et al. (1987) have reported that TGF-beta can inhibit kappa light chain expression of normal BM cells and transformed cloned pre-B cells. This effect is specific for B-cell development since MHC Class II expression was unaffected by TGF-beta. Along with this TGF-beta also prevented maturation of developing B-cells to mitogen responsiveness, while not affecting kappa chain expression of B-cell lymphomas which already secrete Ig. This indicates that TGF-beta regulates early stages in B-cell development,

and is supported further by findings that TGF-beta inhibits IL-7 dependent proliferation of B-cell progenitors from BM (Lee et al., 1989).

The natural production of TGF-beta may be intimately associated with a lack of immune function in so-called "immunologically priveleged" sites. In one example, Clark et al. (1990) described nonspecific suppressor cells with a null phenotype present in the murine decidua during pregnancy which produce an immunosuppressive factor called decidual suppressor factor (DSF). Upon characterization of this factor and comparison of biological activity, it was determined to be TGF-beta-2. The authors suggested that an important function of TGF-beta-2 in murine decidua may be associated with successful pregnancy, and was supported by the finding that the suppressor population was not present in the decidua of mice which spontaneously aborted their fetuses (Clark et al., 1986).

In a second example, the anterior chamber of the eye has long been known as an immunologically priveleged site which will readily accept allografts (Medawar, 1984). Granstein et al. (1990) have reported that aqueous humor contains substances which can inhibit proliferation of thymocytes in the presence of sub-optimal doses of PHA and IL-1 or IL-2. Two factors have been isolated from aqueous humor which mediate this activity. One is a low molecular weight inhibitory factor (< 3.5 kDa) which is not PGE_2. The other is TGF-beta, demonstrated by the ability of anti-TGF-beta antiserum to neutralize the inhibitory activity. Thus TGF-beta may represent a naturally occurring inhibitor of activation which maintains hyporesponsiveness in these priveleged sites.

3.1.8 IL-1 and IL-2 Inhibitors

This section will deal with substances which primarily function to block IL-1 or IL-2 induced activation signals. Some of the previously described inhibitors, such as gangliosides, which inhibit IL-2 driven proliferation, have already been discussed (section 3.1.2). Larrick (1989) presents an excellent review of native IL-1 inhibitors, which typically function to block thymocyte co-mitogenesis. Several of these inhibitors have been described from diverse cell sources, including granulocytes, keratinocytes, transformed B-cell lines, and leukemic or virally infected MØ. Urine can also be a rich source of IL-1 inhibitors, especially during pregnancy and disease states. For example, inhibitors of IL-1 have been described in the urine of pregnant women, of febrile patients with pulmonary tuberculosis, and from patients with monocytic leukemia. TGF-beta, which has been implicated as an immunosuppressive agent active during pregnancy (Clark et al., 1990), has been shown to inhibit IL-1 induced proliferation (Larrick, 1989) and to inhibit production of IL-1 (Chantry et al., 1989b).

Honda et al. (1985) have described an IL-2 inhibitor in normal mouse serum which neutralizes IL-2 activity by interacting with IL-2. This factor is not Ag- or MHC-restricted, is produced by $CD8^+$ Ts cells, and is a protein in the MW range of 50-60 kDa (Male et al., 1985). Kresina (1990) has described a 30-35 kDa inhibitor of IL-2 induced proliferation called contra-IL-2. This factor is produced by a suppressor T cell hybridoma generated from mice suppressed for collagen-induced arthritis. Since IL-2 levels are elevated in rheumatoid arthritis, this factor may be important in controlling IL-2

induced pathogenesis.

3.1.9 Immunosuppressive Factors from Suppressor Cells and Other Sources

Suppressor factors derived from BM have not only been described from this lab but from several others as well. Petrov et al. (1979) described a suppressor factor from murine BM which suppressed Ab synthesis of spleen cells in vitro, primarily by inhibiting proliferation. Release of this factor required cell-cell contact with Ag-stimulated cells. The factor was not MHC-restricted, and ultrafiltration revealed a MW of < 10 kDa. Alley et al. (1983) reported that human BM secreted a suppressor factor after PWM stimulation, called marrow-derived suppressor substance (MDSS). This factor could prevent Ig synthesis of all isotypes of BM cell cultures, and was not genetically restricted. They also show that the PWM-stimulated BM cells were equally suppressive.

Dittmer et al. (1984) described the production of a factor from both rat and dog BM cultures which inhibited Con A induced proliferation of thymocytes. In addition, this factor inhibited growth of some tumor cell lines while not affecting others. While growth of unstimulated thymocytes or BM cells was not affected, it was found that the anti-proliferative effects were associated with cytotoxicity as determined by ^{51}Cr-release. Physically, this factor was not sensitive to heat and trypsin digestion, and its MW was < 12 kDa.

In the rabbit, Maes et al. (1988) reported the constitutive production of a suppressor factor (SF) during 24 hr culture. This > 10 kDa SF was produced by Fc-gamma$^+$ BM cells and suppressed proliferation of Fc-gamma$^-$ BM cells. Both Fc-gamma$^+$ BM cells and SF could inhibit

spleen cell proliferation to Con A, PHA, and PWM, as well as growth of IL-2 dependent T-cells in the presence of IL-2. In addition, SF blocked production or release of IL-2 from Con A stimulated T-cells.

Soluble mediators of immune suppression have also been discovered from neonatal sources. Argyris (1981) described the production of a suppressor factor (SF) from cultured neonatal spleen. This factor was produced by null cells, and suppressed the MLR and generation of CTL. It was not Ag- or MHC-restricted, acted early during the response, and bound readily to peritoneal exudate cells. This factor was resistant to heat and UV treatment, and pronase and trypsin digestion, and was made up of a small and large component. Knaan-Shanzer and van Bekkum (1987) similarly described an immunosuppressive factor (SUF) produced constitutively in culture by thymocytes or spleen cells from neonatal mice and rats. They were able to generate an SUF secreting hybridoma produced by fusion of neonatal spleen cells with the thymoma BW5147. The activities of this factor included suppression of GVHD and the MLR. Like the SF described by Argyris (1981), this factor contained 2 moieties. One was small (< 3 kDa) which could not prevent GVHD but suppressed cell proliferation by interfering with the utilization of IL-2. The other was large (> 100 kDa) which prevented both GVHD and MLR, and acted by preventing IL-2R expression.

Barton (1988) also generated a factor from a hybridoma derived from murine neonatal spleen fused with the non-secreting myeloma FO. The cell which fused with the myeloma was considered a null cell. This factor suppressed Ag-specific proliferative responses of cloned helper or alloreactive T-cells, and did not inhibit IL-2 production or

function. The target in this case was the accessory cell population, and further, this factor inhibited proliferation of thymocytes to IL-1. Like the previous molecules described, this was a large MW factor (>90 kDa), however there was no low MW component. Lastly Jadus and Peck (1986) have reported on production of 3 monocyte-derived protein molecules from the spleens of newborn mice of MW 58, 10.8, and 10 kDa. These molecules suppressed the MLR but were ineffective at preventing GVHD.

NS cell clones derived from the spleens of TLI-treated mice were also reported to secrete a suppressor factor, called NSF (Hertel-Wulff and Strober, 1988). This factor was produced by PMA and calcimycin stimulated NS clones which expressed CD3 and TcR alpha/beta chains (Strober et al., 1989). NSF was not Ag- or MHC-restricted, and inhibited proliferation in the MLR but not mitogen-induced proliferation. It is a protein which is heat and pH stable and is between 50-100 kDa in size.

Many types of Ag-nonspecific factors have been shown to be T-cell products. Perhaps the most classic is the soluble immune response suppressor, or SIRS, which is produced by Con A stimulated spleen cells and T-cell hybridomas (Aune et al., 1983). SIRS is produced by $CD8^+$ T-cells and suppresses Ab synthesis, MLR, and proliferation of both normal and neoplastic cells. There are 2 species of SIRS (21.5 and 14 kDa) and both must be oxidized by H_2O_2 from MØ before they are biologically active. Nakamura et al. (1987) have also described production of a monoclonal nonspecific suppressor factor (MNSF) from a murine T-cell hybridoma. This factor suppresses Ig production by LPS-stimulated B-cells, and unlike SIRS which only acts early this factor

acts early and late. Apparently MNSF is a 70 kDa aggregate made up of 24 and 16 kDa subunits. Lastly, Lau et al. (1990) described a suppressor activating factor (SAF) produced by a mutant T-cell line. This factor suppressed mitogen-activated proliferation, CTL generation, and PWM-stimulated PFC responses. It was characterized as the oxidized product of spermine, spermine dialdehyde (SDA).

Finally, monocytes have been shown to produce a variety of immunosuppressive substances, including prostaglandins (section 3.1.3). Fujiwara and Ellner (1986) have reported that the monocytic cell line U937 produced a factor which inhibited IL-1, IL-2, and PHA-induced blastogenesis of thymocytes. This factor was sensitive to heat and low pH, and was NH_2SO_4 precipitable. The MW of this factor was 85 kDa. Sugimura et al. (1989) also describe a 45 kDa factor (LBIF) which inhibits PHA and IL-1 induced stimulation of thymocytes. This factor arrests T-cells in the G_1 phase of the cell cycle, but does not block IL-2 production or IL-2R expression. Likewise, calcitriol (1,25-dihydroxyvitamin D_3) produced by normal human MØ also arrests T-cells in the G_1 phase of the cell cycle, similarly decreasing expression of the transferrin receptor (Rigby et al., 1990). However, unlike LBIF, calcitriol can inhibit IL-2 production by T-cells. Wilkins and Warrington (1984) also described an inhibitor from Con A-stimulated U937 cells called IDS, which blocks proliferation of mitogen-driven cells and malignant lymphoid cells. This factor is not species specific and is not cytotoxic, and interestingly it appears to be lymphoid specific.

3.2 MATERIALS AND METHODS

3.2.1 Production of BDSF

Bone marrow cells from 8-12 week old mice were prepared by flushing tibias and femurs with sterile BSS using a syringe and a 26-gauge needle according to the method of Oliver and Goldstein (1978). Unseparated cells were cultured at a concentration of 1 X 10^7 cells/ml in 25 cm^2 T-flasks (Nunc) at a volume of 7-10 ml/flask. The culture medium consisted of MEM as prepared for Mishell-Dutton culture (see Section 2.2.2) without adding FBS. The cells were cultured for 24 hr, after which supernatants were harvested by centrifugation of the cell suspensions at 1500 rpm for 7 min. The cells were resuspended in fresh serum-free medium for a further 24 hr of culture, after which the supernatants were harvested. The supernatants were pooled and filtered using a Nalgene 0.45 um filter unit (Nalge Company, Rochester, N.Y.) to remove cellular debris. Viable cell recovery after 24 hr was typically 40-45% of the initial culture, and after 48 hr had dropped to approximately 25%.

Supernatants were passed through a YM 10 filter (nominal cutoff of 10 kDa) by the Amicon ultrafiltration system (Amicon Corp., Oakville, Ont.). The material which passed through the membrane was collected and concentrated on a YM 2 filter (nominal cutoff of 1 kDa) to one-tenth the original volume, and then was dialyzed against at least 50 volumes of PBS to remove low molecular weight molecules, phenol red, and excess salts. After dialysis, the supernatant was filter sterilized using a 0.22 um Millipore filter (Millipore Corp., Bedford, Mass.), aliquoted, and stored at -20^{o}C.

3.2.2 IL-2 Assay

The presence of IL-2 was detected using the murine IL-2 sensitive cell lines CTLL-2 and HT-2, which were maintained using either human IL-2 or rat Cas. For the assay 1 X 10^4 CTLL-2 or HT-2 cells were cultured in 96-well flat-bottomed plates in RPMI containing 5% FBS. Potential IL-2 containing supernatants were added to the cells in 50-100 ul volumes to give a total volume of 200-250 ul. The cells were cultured for 18 hr followed by a 6 hr pulse with ^{3}H-TdR. Incorporation of ^{3}H-TdR was determined by liquid scintillation counting as previously described.

3.2.3 IFN Assay

The presence of IFN-gamma was detected using the murine IFN-gamma sensitive B-cell lymphoma WEHI-279, which was maintained by regular passage in RPMI containing 10% FBS. For the assay, 1 X 10^4 WEHI-279 cells were cultured in 96-well flat-bottomed plates in the presence of potential IFN-gamma containing supernatants in a total volume of 250 ul. The cells were cultured for 48 hr followed by a 24 hr pulse with ^{3}H-TdR. Plates were harvested as previously described and ^{3}H-TdR incorporation was determined by liquid scintillation counting.

3.2.4 IL-3 Assay

Proliferation was assayed on the murine IL-3 dependent cell lines DA-1 and MC-9. To maintain these lines conditioned medium was first prepared from the myelomonocytic leukemia WEHI-3, which constitutively secretes IL-3. Briefly 1 X 10^6 WEHI-3 cells/ml were cultured in T-25 flasks in RPMI containing 10% FBS for 48 hr, and after centrifugation supernatants were harvested, filtered, and stored at-

20°C. The cell lines DA-1 and MC-9 were typically maintained in 10% WEHI-3 conditioned medium (WEHI-3 CM).

For the assay, firstly 1 X 10^4 DA-1 cells were cultured in 96-well flat-bottomed plates in RPMI containing 10% FBS. WEHI-3 CM, which was used as a source of IL-3, was added to the cells so that the total volume was 250 ul/well. The cells were cultured for 18 hr, followed by a 6 hr pulse with ^{3}H-TdR. Secondly, 5 X 10^3 MC-9 cells were cultured in 96-well flat-bottomed plates as for the DA-1 cells. In this case, the length of culture was 72 hr, including a pulse with ^{3}H-TdR for the final 6 hr of culture. In both cases, plates were harvested and ^{3}H-TdR incorporation was determined by liquid scintillation counting.

3.2.5 LAF Assay

The LAF assay detects the presence of IL-1 (previously known as lymphocyte activating factor, LAF) by its ability to synergize with sub-optimal doses of T-cell mitogens to induce proliferation of murine thymocytes. In most cases, normal thymocytes from young animals (4-6 week) were used. However, if potential IL-1 containing preparations contained LPS, thymocytes from young C3H/HeJ mice were used in the assay since these animals do not respond to LPS.

For the assay, 1.5 X 10^6 thymocytes/well were cultured in 96-well flat-bottomed plates in the presence of 1 ug/ml PHA (Difco, Detroit, Michigan) and IL-1 containing preparations. The cells were cultured for 72 hr, including a pulse with ^{3}H-TdR for the final 6 hr of culture. Incorporation of ^{3}H-TdR was determined by liquid scintillation counting.

3.2.6 Preparation of Human IL-1

Human IL-1 was produced from monocytes derived from peripheral

blood lymphocytes (PBL). Briefly, 20 ml of blood was layered onto Ficoll-Hypaque and spun at 2500 rpm for 10 min to isolate PBL. The PBL, contained in the buffy coat layer, were aspirated off and washed 3 times in BSS. The cells were then suspended in RPMI containing 5% FBS at a density of 3 X 10^6 cells/ml and incubated in a petri dish (100 X 15 mm)for 4 hr at 37°C. At this time supernatants containing non-adherent cells were discarded, and the plates were gently washed with BSS. The remaining adherent cells in each dish were then cultured for 24 hr in RPMI containing 5% FBS plus 20 ug/ml of LPS (Sigma). Supernatants were harvested and dialyzed against RPMI at 4°C for 2 days, changing the dialysis medium daily. The supernatants were then filter sterilized, aliquoted, and stored at -20°C.

3.2.7 Maintenance of Cell Lines

All cell lines that were not lymphokine dependent were maintained by regular passage in RPMI including 5-10% FBS. These included WEHI-3 and P388D1 (provided by Dr. P. Flood, Yale University), LBRM-33 (provided by Dr. G. Mills, Toronto General Hospital), WEHI-279 and 11B11 (provided by Dr. G. Strejan, UWO), and YAC-1 (ATCC, Rockville Pike, Md.). The IL-2 dependent cell lines CTLL-2 and HT-2 were kindly provided by Dr. G. Strejan (UWO) and Dr. P. Flood (Yale), respectively, and maintained as described in section 3.2.2. The IL-3 dependent cell lines DA-1 and MC-9 were also provided by Dr. P. Flood (Yale), and maintained as described in section 3.2.4. Stocks of frozen cells were routinely kept in liquid nitrogen when not required for experimentation.

3.3 RESULTS

3.3.1 Suppression by Bone Marrow Derived Suppressor Factor, BDSF

BDSF prepared from BM cultures of C57Bl/6 mice was added to cultures of syngeneic spleen cells in a PFC or MLR response. Figure 3.1 demonstrates that BDSF suppressed both of these responses in a dose-dependent manner. In both of these systems, BDSF was added to the cultures at the initial time of plating. To determine whether BDSF acted early or late during the generation of the response, BDSF was added to an MLR culture at various time intervals after the initiation of culture. The results in Figure 3.2 demonstrate that BDSF was most effective when added at culture initiation, and suppression progressively decreased when BDSF was added 1 and 2 days after culture initiation.

3.3.2 Effect of BDSF on Cellular Proliferation

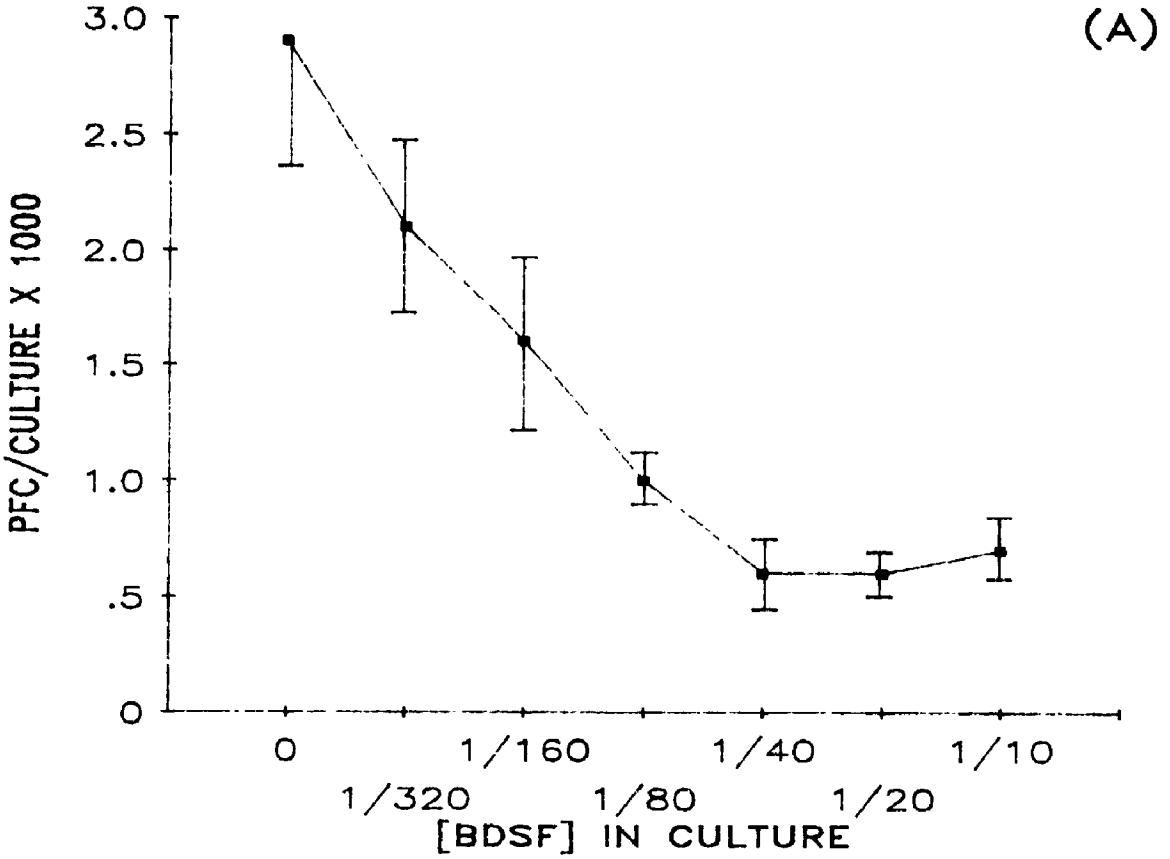
Duwe and Singhal (1978) had previously shown that BDSF did not inhibit proliferation of thymocytes, and was mitogenic for spleen and BM cells. Table 3.1 confirms the previous findings by demonstrating the ability of several preparations of BDSF to induce proliferation of BM cells. Since other groups had described factors derived from BM which inhibited proliferation of tumor cells (Dittmer et al., 1984), BDSF was tested for this activity. Table 3.2 demonstrates that proliferation of a monocytic leukemia (P388D1), B-cell hybridoma (11B11), T-helper cell line (LBRM-33), and thymic lymphoma (YAC-1) were all unaffected by BDSF. However proliferation of the myelomonocytic leukemia WEHI-3 was inhibited by BDSF, as demonstrated in Table 3.2 and confirmed in Table 3.3 under slightly different culture conditions.

FIGURE 3.1

Suppression of the PFC Response and MLR by BDSF

(A) BDSF from C57Bl/6 mice was prepared as indicated in Materials and Methods section 3.2.1. Dilutions of BDSF were added at culture initiation to 10^7 C57Bl/6 spleen cells in a PFC response. PBS was added to separate wells as a control. The background response of unstimulated cells was 131 ± 70 PFC/culture.

(B) BDSF from C57Bl/6 mice was added to an MLR consisting of 2 X 10^5 mitomycin C treated Balb/c spleen cells. PBS was added to separate wells as a control. The background response of cultures stimulated with autologous cells was 5,177 ± 1,720 CPM. In both panels vertical bars represent standard deviation.


(A)
3.0
2.5
2.0
1.5
1.0
.5
0
PFC/CULTURE X 1000
0
1/320
1/160
1/80
1/40
1/20
1/10
[BDSF] IN CULTURE

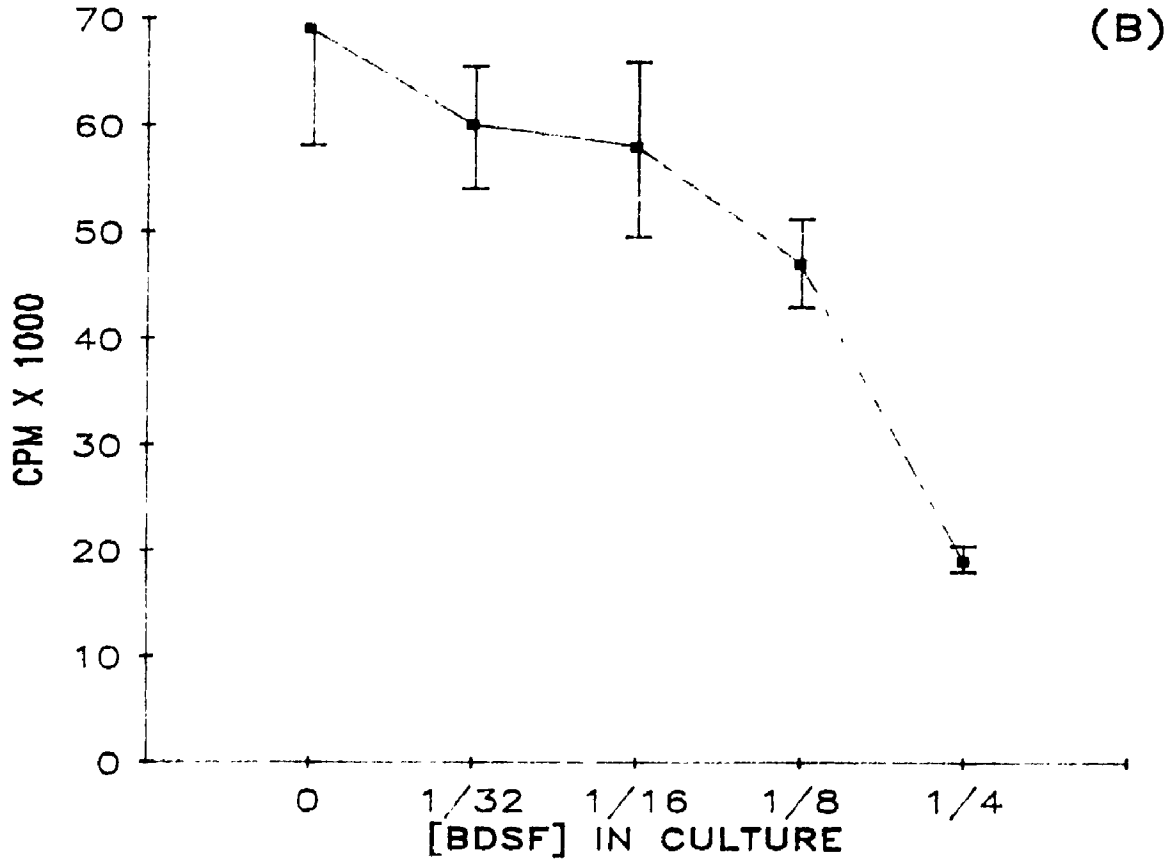

(B)
70
60
50
40
30
20
10
0
CPM X 1000
0
1/32
1/16
1/8
1/4
[BDSF] IN CULTURE

FIGURE 3.2

Kinetics of Suppression by BDSF in the MLR

A predetermined maximal dose of BDSF was added to an MLR culture at the days indicated. The MLR consisted of 2 X 10^5 C57Bl/6 spleen cells cultured with 2 X 10^5 Balb/c spleen cells. As a control, PBS was added to separate wells at the same time that BDSF was added to culture. The background response of C57Bl/6 responders cultured with autologous stimulators was 8,147 ± 2,149 CPM. Vertical bars represent standard deviation.

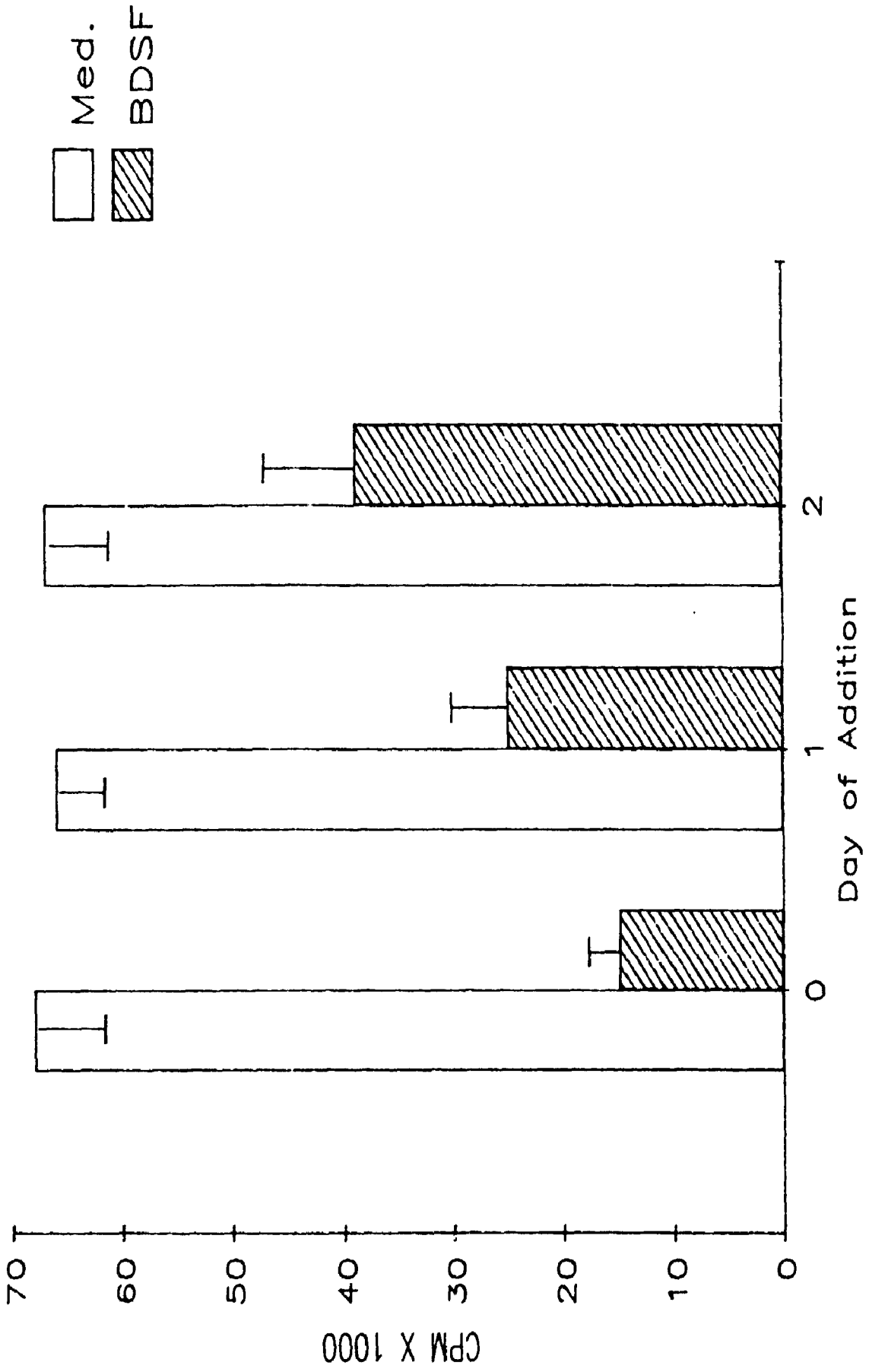

Med.
BDSF
CPM X 1000
70
60
50
40
30
20
10
0
0
1
2
Day of Addition

TABLE 3.1

Effect of BDSF on BM Cell Proliferation

Experiment	[BDSF] in Culture	CPM ± S.D.[a]	P Value
1	—	4,559 ± 868	—
	1/5	8,767 ± 1,562	<.005
	1/25	6,166 ± 166	<.01
	1/125	4,473 ± 486	N.S.[b]
2	—	4,559 ± 868	—
	1/5	8,348 ± 871	<.0005
	1/25	5,188 ± 434	N.S.
	1/125	4,775 ± 505	N.S.
3	—	3,298 ± 199	—
	1/10	10,988 ± 1,820	<.0005
	1/50	7,047 ± 1,192	<.0005
	1/250	4,343 ± 467	<.005

[a] BM cells were cultured at a cell density of 5 X 10^5 cells/well in 96-well flat-bottomed plates for 24 hr followed by a 24 hr pulse with 3H-TdR.

[b] N.S. refers to not significant.

TABLE 3.2

Effect of BDSF on Proliferation of Continously Growing Cell Lines

Cell Line	[BDSF] in Culture	CPM ± S.D.[a]
P388D1[a]		
(Monocytic Leukemia)	—	15,597 ± 2,848
	1/5	17,777 ± 2,098
	1/10	14,823 ± 3,002
	1/20	13,076 ± 1,693
11B11[b]		
(B-cell Hybridoma)	—	324,478 ± 23,909
	1/5	303,618 ± 15,234
	1/10	314,557 ± 15,732
	1/20	293,041 ± 2,981
LBRM-33[c]		
(T-helper cell line)	—	2,279 ± 304
	1/5	2,057 ± 78
	1/10	1,934 ± 90
	1/20	2,007 ± 323
YAC-1[d]		
(Thymic Lymphoma)	—	665,692 ± 8,720
	1/5	693,792 ± 26,944
	1/10	699,540 ± 22,815
	1/20	697,718 ± 23,505
WEHI-3[d]	—	433,661 ± 20,629
(Myelomonocytic Leukemia)	1/5	119,384 ± 4,639
	1/10	190,195 ± 16,154
	1/20	269,654 ± 13,400

[a] P388D1 cells were plated at a density of 10^4 cells/well in 96-well flat-bottomed plates for 24 hr, followed by a 24 hr pulse with ^{3}H-TdR.

[b] 11B11 hybridoma cells were cultured at a density of 10^4 cells/well for 24 hr, and ^{3}H-TdR was added for the final 6 hr of culture.

[c] LBRM-33 cells were cultured at 10^4 cells/well for 24 hr, followed by a 24 hr pulse with ^{3}H-TdR.

[d] YAC-1 and WEHI-3 cells were cultured at densities of 10^5 cells/well for 24 hr, followed by a 24 hr pulse with ^{3}H-TdR.

TABLE 3.3

BDSF Selectively Inhibits Proliferation of the WEHI-3 Cell Line

[BDSF] in Culture	CPM ± S.D.[a]	P Value
Exp. 1		
—	78,168 ± 21,669	—
1/10	31,248 ± 4,748	<.005
1/20	28,160 ± 9,925	<.005
1/40	37,667 ± 3,451	<.01
1/80	44,775 ± 5,608	<.025
1/160	55,443 ± 14,541	N.S.
1/320	64,248 ± 13,243	N.S.
Exp. 2		
—	72,589 ± 3,144	—
1/10	25,687 ± 3,362	<.0005
1/20	28,915 ± 3,274	<.0005
1/40	36,573 ± 6,960	<.0005
1/80	39,831 ± 4,951	<.0005
1/160	40,490 ± 3,439	<.0005
1/320	54,541 ± 2,012	<.005

[a] WEHI-3 cells were cultured at a density of 10^4 cells/well in 96-well flat-bottomed plates for 24 hr, including a pulse with ^{3}H-TdR during the last 6 hr of culture.

TABLE 3.4

Production of IL-3 by the Myelomonocytic Cell Line

WEHI-3 is not Affected by BDSF

[BDSF] in[a] Culture	Proliferation of DA-1[b] (CPM ± S.D.)
-	64,498 ± 14,135
1/10	103,656 ± 6,067
1/20	87,796 ± 9,649
1/40	85,258 ± 9,527
1/80	71,976 ± 11,073

[a] 1 X 10^4 WEHI-3 cells/well were cultured in 96-well flat-bottomed for 24 hrs and then supernatants were harvested and tested for IL-3 activity. BDSF was added at the indicated doses.

[b] IL-3 was detected using the cell line DA-1 as described in Materials and Methods section 3.2.4.

Production of IL-3 was also examined using the constitutive IL-3 producing cell line WEHI-3. The cell line was cultured in the presence or absence of BDSF, and the supernatants were tested on DA-1. Table 3.4 shows that BDSF had no effect on IL-3 production by WEHI-3, and in fact enhanced it slightly.

3.3.3 Effect of BDSF on Con A-Induced Proliferation and Lymphokine Production

Duwe and Singhal (1978) had also reported that BDSF did not inhibit the proliferative response of spleen cells to the T-cell mitogens Con A and PHA, and to the B-cell mitogen LPS. This data was confirmed here in Table 3.5 demonstrating that BDSF could not block proliferation of spleen cells to a maximal dose of Con A (2.5 ug/ml). The possibility existed that BDSF could not inhibit mitogenic proliferation due to the large magnitude of the response. However, even in the presence of a minimal dose of Con A (0.25 ug/ml), BDSF was ineffective at suppressing this response.

In order to induce the production of IL-2 and IFN-gamma, spleen cells from C57Bl/6 mice were cultured in the presence of Con A (2.5 ug/ml) for 48 hr. Supernatants from these cultures typically contained both IL-2 and IFN-gamma as assayed on the IL-2 dependent cell line CTLL-2 and IFN-gamma sensitive cell line WEHI-279, respectively. When BDSF was added to spleen cell cultures in the presence of Con A, there was no effect on either IL-2 or IFN-gamma production as assessed by proliferation of the appropriate cell lines in the presence of IL-2 and IFN-gamma containing supernatants (Tables 3.6 and 3.7). This is in agreement with the findings in Table 3.5 that BDSF did not inhibit Con A-induced proliferation of spleen cells. Furthermore, to examine the possibility that BDSF might contain IFN-gamma, WEHI-279 cells were

TABLE 3.5

Effect of BDSF on Con A Induced Proliferation of Spleen Cells

Dose of Con A[a]	[BDSF] in Culture	CPM ± S.D.
2.5 ug/ml	—	252,410 ± 11,303
	1/5	246,996 ± 6,304
	1/10	267,314 ± 39,491
	1/20	284,947 ± 13,362
	1/40	292,351 ± 11,636
0.25 ug/ml	—	8,802 ± 1,320
	1/5	12,578 ± 2,133
	1/10	11,690 ± 1,192
	1/20	9,671 ± 764
	1/40	11,573 ± 308

[a] Spleen cells were cultured with BDSF and Con A as described in Materials and Methods section 2.2.4.

TABLE 3.6

BDSF Does Not Block the Production of IL-2

by Spleen Cells Stimulated with Con A

Pretreatment Dose of BDSF	Con A[a] (2.5 ug/ml)	Proliferation of CTLL-2[b] (CPM ± S.D.)
-	-	474 ± 115
-	+	4,743 ± 790
1/10	+	3,293 ± 747
1/20	+	4,457 ± 816
1/40	+	5,065 ± 844
1/80	+	6,714 ± 1,593

[a] Spleen cells were cultured with Con A at 2 X 10^6 cells/well in 24-well plates for 48 hrs for the production of IL-2. BDSF was added at the doses indicated.

[b] The presence of IL-2 was assayed on the IL-2 sensitive cell line CTLL-2 as described in Materials and Methods section 3.2.2.

TABLE 3.7

BDSF Does Not Block the Production of IFN-gamma

by Spleen Cells Stimulated with Con A

Pretreatment Dose of BDSF	Con A[a] (2.5 ug/ml)	Proliferation of WEHI-279[b] (CPM ± S.D.)
-	-	407,687 ± 10,820
-	+	125,109 ± 6,251
1/10	+	114,401 ± 5,143
1/20	+	113,126 ± 14,314
1/40	+	111,888 ± 5,526
1/80	+	111,860 ± 6,875

[a] Spleen cells were cultured with Con A at 2 X 10^6 cells/well in 24-well plates for 48 hrs for the production of IFN-gamma. BDSF was added at the doses indicated.

[b] The presence of IFN-gamma was assayed on the IFN-gamma sensitive cell line WEHI-279 as described in Materials and Methods section 3.2.3.

TABLE 3.8

Lack of IFN-gamma Activity by BDSF

[BDSF] in Culture	Proliferation of WEHI-279[a] (CPM ± S.D.)
-	249,725 ± 23,797
1/4	219,713 ± 13,015
1/8	234,958 ± 4,122
1/16	231,350 ± 8,770
1/32	236,493 ± 7,836
1/64	236,632 ± 10,697

[a] IFN-gamma was detected using the IFN-gamma sensitive cell line WEHI-279 as described in Materials and Methods section 3.2.3.

cultured in the presence of increasing doses of IFN-gamma and cell proliferation was assayed after 72 hr. As demonstrated in Table 3.8, BDSF contained no significant amounts of IFN-gamma.

3.3.4 Effect of BDSF on Lymphokine Driven Responses

BDSF was tested for its ability to inhibit proliferation of murine thymocytes directly due to human IL-1. In this case (Table 3.9) the LAF assay was used, without the addition of a sub-optimal dose of PHA. The results demonstrate that human IL-1 by itself induced a vigorous proliferative response compared to unstimulated cells, and that increasing doses of BDSF had no effect on this response.

There have been reports from several labs of substances which could inhibit proliferation of IL-2 dependent cell lines, including gangliosides (Robb, 1986). BDSF was tested for the ability to suppress IL-2 driven proliferation using 2 cell lines, HT-2 and CTLL-2, both dependent upon IL-2 for growth. Figure 3.3 demonstrates that BDSF did not inhibit proliferation of both the HT-2 and CTLL-2 cell lines in the presence of rat Cas. Due to the heterogeneity of lymphokine production associated with rat Cas, the experiment was repeated using either purified human IL-2 or murine recombinant IL-2. Furthermore, only the CTLL-2 line was used in the assay since only IL-2 will stimulate its proliferation. This is in contrast to HT-2, which can proliferate in the presence of both IL-2 and IL-4 (Tartakovsky et al., 1989). Table 3.10 demonstrates that BDSF had no effect on either human or murine IL-2 driven proliferation of CTLL-2, and that BDSF added alone had no effect on background proliferation of CTLL-2.

Similar to the previous section, BDSF was tested for the ability to inhibit proliferation of 2 cell lines dependent on IL-3, DA-

TABLE 3.9

Thymocyte Proliferation in the Presence of Human IL-1 and BDSF

[BDSF]in Culture	CPM ± S.D.[a]
—	21,548 ± 4,632
1/4	33,025 ± 4,439
1/8	23,485 ± 6,164
1/16	20,417 ± 5,715
1/32	17,178 ± 9,446
1/64	19,863 ± 8,237

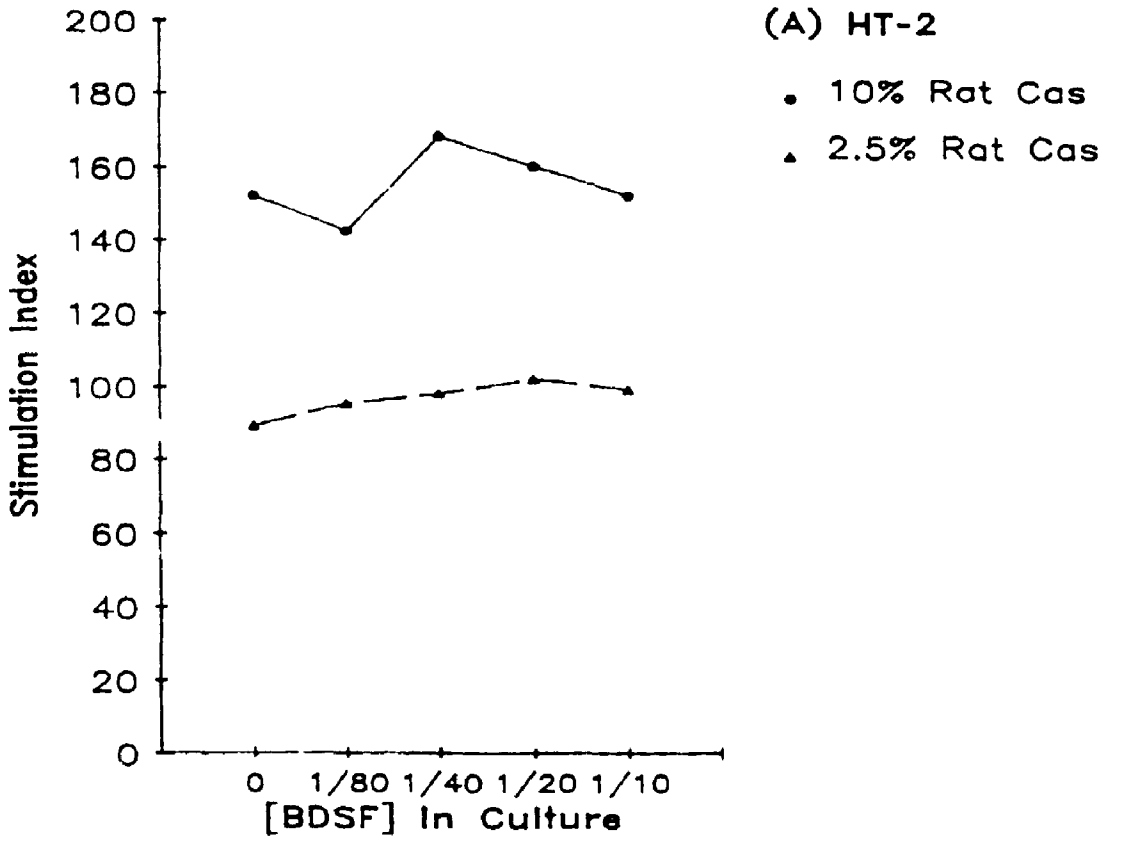
[a] Thymocytes were stimulated in the LAF assay with a 1/20 dilution in culture of human IL-1 as a direct mitogenic agent. BDSF was added in the doses indicated. The background response of unstimulated thymocytes was 211 ± 88 CPM.

FIGURE 3.3

Effect of BDSF on Proliferation of the IL-2 Dependent Cell Lines HT-2 and CTLL-2

(A) The proliferation of HT-2 cells in the presence of rat Cas was performed as described in Materials and Methods section 3.2.2. The stimulation index was calculated as proliferation in the presence of rat Cas divided by background proliferation of HT-2. Background proliferation of HT-2 was 997 ± 287 CPM.

(B) The proliferation of CTLL-2 cells in the presence of rat Cas was performed as described in section 3.2.2. Stimulation index was calculated as in (A). The background proliferation of CTLL-2 cells was 152 ± 56 CPM.


(A) HT-2
• 10% Rat Cas
▲ 2.5% Rat Cas
Stimulation Index
200
180
160
140
120
100
80
60
40
20
0
0 1/80 1/40 1/20 1/10
[BDSF] In Culture

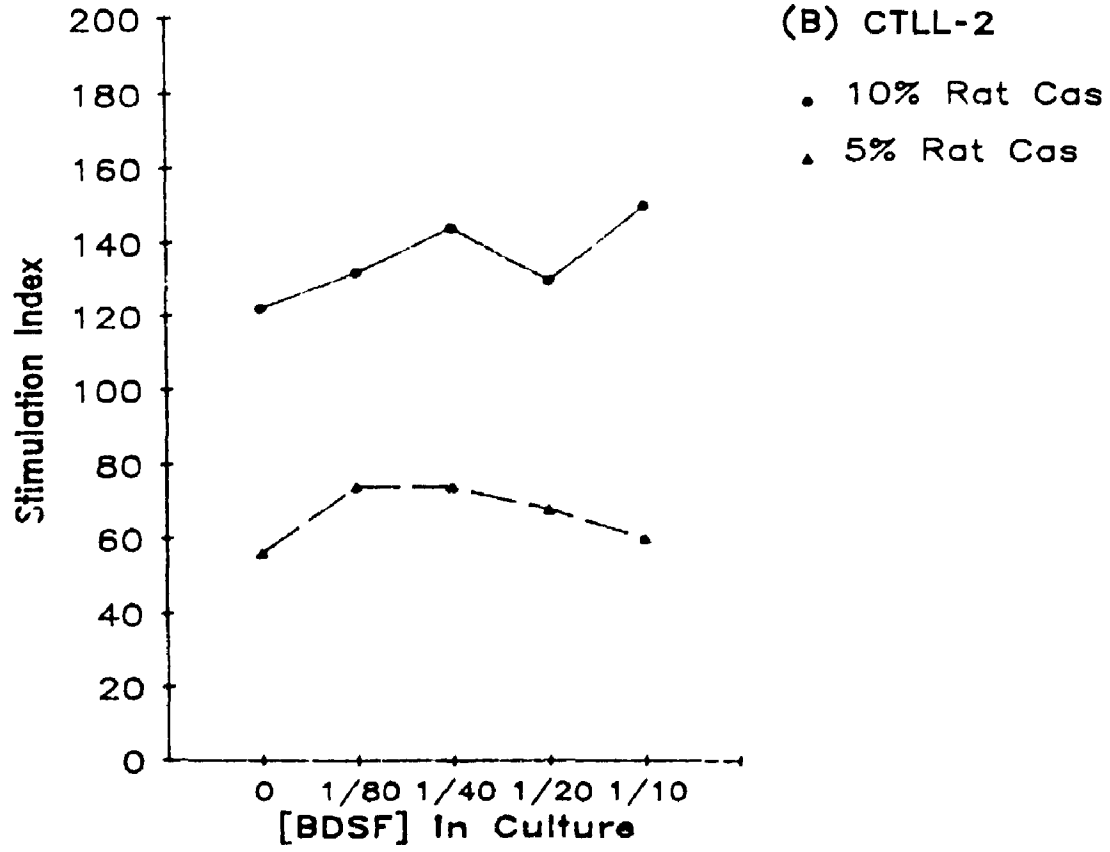

(B) CTLL-2
• 10% Rat Cas
▲ 5% Rat Cas
Stimulation Index
200
180
160
140
120
100
80
60
40
20
0
0 1/80 1/40 1/20 1/10
[BDSF] In Culture

TABLE 3.10

BDSF Does Not Block IL-2 Dependent Proliferation of CTLL-2

[IL-2] Added (U/ml)	[BDSF] in Culture	CPM ± S.D.[a]
Human (30)	--	72,050 ± 3,882
"	1/4	54,224 ± 1,433
"	1/8	68,368 ± 2,681
"	1/16	67,689 ± 1,823
"	1/32	62,321 ± 1,155
Murine (50)	--	88,342 ± 4,666
"	1/4	84,389 ± 2,047
"	1/8	95,051 ± 3,509
"	1/16	101,288 ± 3,209
"	1/32	101,560 ± 6,957
--	--	280 ± 100
--	1/4	273 ± 41

[a] Proliferation of CTLL-2 is described in Materials and Methods section 3.2.2.

TABLE 3.11

Proliferation of the IL-3 Dependent Cell Lines

DA-1 and MC-9 is not Inhibited by BDSF

[BDSF] in Culture	(CPM ± S.D.)[a]
DA-1	
-	167,737 ± 10,943
1/5	165,382 ± 11,668
1/10	184,486 ± 17,339
1/20	190,622 ± 4,922
1/40	185,307 ± 18,944
MC-9	
-	79,914 ± 9,486
1/5	109,506 ± 11,933
1/10	82,281 ± 7,678
1/20	73,344 ± 7,005
1/40	78,539 ± 8,110

[a] The amount of IL-3 in culture used to stimulate the cells was 1/5 for DA-1 and 1/50 for MC-9. Proliferation due to BDSF alone was 8,158 ± 1,197 for DA-1 cells and 1,615 ± 832 for MC-9 cells. In the absence of IL-3 the background response of DA-1 cells was 9,021 ± 1,310 CPM and of MC-9 cells was 920 ± 222 CPM.

1 and MC-9. As indicated in Table 3.11, BDSF did not inhibit the proliferation of either line in the presence of IL-3.

3.3.5 BDSF Blocks Production of IL-2 in the MLR

The generation of the MLR specifically involves the expansion of Ag-specific Th-1 cells which proliferate as a result of antigenic recognition and elaboration of IL-2 (Ilonen and Karttunen, 1984). Since BDSF consistently suppressed proliferation in the MLR, IL-2 production was determined in cultures treated with BDSF. Table 3.12 clearly demonstrated that decreased proliferation in the presence of BDSF was associated with decreased levels of IL-2 in the MLR culture supernatants.

Consistent with this finding was the ability of exogenously added IL-2 to partially or completely reverse BDSF-mediated suppression in the MLR. Table 3.13 demonstrates that while human IL-2 reversed suppression in the MLR, addition of either human IL-1 or murine IL-3 had no effect, in spite of the sub-optimal dose of BDSF used. This was supported further in Table 3.14 by the ability of murine recombinant IL-2 to reverse the BDSF suppressed response in two separate experiments. Collectively, the results suggest that BDSF inhibits proliferation in the MLR by causing a deficit of IL-2 production necessary for proliferation of Ag-specific responding cells. Production of IL-2 early during activation is necessary for transition of cells through cell cycle. Table 3.15 demonstrates that addition of IL-2 to the MLR had a maximal effect during the first 24 hrs in culture, consistent with optimal suppressive activity of BDSF (Figure 3.2).

TABLE 3.12

BDSF Blocks Production of IL-2 in the MLR

[BDSF] in[a] the MLR	MLR Response (CPM ± S.D.)	CTLL-2 Proliferation[b] (CPM ± S.D.)
—	43,077 ± 5,622	9,174 ± 832
1/4	10,335 ± 1,137	3,334 ± 243
1/8	25,034 ± 1,304	3,761 ± 364
1/16	29,078 ± 6,626	4,056 ± 242
1/32	31,910 ± 2,679	5,220 ± 504
1/64	32,085 ± 5,050	5,660 ± 467
1/128	39,114 ± 5,845	7,080 ± 1,407

[a] BDSF was added to a C57Bl/6 vs. Balb/c MLR in the doses indicated and proliferation was determined after 96 hr of culture.

[b] Supernatants from a duplicate C57Bl/6 vs. Balb/c MLR were harvested after 96 hr of culture and added to the IL-2 dependent cell line CTLL-2. As a control, CTLL-2 cells cultured with 30 U/ml human IL-2 proliferated at a rate of 65,322 ± 4,386 CPM.

TABLE 3.13

Reconstitution of the BDSF Suppressed Response in the MLR by IL-2

Lymphokine[a] Added	CPM ± S.D.	P Value
--	105,750 ± 11,557	--
BDSF	75,115 ± 4,422	<.005[b]
Human IL-1		
1/40	72,586 ± 2,782	--
1/80	84,028 ± 5,295	<.025[c]
1/160	78,260 ± 7,989	--
Human IL-2		
1/10	131,884 ± 7,264	<.0005[c]
1/100	115,575 ± 4,252	<.0005[c]
1/1000	104,093 ± 5,270	<.0005[c]
Murine IL-3		
1/10	73,888 ± 5,478	--
1/100	73,861 ± 5,581	--
1/1000	79,296 ± 2,990	--

[a] The amount of lymphokine represents the final dilution in culture.

[b] P value as compared to the control of medium only in culture.

[c] P value as compared to BDSF alone added to culture.

TABLE 3.14

Reconstitution of the BDSF Suppressed Response in the MLR by Murine Recombinant IL-2

Amount IL-2 Added	CPM ± S.D.	P Value
Exp. 1		
—	48,865 ± 18,270	—
BDSF only	22,160 ± 5,203	<.025[a]
50 U	51,042 ± 3,379	<.0005[b]
25 U	20,603 ± 1,509	N.S.[b]
12.5 U	17,228 ± 1,340	N.S.[b]
Exp. 2		
—	101,652 ± 4,579	—
BDSF only	14,437 ± 2,311	<.0005[a]
200 U	32,009 ± 7,445	<.005[b]
100 U	29,872 ± 1,283	<.0005[b]
50 U	19,968 ± 4,305	<.05[b]

[a] P value as compared to the control of medium only in culture.

[b] P value as compared to BDSF alone added to culture. N.S. refers to not significant.

TABLE 3.15

Kinetics of IL-2 in the MLR

Day IL-2 Added	[IL-2] Added (Units)	CPM ± S.D.[a]	P Value
--	--	67,765 ± 7,886	--
0	75	237,481 ± 1,112	<.0005
	37.5	166,084 ± 13,110	<.0005
1	75	97,073 ± 8,253	<.005
	37.5	76,359 ± 15,587	N.S.[b]
2	75	48,896 ± 4,529	<.005
	37.5	59,356 ± 12,256	N.S.

[a] The MLR consisted of 2 X 10^5 C57Bl/6 spleen cells cultured with 2 X 10^5 Balb/c stimulator cells. The background response of C57Bl/6 spleen cultured with autologous stimulators was 3,008 ± 1,246.

[b] N.S. refers to not significant.

3.4 DISCUSSION

Duwe and Singhal (1978) initially observed that BM cells could suppress Ab responses across cell impermeable membranes. This suggested that BM cells elaborated a soluble mediator of suppression. Fractionation of supernatants from BM cell cultures revealed that the suppressive factor resided in the 1-10 kDa range, and later in the range of 1-3.5 kDa (McGarry et al., 1982). This activity is now referred to as bone marrow derived suppressor factor, BDSF. The studies here confirm the ability of BDSF to suppress the murine Ab response in vitro, and further demonstrate suppression of the MLR. Furthermore, as in previous studies in the Ab response suppression was also maximal at culture initiation in the MLR. Thus the results demonstrate that BDSF can effectively suppress both humoral and cellular responses in the same manner.

Previous studies by Duwe and Singhal (1978) had shown that BDSF did not inhibit mitogen-induced proliferation and by itself was mitogenic for both spleen and BM cells. The results here confirm the mitogenicity of BDSF on BM cells, and also show that Con A induced proliferation of spleen cells was not affected by BDSF, in the presence of either optimal or sub-optimal doses of Con A. Many immunosuppressive agents have been described which, unlike BDSF, are potent inhibitors of mitogen-induced proliferation. Several of these include gangliosides (Whisler and Yates, 1980; Ladisch et al., 1983; Marcus et al., 1987), the arachidonic acid metabolites prostaglandins and leukotrienes (Wile et al., 1984; Rola-Pleszczynski, 1985), calcitriol (Rigby et al., 1990), tumor cell products (Wilkins and

Warrington, 1984; Fujiwara and Ellner, 1986; Sugimura et al., 1989; Cornelius and Normann, 1988; Santoli et al., 1986; Stallcup et al., 1984; Sawada et al., 1984), IFN (Weinstein et al., 1977) and BM derived products (Dittmer et al., 1984; Maes et al., 1988).

Interestingly Hertel-Wulff and Strober (1988) described a suppressor factor, called NSF, from cloned NS cells, which like BDSF inhibited the MLR but not mitogen-induced proliferation of spleen cells. Consistent with the inability of BDSF to affect Con A-induced spleen cell proliferation was the lack of effect on IL-2 and IFN-gamma production by the same cells. It is possible that distinct events associated with mitogen-driven activation pathways are not affected by either BDSF or NSF preparations. While NSF is a 50-100 kDa protein, and BDSF a low MW lipid, it is apparent that their biological activity is similar. The possibility exists that NSF may be a BDSF-like lipid molecule which has attached to a high MW carrier, since NSF was insensitive to heating. In support of this, Cornelius and Normann (1988) described production of an immunosuppressive lipid mediator soluble in aqueous solutions as a result of its association with a high MW carrier protein. Although BDSF is a lipid, it is also readily soluble in aqueous culture, perhaps as a result of its high polarity (McGarry et al., 1982). BDSF may also be able to form a complex with a carrier protein to increase its solubility in aqueous media. It is likely that this would be a low MW carrier, since BDSF is routinely recovered in a preparation of molecules of MW between 1-10 kDa.

Dittmer et al. (1984) described a factor from unprocessed supernatants of rat and dog BM cultures which inhibited the response of rat thymocytes to Con A. In addition, this material inhibited growth

of some transformed cell lines, which included HeLa cells, EL-4 thymoma, Sarcoma 180, and Balb/c K3T3 cells. BDSF was similarly tested for its ability to inhibit proliferation of both tumor derived and culture derived cell lines. There was no effect on any of the lines tested, with one exception. BDSF consistently inhibited growth of the myelomonocytic leukemia WEHI-3. While the selective suppression on this line is unknown, it is possible that specific intracellular events associated with activation/transformation of WEHI-3 are sensitive to BDSF-induced inhibitory effects. This is consistent with the results of Dittmer et al. (1984) showing that not all cell lines were sensitive to the growth inhibitory factor in their system. Alternatively, it may suggest that BDSF has a growth inhibitory effect on hematopoietic cells at a particular stage of maturation. WEHI-3 is a myelomonocytic leukemia which is believed to have arisen from neutrophil-macrophage progenitors (Schrader and Crapper, 1983; Ymer et al., 1985), while P388D1, for example, represents a mature macrophage tumor line (Mizel et al., 1978). This introduces the potential regulatory function of BDSF on hematopoietic cell development in addition to the observed effects on peripheral immune responses.

The fact that WEHI-3 arose as a result of activation by a retrovirus (Ymer et al., 1985) may shed light onto the ability of BDSF to selectively inhibit cell growth, and possibly inhibit limited transformation events of other cell types. Although proliferation of WEHI-3 was inhibited by BDSF, the constitutive production of IL-3 by this cell line was not affected. This suggests that proliferation of WEHI-3 is not coupled to synthesis and secretion of IL-3. The ability of WEHI-3 to constitutively secrete IL-3 is linked to insertion of an

intracisternal A particle genome, including its LTR, in proximity to the promoter of the IL-3 gene (Ymer et al., 1985). It is postulated that this activation event was important in the evolution of this particular leukemia, such that WEHI-3 may have initially been an IL-3 dependent progenitor which gained the ability to maintain autocrine growth. However with time WEHI-3 apparently lost the requirement for IL-3 dependence and hence autostimulatory capability (Schrader et al., 1987). BDSF was unable to inhibit proliferation of two IL-3 dependent cell lines, DA-1 and MC-9, suggesting that potential activation events associated with IL-3 are not affected by BDSF.

Many inhibitors of IL-1 have been described which can interfere with IL-1 driven proliferation of thymocytes (Larrick, 1989). These include TGF-beta and substances found in urine during pregnancy and disease states. However, BDSF was unable to inhibit thymocyte proliferation in the presence of IL-1. Several agents have also been described which can interfere at several levels of IL-2 mediated activation. For example, calcitriol (Rigby et al., 1990), the fatty acids arachidonic and dihomogamma linolenic acid (Santoli and Zurier, 1989), and BM derived SF (Maes et al., 1988), can all block IL-2 production by mitogen or phorbol ester stimulated T-cells. Several factors have been described which can inhibit growth of IL-2 dependent cell lines or IL-2 responsive cells by interfering with the binding of IL-2 to its receptor. These include gangliosides (Merritt et al., 1984; Robb, 1986), TGF-beta (Kerhl et al., 1986a), mouse serum (Honda et al., 1985), Ts derived molecules (Kresina, 1990), BM derived SF (Maes et al., 1988), and neonatally derived SUF (Knaan-Shanzer and van Bekkum, 1987). Lastly, some agents such as the neonatal product SUF

(Knaan-Shanzer and van Bekkum, 1987) and TGF-beta (Kerhl et al., 1986a) can inhibit IL-2R expression on responding cells. Since BDSF was able to inhibit proliferation in the MLR, which is largely an IL-2 driven response (Ilonen and Karttunen, 1984), it was logical to examine either production or responsiveness to IL-2. Under no circumstances was BDSF able to block proliferation of IL-2 dependent cell lines in the presence of IL-2. This suggested that BDSF did not interfere with the ability of IL-2 to activate its target to proliferate, and thus presumably did not bind to IL-2 or IL-2R.

The next approach therefore involved examination of IL-2 production. As previously discussed, BDSF was unable to inhibit IL-2 production of Con A-stimulated spleen cells, which was not surprising since murine BDSF could not inhibit Con A-induced mitogenesis. By contrast, several other agents, such as unsaturated fatty acids (Santoli and Zurier, 1989), calcitriol (Rigby et al., 1990), and rabbit BM derived SF (Maes et al., 1988), all inhibit mitogen-induced proliferation and production of IL-2. However, upon examination of MLR culture supernatants it was determined that BDSF could in fact inhibit IL-2 production. This lack of IL-2 production was closely correlated with decreased proliferation in the MLR, and was further supported by the findings that addition of exogenous IL-2 to BDSF-treated MLR cultures could partially or completely reverse suppression. A lack of IL-2 production in the presence of antigenic stimulation would ultimately lead to hyporesponsiveness by that clone, since IL-2 is known to be essential for proliferation and maintenance of the response (Cantrell and Smith, 1984; Smith, 1988). Malkovsky and Medawar (1984) have proposed that responsiveness to Ag is determined by the

availability or effectiveness of IL-2. This hypothesis is supported by the findings that 1) tolerance to hapten-modified self antigens can be reversed by simultaneous injection of Con A, which induces IL-2 production, and 2) tolerance to allogeneic grafts, induced by injection of allogeneic cells into neonates, can be reversed by injection of IL-2 at the time of grafting (Malkovsky and Medawar, 1984).

Schwartz (1990) has recently confirmed the above findings that lack of IL-2 production leads to anergy of T-helper clones. He proposes that T-cell responsiveness is dependent upon engagement of the TcR by antigen (or anti-TcR Ab) and a costimulatory signal provided by antigen-presenting cells (APC). The nature of this costimulatory signal is vague, and no known lymphokines, including IL-1, can provide costimulation. However, the delivery of this second signal is essential for IL-2 production, and in the absence of costimulation there is a 95% decrease in messenger RNA coding for IL-2. This 2 signal model for T-cell activation resembles that of Bretscher and Cohn (1970) for B-cell activation, in which B-cell responses are dependent upon Ig-receptor occupancy and delivery of a second signal by Ag-specific T-cells. In both models, occupancy of the Ag receptor in the absence of the second signal results in clonal anergy.

It is proposed here that BDSF inhibits IL-2 production in an Ag-driven response, thereby preventing events necessary for clonal expansion. The mechanism by which BDSF prevents IL-2 production is unknown, and may involve either interference with T-cell activation by APC or inhibition of events associated with IL-2 production. Interestingly Mortari (1989) demonstrated that human BDSF could inhibit PMA-stimulated IL-1 production by the human histiocytic cell line U937.

Perhaps interference with IL-1 production in the MLR by murine BDSF may also contribute to insufficient activation. Sprent et al. (1990) have postulated that low avidity interactions between T-cell and APC results in only partial triggering and limited IL-2 production, leading to clonal anergy. It is possible that BDSF may influence the avidity of this interaction by altering either Ag-processing or the expression of accessory molecules on the APC, or by inhibiting costimulatory signals as described by Schwartz (1990). Alternatively, BDSF may prevent intracellular signals, such Ca^{++} flux and kinase activity, from activating the IL-2 gene. In either case, inhibition of IL-2 production by BDSF leads to unresponsiveness, which could potentially influence both cellular and humoral responses which are dependent upon T-cell help.

CHAPTER 4

THE IMMUNOSTIMULATORY ACTIVITY OF BONE MARROW DERIVED ENHANCING FACTOR, BDEF

4.1 INTRODUCTION

The production of both positive and negative regulators of immune function by antigen- and mitogen-stimulated cells has been studied extensively (Waksman and Namba, 1976; Germain and Benacerraf, 1980). The previous chapter dealt with one such negatively signalling mediator, BDSF, derived from unstimulated murine BM cells. This chapter describes the activities associated with a positive regulatory mediator derived from murine BM cells, called bone marrow derived enhancing factor, BDEF. This factor was initially described by Duwe and Singhal (1978) as a constitutively produced molecule which enhanced Ab synthesis in vitro, and induced proliferation of thymocytes. The data presented in this chapter extend the initial findings, suggest a potential target of BDEF, and provide partial biochemical characterization of this molecule.

Since the early studies describing the production of soluble mediators of immune function, many similar molecules have been discovered, now collectively known as cytokines. The production and activities of many of these are well characterized, and advances in recombinant DNA technology have led to the cloning of genes for most cytokines (Miyajima et al., 1988). Unlike BM derived BDEF, most of these mediators are synthesized by mature cells in secondary lymphoid organs after antigen or mitogen stimulation. Mosmann and Coffman (1987) have recently proposed that T-helper cells, which are the primary source of T-cell derived cytokines, can be divided into 2 subsets based on the lymphokines which they secrete. These are designated T_{H1} and T_{H2}, which display the following lymphokine profile

upon activation: 1) T_{H1}: IL-2, IFN-gamma, lymphotoxin, GM-CSF, tumor necrosis factor, and IL-3; 2) T_{H2}: IL-4, IL-5, IL-3, GM-CSF, and tumor necrosis factor (Mosmann and Coffman, 1987; Coffman et al., 1988). T_{H1} cells mediate delayed-type hypersensitivity, while T_{H2} cells are better at providing help in Ab responses. Although both subsets secrete similar cytokines (ie. IL-3), the primary criterion for distinguishing the two is IL-2 and IFN-gamma synthesis by T_{H1}, and IL-4 and IL-5 synthesis by T_{H2}. The rest of this section will be devoted to a brief review of these and several other important molecules.

4.1.1 Interleukin 1 (IL-1)

The ability of MØ to produce IL-1 was first reported by Hoffman and Dutton (1971), who described restoration of Ab responses in MØ-depleted cultures by the addition of supernatants from cultured MØ. Owing to its ability to induce proliferation of thymocytes directly or in synergy with T-cell mitogens, IL-1 was initially referred to as Lymphocyte Activating Factor (LAF) by Gery and Waksman (1972). Further work demonstrated that LAF (IL-1) could enhance Ab responses to SRBC in vitro, and could also restore PFC responses from both T-depleted and nude mouse spleen cell cultures (Wood and Gaul, 1974; Koopman et al., 1978). LAF and related molecules were subsequently coined interleukin 1 (IL-1) at the 2nd International Lymphokine Workshop, based on biological activity and physical characteristics (Aarden et al., 1979).

The most common source of IL-1 is the MØ. Optimal production requires stimulation by one of several agents, including Ag, LPS, activated T-cells, colony stimulating factor, yeast cell wall (zymosan), muramyl dipeptide, immune complexes, C5a, silica crystals,

and phorbol esters (ie. PMA), to name a few (Unanue et al., 1976; Durum et al., 1985). Production of IL-1 is not restricted to MΦ, but also includes keratinocytes, kidney mesangial cells, corneal epithelium, stimulated B-cells, NK cells, fibroblasts, astrocytes, glioma cells, endothelial cells, and some T-cell lines (Durum et at., 1985; Dinarello, 1988). However, most recent work on IL-1 biology and biochemistry has come from utilization of the murine monocytic leukemia, P388D1, which produces large quantities of IL-1 after stimulation with LPS or phorbol ester (Durum et al., 1985).

While much of the biology of IL-1 has focussed on its immunological activities, it can also affect hematopoietic, neurologic, and metabolic systems (Dinarello, 1988). For example, IL-1 has been shown to augment BM production of growth factors, increase non-specific resistance and tumor killing, and induce neutrophilia and lymphopenia. Functions on CNS include induction of fever, sleep, and release of pituitary hormones which systemically affect bodily functions. IL-1 affects metabolism by inducing catabolism of muscle, bone, and cartilage, and also acts as a mediator of inflammation, inducing prostaglandin release and production of acute phase proteins from hepatocytes (Durum et al., 1985; Dinarello, 1988). The activities described above demonstrate that IL-1 is the most pleiotropic of the interleukins.

Two distinct forms of IL-1 have been described, which are IL-1 beta (Auron et al., 1984) and IL-1 alpha (Lomedico et al., 1984). Both are coded for by separate genes on chromosome 2, each containing 7 exons (Dinarello, 1988). IL-1 beta is generally produced in greater amounts, as evidenced by greater amounts of mRNA for IL-1 beta in

activated MØ. Both IL-1 alpha and beta are initially produced as an intracellular precursor polypeptide (MW 31kDa) which is cleaved by serine proteases into the mature dominant peptide (MW 17.5 kDa), as well as several other smaller peptides which are secreted to the exterior of the cell. Membrane forms of IL-1 also exist, including the 31 kDa precursor and a 22 kDa cleavage product. However, whether this form of IL-1 is biologically active remains a point of controversy (Kurt-Jones et al., 1985; Minnich-Carruth et al., 1989). Although IL-1 alpha and beta share only slight homology, they both bind to the same receptor on target cells, and their biological properties are identical.

The biological activities of IL-1 within the immune system are also pleiotropic (Durum et al., 1985; Dinarello, 1988). IL-1 has mainly been implicated in T-cell activation, functioning to induce lymphokine production (ie. IL-2) and lymphokine receptor expression. B-lymphocytes are also responsive to IL-1, especially in synergy with other lymphokines (ie. IL-4 and IL-6) required for proliferation and differentiation. NK cells demonstrate increased tumoricidal potential in the presence of IL-1, and IL-1 induced production of cytokines by NK cells augments NK activity. Lastly, IL-1 induces its own production in MØ as well as production of CSFs. Furthermore, IL-1 induces release of PGE_2, which acts as a potential negative feedback loop, since PGE_2 increases intracellular cAMP levels, resulting in decreased IL-1 production by MØ (Chantry et al., 1989).

4.1.2 Interleukin 2 (IL-2)

Initial reports demonstrated that the products of Con A-

stimulated murine spleen cells were essential for mitogen-induced proliferation of thymocytes. This mitogenic signal was initially given the names Thymocyte Stimulation Factor, TSF (Chen and DiSabato, 1976) and Costimulator (Paetkau et al., 1976). Subsequently Con A stimulated supernatants were shown to be an essential growth factor for the long term maintenance of cytotoxic T-cells, and were denoted T-cell growth factor, TCGF (Gillis and Smith, 1977; Gillis et al., 1978). This important finding led to the birth of methodologies allowing T-lymphocytes to be cloned and maintained indefinitely in culture. Paetkau et al. (1976) demonstrated that MØ were required for the production of the activity, and that in fact it was derived from T-cells which were Lyt-1^+2^-, characteristic of the helper phenotype. After assessment of the biological and biochemical properties of TSF, costimulator, and TCGF, they were found to be identical, and hence were designated interleukin 2, IL-2 (Aarden et al., 1979).

Early studies on IL-2 described several immunological effects, including thymocyte co-mitogenicity, generation of CTL, PFC enhancing activity on normal, T-depleted, and nude spleen cell cultures, induction of IFN-gamma synthesis, and stimulation of NK activity (Farrar et al., 1982). More recently, IL-2 has also been shown to induce proliferation of activated B-lymphocytes and also oligodendrocytes (Zubler et al., 1984; Hamblin, 1988). However, it still remains that the primary function of IL-2 is to deliver a mitogenic signal to T-cells after antigenic stimulation, driving T cells through the G_1 phase of the cell cycle to DNA synthesis (Miyajima et al., 1988). Immature thymocytes also express IL-2 receptor and it is now evident that IL-2 is required for T-cell differentiation events

taking place in the thymus (Ceredig, 1986; Jenkinson et al., 1987; Tentori et al., 1988). The IL-2 receptor (IL-2R) is composed of 2 chains, the low affinity IL-2R alpha (p55) and intermediate affinity IL-2R beta (p75), and non-covalent association of both chains results in a high affinity IL-2R (Smith, 1988). Binding of IL-2 to either the intermediate (p75) or high affinity receptor leads to phosphorylation of tyrosine residues on several intracellular substrates. This step is presumed to be an essential component of IL-2 induced proliferation (Saltzman et al., 1988).

The production of IL-2 has not only been derived from Con A-stimulated spleen cells, but also T-cell clones and T-cell tumor lines such as the lymphoma, EL-4. An activation signal is required for production of IL-2 by these sources, typically Con A or antigen, and with EL-4, Con A and phorbol esters (Farrar et al., 1982). Human and mouse IL-2 have been extensively characterized and the genes coding for them have been cloned (Arai et al., 1986). The MW of human IL-2 is 15 kDa, while that of murine IL-2 is 20-35 kDa owing to extensive glycosylation differences (Gillis, 1983; Arai et al., 1986).

4.1.3 Interleukin 3 (IL-3)

IL-3 was initially described as a product of activated T cells which could induce expression of the enzyme 20 alpha-hydroxysteroid dehydrogenase (20 alpha-SDH) in cultured spleens of nude mice, an event presumed to be involved in early T-cell differentiation (Ihle et al., 1981). IL-3 has been purified and the gene which codes for it has been cloned. The gene encodes a protein, which exists as a monomer, with a MW of 28 kDa (Ihle, 1989). Its activities are directed towards a wide

spectrum of hematopoietic cells, and thus IL-3 has been commonly referred to as multi-CSF or panspecific hemopoietin (Schrader, 1986). The many activities of IL-3 include multi-CSF activity, burst-promoting activity, CFU-S stimulation, induction of Thy-1 expression, and ability to maintain long-term growth of hematopoietic progenitor cell lines and mast cell lines (Miyajima et al., 1988).

Much of the work on IL-3 has suggested its importance in regulating events associated with myeloid growth and differentiation, and possible transformation associated with myeloid leukemia (Ihle, 1989). While IL-3 is classically produced by activated T-cells, the myelomonocytic murine leukemia WEHI-3 produces large quantities of IL-3 constitutively. It has been proposed that this cell line arose from a progenitor which acquired the ability to produce and utilize IL-3, inducing transformation and the more mature leukemic phenotype (Schrader and Crapper, 1983). However, the precise role of IL-3 in myeloid differentiation is not clear, and there is no evidence that BM cells are capable of producing IL-3 (Kodama et al., 1986; Ihle, 1989). Differentiation of early B and T cell progenitors may also be affected by IL-3. IL-3 dependent cell lines have been generated which, when injected into the appropriate hosts, can differentiate along B and T cell lineages, rearranging genes for the appropriate Ag-receptors (reviewed by Ihle, 1989).

4.1.4 Colony Stimulating Factors (CSFs)

The ability of IL-3 to affect several hematopoietic lineages has led to the designation of multi-CSF, as described in the previous section. However, many specific CSFs exist which stimulate committed

progenitor cells to differentiate. These include macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), and erythropoietin (EPO). GM-CSF is a glycoprotein of MW 23 kDa which most closely resembles IL-3 in its ability to affect multiple lineages of myeloid cells (Miyajima et al., 1988). Like IL-3, GM-CSF can also be produced by activated T-cells, however stromal cells, MØ, and endothelial cells also produce GM-CSF after stimulation by IL-1 or tumor necrosis factor. GM-CSF and IL-3 both map to the same chromosome, 11 in the mouse, and 5 in the human (Ihle, 1989).

M-CSF is a large protein (MW 45-70 kDa) which exists as a dimer (Nicola, 1987). There are 2 forms of M-CSF, one which is secreted, and a lower MW form which is associated with the cell membrane. The receptor for M-CSF is encoded for by the proto-oncogene c-fms, which displays tyrosine kinase activity (Sherr et al., 1985). Phosphorylation of specific substrates at tyrosine residues appears to be requisite for the growth promoting activity after binding of M-CSF to its receptor (Ihle, 1989). In addition to promoting growth of committed MØ progenitors, M-CSF can also augment Ab responses via its ability to activate MØ and stimulate production of IL-1 (Moore et al., 1980).

G-CSF is a 25 kDa glycoprotein defined by its ability to give rise to granulocytes from committed progenitors (Nicola, 1987). Like GM-CSF and IL-3, G-CSF has been mapped to murine chromosome 11, suggesting that these factors may have evolved from a common growth factor (Ihle, 1989). Furthermore, G-CSF is able to support limited multilineage colony growth, inducing granulocyte-macrophage and blast colony formation (Ihle, 1989).

Erythropoietin (EPO) is a 34 kDa glycoprotein which can promote proliferation and differentiation of erythroid lineage cells. It has been described in the urine of patients with aplastic anemia as a substance which could increase the RBC count in anemic rats and induce erythroid colony formation (Goldwasser et al., 1985).

4.1.5 Interleukin 4 (IL-4)

IL-4 was initially described as a costimulator of B-cell proliferation in the presence of anti-Ig, which was distinct from IL-2 (Howard et al., 1982). The activity could be produced from PMA-stimulated EL-4 thymoma, and was initially called B-cell growth factor (BCGF) and later B-cell stimulatory factor 1 (BSF-1) before its eventual designation as IL-4 (Paul, 1987).

Initially IL-4 was thought to resemble the B-cell analogue of IL-2 as a B-cell growth factor which could drive cells through the G_1 phase of the cell cycle after antigenic stimulation (Paul, 1987). However, it is now apparent that IL-4 acts at several stages of B-cell activation. For example, IL-4 induces expression of class II MHC and CD23 molecules on resting B-cells, as well as up-regulating expression of its own receptor. In addition, treatment of resting cells with IL-4 also allows more prompt entry into S phase upon subsequent stimulation, and induces B-cells to switch to IgG1 and IgE production upon stimulation with LPS (Paul, 1987; Miyajima et al., 1988).

The activities of IL-4 are not restricted to B-cell activation. IL-4 has been shown to induce proliferation of immature thymocytes stimulated with PMA. Also, similar to its effects on B-cells, IL-4 can act on both resting and activated T-cells, inducing them to divide in

conjunction with or after stimulation (Paul, 1987; Miyajima et al., 1988). IL-4 can also act on hematopoietic cells of several lineages. For example, it can synergize with EPO to induce growth of immature erythroid colonies and affects myelomonocytic and megakaryocytic precursors and mast cells (Paul, 1987). It can also activate MΦ resulting in either enhanced antigen presentation or cytotoxic capabilities (Paul, 1987; Miyajima et al., 1988).

4.1.6 Interleukin 5 (IL-5)

IL-5 was initially described by Schimpl and Wecker (1972) as a T-cell product after mitogenic or allogeneic stimulation. This factor was called T-cell replacing factor, TRF, for its ability to restore the responses of T-depleted and nude mouse spleen cells (Askonas et al., 1974). It was further shown that TRF acted late in the response to provide activated B-cells with a maturation signal resulting in Ig-production. TRF shared some similarities with IL-2, however was not able to stimulate proliferation of thymocytes (Aarden et al., 1979). IL-5 was also referred to as BCGF-II at one time, specifically enhancing IgM and IgA production by activated B-cells (Miyajima et al., 1988). As well as its effects on B-cells, IL-5 can also act as a CSF for eosinophils, previously thought to be mediated by eosinophil differentiation factor (Sanderson et al., 1986). Takatsu et al. (1988) have also shown that IL-5 can induce differentiation of CTL, therefore indicating that IL-5 is not restricted only to B-lymphocytes. The apparent MW of IL-5 is 50 kDa, and it has been determined that IL-5 in its native form exists as a homodimer, in both humans and mice.

4.1.7 Interleukin 6 (IL-6)

IL-6, like all of the lymphokines described, is able to mediate multiple activities. It is perhaps the most pleiotropic lymphokine, with the exception of IL-1. In the past, IL-6 was referred to as B-cell stimulatory factor 2 (BSF-2), IFN-beta-2, hybridoma growth factor (HGF), T-cell activating factor (TAF), cytotoxic T-cell differentiation factor (CDF), and hepatocyte stimulating factor. It can also be a costimulator of thymocyte proliferation, synergize with IL-3, synergize for proliferation of myeloid leukemic blasts, and induce fever (Sehgal et al., 1987; Wong and Clark, 1988). Interestingly, human IL-6 cDNA derived from a human T-cell line (Hirano et al., 1986) shares sequence similarities with G-CSF, especially at the amino terminus (Yasukawa et al., 1987). Both IL-1 and TNF (alpha and beta) can augment transcription of the gene encoding IL-6 in fibroblasts, and Wong and Clark (1988) caution that interpretation of many activities to IL-1 may be indirect, in fact involving IL-6. The gene for IL-6 has been cloned in both mice and humans, coding for glycoproteins with sizes ranging from 21-34 kDa. It has been suggested that because of their pleiotropic effects, and diversity of targets, that both IL-1 and IL-6 may represent central components within the cytokine network typically active in regulating the activities of hematopoietic and lymphoid cells (Wong and Clark, 1988).

4.1.8 Interleukin 7 (IL-7)

IL-7 was initially discovered as the product of a transformed stromal cell line which was used to maintain B-lineage lymphocytes in long-term cultures (Namen et al., 1988). The function of IL-7 appears

to be maintaining proliferation of both $B220^-$ (pro-B) and $B220^+$ (pre-B) cells in long-term cultures of BM cells. The gene for IL-7 has been cloned and codes for a molecule with an apparent mass of 14.9 kDa. In its native form IL-7 is a glycoprotein which is 25 kDa in size. Interestingly, messenger RNA for IL-7 has been detected in both spleen and thymus, although no biologically active IL-7 can be detected in culture supernatants of spleen and thymus cells (Namen et al., 1988). However, IL-7 can act as a costimulator of mitogen-induced proliferation of mature T-cells (Morrissey et al., 1988) and of thymocytes (Chantry et al., 1989a). The importance for the presence of IL-7 in thymus has recently been supported by observations that IL-7 maintains growth of early lineage T-cells in fetal thymus organ cultures (Watson et al., 1989). Thus IL-7 may represent a cytokine which is an obligatory requirement for early differentiation events in lymphoid progenitor cells.

4.1.9 Interleukin 8 (IL-8)

IL-8 refers to a group of previously described cytokines which act as attractant and activating factors for neutrophils (Westwick et al., 1989, Leonard, 1990). The previous designations included MDNCF (monocyte-derived neutrophil chemotactic factor), NAF (neutrophil activating factor), MONAP (monocyte-derived neutrophil-activating protein), LYNAP (lymphocyte-derived neutrophil-activating peptide), GCP (granulocyte chemotactic factor), and NAP-1 (neutrophil attractant/activation protein-1). In each case, the active ingredient is a single peptide of 72 amino acids. The major source of IL-8 is monocytes, and production can be stimulated by IL-1 alpha and beta,

TNF-alpha, IL-3, and GM-CSF. Production has also been reported from human T-cells stimulated with mitogen, from some human T-cell lines, endothelial cells, and fibroblasts. Although T-cells possess receptors for IL-8, the major effect appears to be directed towards neutrophils (Leonard, 1990).

4.1.10 Interferon-gamma (IFN-gamma)

IFN-gamma, previously referred to as immune IFN, is the product of activated T-helper cells, although NK cells can also produce IFN-gamma (Kashara et al., 1983; Coffman et al., 1988). While suppression of immune activation is one feature of IFN-gamma (reviewed in section 3.1.6), it is conversely able to enhance in vivo PFC responses, regulate isotype expression, activate NK cells, and induce differentiation of CTLs (Coffman et al., 1988; Handa et al., 1983; Maraskovsky et al., 1989). Perhaps its most profound effect is its ability to stimulate B-cells to synthesize IgG2a, while inhibiting synthesis of IgG3, IgG1, IgG2b, and IgE (Snapper and Paul, 1987). Furthermore, while IL-4 can induce synthesis of IgG1 and IgE, this can be inhibited by IFN-gamma (Coffman et al., 1987).

4.1.11 Tumor Necrosis Factor-alpha (TNF-alpha) and Lymphotoxin (LT)

TNF-alpha was originally discovered in the sera of mice injected with BCG and endotoxin, and was characterized by its ability to inhibit development of a Meth A sarcoma in vivo (Carswell et al., 1975). It has also been referred to as cachectin due to its ability to induce wasting (Beutler and Cerami, 1989). TNF-alpha is produced by mitogen-activated macrophages and lymphocytes, and its activities

include cytotoxicity on transformed cells in vitro (Sugarman et al., 1985), stimulation of IL-1 synthesis from endothelial cells (Nawroth et al., 1986), induction of thymocyte proliferation (Ranges et al., 1988), activation of osteoclasts (Dinarello, 1988), and stimulation of hematopoietic growth factors from fibroblasts (Zucali et al., 1988). The ability of TNF-alpha to induce thymocyte proliferation may be associated with its ability to enhance T-cell proliferative responses to IL-2 (Scheurich et al., 1987). By contrast, Kashiwa et al. (1987) have shown that TNF-alpha inhibits PWM-induced B-cell differentiation in humans.

LT possesses activities very similar to TNF-alpha, however it is only produced by mitogen-activated T-lymphocytes. In fact, due to its structural similarities with TNF-alpha and similar biological activities, LT is commonly referred to as TNF-beta (Sugarman et al., 1985). TNF-alpha and LT are located close together on human chromosome 6, and share the same receptor on target cells (Miyajima et al., 1988). The MW of TNF-alpha after gel filtration is 45 kDa, however SDS-PAGE reveals a monomeric protein of 17 kDa (Beutler and Cerami, 1989). Similarly, LT purifies as a protein of MW 60-70 kDa by gel filtration, and after SDS-PAGE as a monomeric protein of 20-25 kDa (Paul and Ruddle, 1988).

4.2 MATERIALS AND METHODS

4.2.1 Preparation of BDEF

Bone marrow cells were cultured as described in Materials and Methods section 3.2.1 for the production of BDSF. Supernatants obtained from culture of BM cells in serum-free medium were concentrated at 4°C to one-tenth the original volume using a YM-10 filter (nominal cutoff of 10 kDa) by Amicon ultrafiltration. The retentate, of molecular weight greater than 10 kDa, was dialyzed against at least 50 volumes of sterile PBS to remove low molecular weight molecules. After dialysis, the supernatant was filter sterilized, aliquoted, and stored at -20°C.

4.2.2 Cycloheximide Treatment of BM Cultures

BM cells were treated with different doses of cycloheximide (Sigma Chemical Co., St. Louis, MO) to block protein synthesis. Briefly, BM cells were cultured in 10 ml volumes in the presence or absence of cycloheximide (10 ug/ml) at 10^7 cells/ml, and supernatants were processed as usual for the production of BDEF. After the first 24 hr of culture, the cells were harvested and centrifuged, supernatants were kept, and the cell pellet was resuspended in methionine-free alpha MEM (Gibco). The cells were kept in culture for 1 hr in this medium to starve them of methionine. After 1 hr the cells were centrifuged and resuspended in fresh serum-free medium with or without cycloheximide for a further 24 hr. To determine the level of protein synthesis, a 0.5 ml aliquot of each "starved" cell suspension was centrifuged and resuspended in methionine-free medium containing ^{35}S-methionine (25 uCi/sample) plus 2% FBS. Each sample was incubated at 37°C for 30 min,

and then was mixed with 2.5 ml of a 10% Trichloroacetic acid (TCA) solution and kept on ice for 30 min. After this incubation each sample was individually harvested on to Whatman glass fiber filter discs by vacuum filtration, and washed 3 times with equal volumes of cold 10% TCA, followed by 3 washes with equal volumes of 95% ethanol. The discs were dried in a drying oven for at least 1 hr, then were solubilized in scintanalyzed toluene for liquid scintillation counting. Control cells cultured in the absence of cycloheximide incorporated 51,479 CPM, while cells cultured in the presence of 10 ug/ml cycloheximide incorporated 7,474 CPM, indicating a net decrease in protein synthesis of 85%.

4.2.3 Enumeration of TNP-Specific Plaques

Antibody synthesis in vitro was performed as described in Materials and Methods section 2.2.3. Direct IgM PFC were enumerated according to the double slide method described by Cunningham and Szenberg (1968). Hemolytic plaques were examined under a direct light source. TNP-specific plaques were enumerated in the same manner in the presence of a rabbit anti-mouse immunoglobulin facilitating serum (Cedarlane Labs, Hornby, Ont.). The net anti-TNP PFC response was determined by subtracting the background response to uncoupled SRBC from the total response.

TNP-SRBC were prepared according to the method of Rittenberg and Pratt (1969). Briefly, one ml of packed SRBC were added dropwise to 7 ml of 0.28 M cacodylate buffer (pH 6.9) containing 20 mg of 2,4,6-trinitrobenzenesulphonic (TNBS) acid and incubated at room temperature for 10 min. The reaction was stopped by adding cold BSS, centrifuging, and washing at least 4 times in cold BSS containing 0.63 mg/ml glycyl glycine to remove free TNBS. Coupling was assessed in a

hemagglutination assay using a rabbit anti-TNP-KLH Ab kindly provided by Dr. G. Strejan, with a titre of 1:200 indicating proper coupling.

4.2.4 Peanut Agglutinin Separation of Thymocytes

Peanut agglutinin (PNA) was purchased from Sigma (St. Louis, MO). PNA separation was carried out by a panning technique originally described by Caplan and Rothenberg (1984). PNA was dissolved at a concentration of 20 ug/ml in carbonate buffer, pH 6.0, which consisted of 0.159 g sodium carbonate (Na_2CO_3), 0.293 g sodium bicarbonate ($NaHCO_3$), and 0.02 g sodium azide (NaN_3) per 100 ml of double distilled water. The PNA solution was used to coat 10 cm plastic bacteriological plates (Canlab, Toronto, Ont.). Briefly, 4 ml of the PNA solution was added to the plates for 1-2 hr at room temperature. After coating, the plates were washed with PBS, and used immediately or stored overnight at 4°C after adding PBS containing 1% FBS and 1 mM NaN_3.

For cell separation, thymocytes were prepared as a single cell suspension in PBS containing 5% FBS and 1 mM NaN_3. Each dish was coated with 4 ml of thymocytes at a density of 10^7 cells/ml, and allowed to adhere for 90 min at 4°C. After the incubation, non-adherent cells (PNA^-) were removed by gently swirling and decanting the cells. Plates were washed with PBS and again non-adherent cells were harvested. PNA^- cells were washed in PBS and resuspended in tissue culture medium. To elute the adherent cells (PNA^+), the plates were incubated for 5-10 min with 0.2 M D-galactose at room temperature. After incubation the plates were flushed vigorously to remove adherent cells, and this step was repeated one more time, followed by a final wash with PBS. The PNA^+ cells were washed 3 times to remove D-galactose, and then were resuspended in tissue culture medium. The

PNA^- cells were typically no more than 7-20% of the starting number of cells, while PNA^+ cells represented the majority.

4.2.5 Costimulator Assay

This assay was performed according to the method of Shaw et al. (1978). Balb/c thymocytes were cultured at a density of 1 X 10^5 cells/well in 0.2 ml of complete medium in Nunc 96-well round-bottomed plates. Con A was added at a suboptimal dose of 5 ug/ml to give maximal stimulation in the presence of human IL-2. After 72 hr of culture, the wells were pulsed with 2 uCi of [^{3}H] thymidine for 4 hr, and incorporation was determined by liquid scintillation counting.

4.2.6 Enzyme Linked Immunosorbent Assay (ELISA)

Samples to be tested in the ELISA were first suspended in PBS. Protein suspensions were plated in 96 well flat-bottomed plates (Nunc) at a concentration of 2.5 ug protein/well in 100 ul volumes and incubated overnite at 4°C. All conditions were plated in quadruplicate. After incubation, plates were washed 3 times in washing buffer (0.01 M PBS containing 0.05% Tween-20, 0.02% NaN_3, and 0.2% gelatin), and then were blocked for 2 hr at room temperature by addition of blocking buffer (0.01 M PBS containing 1% gelatin). After incubation, the plates were washed once with blocking buffer, and then washing buffer containing 0.1 ug/ml of anti-p60 Ab was added to each well and incubated overnight at 4°C. Anti-p60 was prepared as previously described by Mortari et al. (1989) and was reactive with human BDEF. After incubation, the plates were washed 3 times in washing buffer. An alkaline phosphatase linked goat anti-rabbit Ab (IgG specific) was added to each well at a 1/2000 dilution in a total volume of 100 ul, and plates were then incubated for 3 hr at room

temperature. After incubation, plates were washed 3 times in washing buffer. Lastly, 100 ul of the substrate p-nitrophenyl phosphate disodium (Sigma) was added in substrate buffer at a concentration of 1 mg/ml. Substrate buffer consisted of 4.9 ml diethanolamine, 0.5 ml of 2% NaN_3 and 5 mg $MgCl_2$-$6H_2O$ topped to 50 ml with H_2O to a final pH of 9.8. After 30 min, the reaction was stopped by addition of 50 ul of 3 M NaOH and the plate was scanned at 405 nm on a Multiskan microplate reader (Flow Laboratories, Mississauga, Ont.).

4.2.7 Ammonium Sulfate Precipitation

Saturated ammonium sulfate (SAS) was prepared according to the method of Good et al. (1980). Briefly, 900 g of ammonium sulfate was added to 1 litre of double distilled water, heated until the ammonium sulfate dissolved, and filtered while the solution was hot. The solution was cooled to room temperature, and adjusted to pH 7.4 with ammonium hydroxide. SAS was routinely kept at room temperature, and ammonium sulfate crystals were always present in the bottle.

To precipitate BDEF, equal volumes (3 ml) were precipitated in either 45 or 90% SAS overnight at 4°C. The precipitated material was collected by centrifugation for 30 min at 10,000 rpm and resuspended in the original volume of PBS (3 ml). The samples were dialyzed against several volumes of PBS for 48 hr to remove contaminating ammonium sulfate, then were filtered and tested for biological activity.

4.2.8 HPLC Fractionation

High performance liquid chromatography (HPLC) was carried out using a Waters M-45 HPLC unit (Waters Associates, Milford, MA). Fractionation of BM preparations was achieved by sequential gel filtration using a Waters I-60 column proceeded by a Waters I-300

column. Prior to chromatography, whole BM supernates were lyophilized, resuspended in double distilled water, and dialyzed against 1000 volumes of PBS for 48-72 hr at 4°C. Dialysis was carried out using low MW dialysis membranes (cutoff of 1 kDa) purchased from Spectrapor (Los Angeles, CA). Protein determinations of the dialyzed material were carried out using the micro-Lowry assay (Lowry et al., 1951).

The buffer used during gel filtration was 0.15 M PBS containing 0.02% NaN_3, which was degassed before use. Initially, an analytical run was performed by injecting a small quantity (20 ul) of sample to determine the protein profile. Profiles were plotted on a recorder after protein content was monitored at 280 nm. This allowed for fractions to be collected according to the number of peaks that were present. For fractionation, samples were injected in 0.5-1.0 ml quantities containing 1-2 mg of total protein. Identical fractions were collected after several runs, pooled, and lyophilized. The individual fractions were then resuspended in 1.5 ml of double distilled water and dialyzed against normal PBS to remove NaN_3. Fractions were then filtered and used in a biological assay to determine activity.

4.2.9 DEAE Cellulose Ion Exchange Chromatography

DEAE cellulose, an anion exchange resin, was obtained from Sigma Chemical Co. (St. Louis, MO). Preparation of DEAE cellulose and column chromatography were both performed at room temperature. DEAE cellulose was initially swollen in 0.5 N HCl and allowed to settle. The HCl was removed and replaced with double distilled water and again allowed to settle. This step was repeated several times until the pH reached 5.0, then the cellulose was resuspended in 0.5 N NaOH, allowed

to settle, and washed with double distilled water until the final pH was 7.5. Finally, the DEAE cellulose was packed into the barrel of a 10 cc syringe, such that the bed volume was approximately 4 ml. The column was then equilibrated by passage of several volumes of low ionic strength buffer (0.0035 M NaCl). Prior to column chromatography, 14 ml of Amicon prepared BDEF was lyophilized, resuspended in 3 ml PBS, and dialyzed extensively against 0.0035 M NaCl buffer. Two ml was then loaded into the column, and protein was eluted using an increasing ionic strength buffer (linear gradient ranging from 0.0035 M to 1.0 M NaCl). The fractions, collected in 2 ml volumes, were dialyzed against PBS, filter sterilized, and tested for biological activity.

4.3 RESULTS

4.3.1 Immunoenhancing Activity of BDEF

The data presented in this chapter describes the ability of Bone Marrow Derived Enhancing Factor, BDEF, to augment immune responses. Duwe and Singhal (1978) had previously reported that enhancing factor preparations from cultured BM cells of MW > 20 kDa could enhance the primary in vitro PFC response to SRBC. In this study, Figure 4.1 demonstrates that BDEF (MW > 10 kDa) enhanced the Ab response to SRBC, as well as proliferation to alloantigen in the MLR. In both systems, enhancement was dose-dependent such that BDEF was active within a defined dose range.

Fractionation of BM by elutriation as previously described in Results section 2.3.6 (Chapter 2) demonstrated that enhancing activity was associated with small, high density cells typically of lymphoid morphology. When elutriated BM cell fractions were cultured as described in section 3.2.1, and tested directly without Amicon filtration in a PFC response, the fraction 1 supernatants contained BDEF activity (Table 4.1). This was consistent with the ability of fraction 1 cells to enhance PFC and MLR responses as shown in Table 2.1 and Figure 2.8. The ability of supernatants from enriched cells to enhance the PFC response confirms previous findings by Duwe and Singhal (1978) that whole BM culture supernatants could augment PFC responses in vitro.

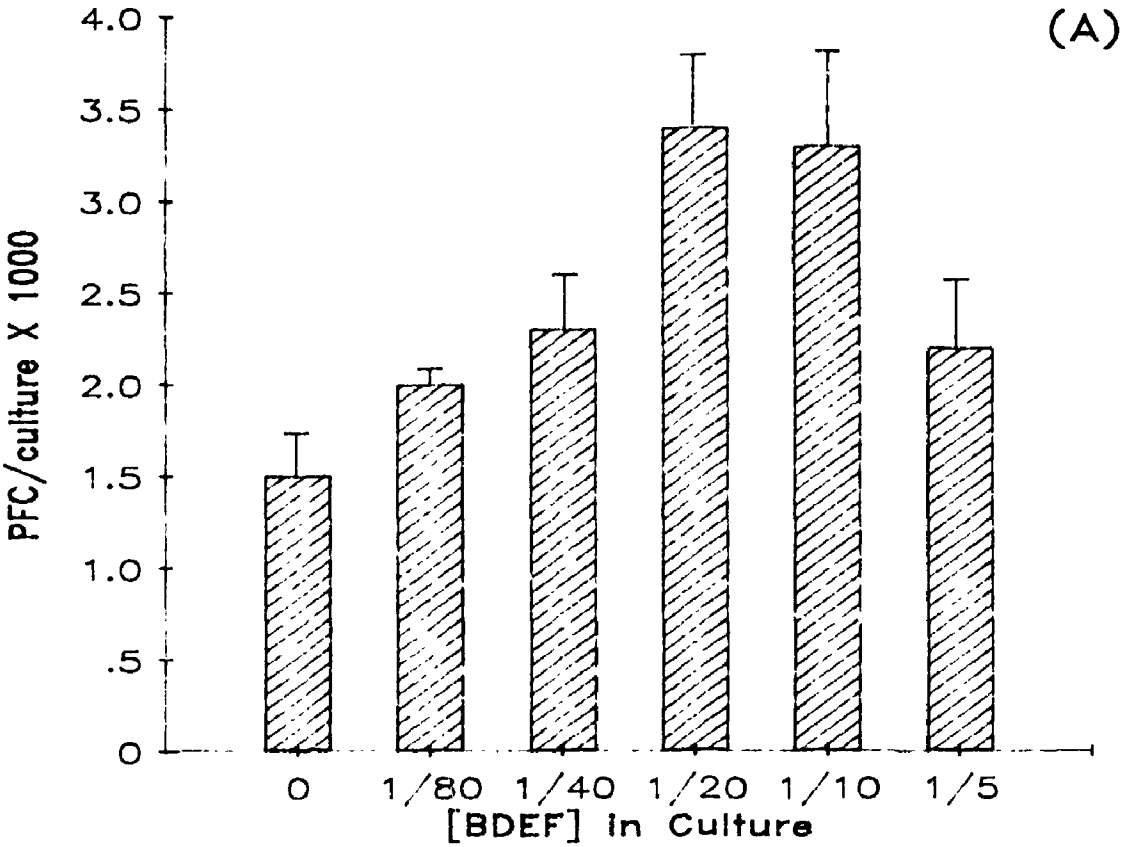
Table 4.2 demonstrates that BDEF only enhanced the response to a single antigen in culture, and did not activate other Ag-specific clones due to a bystander effect. This suggested that BDEF functions

FIGURE 4.1

Enhancement of the PFC Response and MLR by BDEF

(A) The PFC consisted of Balb/c spleen cells cultured in the presence of SRBC with or without BDEF produced from Balb/c mice. The background response of spleen cells in the absence of SRBC was 356 ± 36 PFC/culture.

(B) The MLR consisted of 5×10^5 Balb/c spleen cells plated with 5×10^5 irradiated C57Bl/6 spleen cells as a source of stimulators. The background response of C57Bl/6 spleen cells in the presence of autologous stimulators was 12,891 ± 1,353 CPM. In both panels (A) and (B) vertical bars represent standard deviation.


(A)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0
PFC/culture X 1000
0
1/80
1/40
1/20
1/10
1/5
[BDEF] in Culture

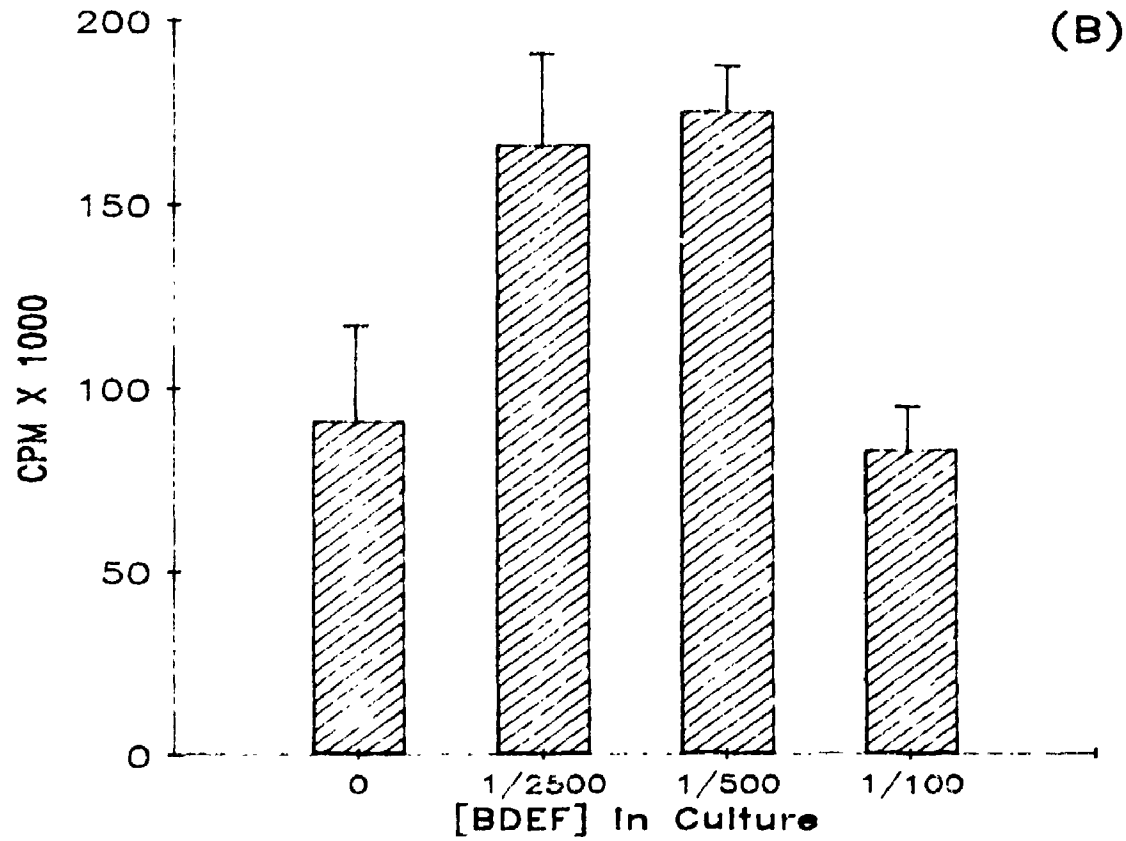

(B)
200
150
100
50
0
CPM X 1000
0
1/2500
1/500
1/100
[BDEF] in Culture

TABLE 4.1

Production of BDEF by the Enhancing Cell Fraction of BM After Elutriation

Preparation added to culture[a]	PFC/culture ± S.D.	% shift from control	P Value
--	3,344 ± 224	--	--
Fraction 1			
1/10	5,531 ± 554	+ 65	<.0005
1/20	4,516 ± 365	+ 35	<.001
1/40	3,156 ± 561	- 6	N.S.[b]
Fraction 2			
1/10	3,891 ± 208	+ 16	<.01
1/20	4,219 ± 640	+ 26	<.025
1/40	3,516 ± 368	+ 5	N.S.[b]
Fraction 3			
1/10	3,969 ± 530	+ 19	<.05
1/20	2,875 ± 358	- 14	<.05
1/40	3,673 ± 569	+ 10	N.S.[b]

[a] Elutriated cell fractions were cultured at a density of 10^7 cells/ml in serum-free medium as described for the production of both BDSF and BDEF (Materials and Methods section 3.2.1). Cell supernatants were tested directly for activity in a PFC assay. The dilutions indicated refer to the final concentration of supernatant in culture. The background response of unstimulated cells was 344 ± 79 PFC/culture.

b N.S. refers to not significant.

TABLE 4.2

BDEF is Antigen-Dependent in its Activity

Culture Conditions[a]	PFC/culture ± S.D.[b] SRBC	Ox RBC
Exp. 1		
Neg. Control	375 ± 75	19 ± 11
SRBC	8,906 ± 1,211	150 ± 65
Ox RBC	330 ± 90	1,266 ± 117
SRBC + BDEF	22,688 ± 2,636[c]	356 ± 98
Ox RBC + BDEF	281 ± 94	2,419 ± 307[d]
Exp. 2		
Neg. Control	212 ± 74	362 ± 161
SRBC	3,563 ± 497	463 ± 120
Ox RBC	212 ± 100	1,300 ± 200
SRBC + BDEF	10,637 ± 905[c]	575 ± 91
Ox RBC + BDEF	385 ± 116	2,263 ± 290[e]

[a] Mishell-Dutton cultures were set up as described in Materials and Methods section 2.2.3.

[b] PFC were enumerated according to the double slide method of Cunningham and Szenberg (1968).

[c] P value was <.0005 as compared to the SRBC control.

[d] P value was <.0005 as compared to the Ox RBC control.

[e] P value was <.001 as compared to the Ox RBC control.

TABLE 4.3

Inability of BDEF to Act as a Polyclonal Activator

Preparation added to culture[a]		PFC/culture ± S.D.		
LPS (ug/ml)	BDEF	Ox RBC	SRBC	TNP-SRBC
—	—	6 ± 13	38 ± 26	281 ± 55
100	—	295 ± 54	450 ± 74	638 ± 125
50	—	167 ± 41	300 ± 66	700 ± 170
10	—	263 ± 65	256 ± 83	638 ± 211
5	—	263 ± 60	319 ± 97	619 ± 138
—	1/10	38 ± 35[b]	21 ± 25[b]	237 ± 75[b]

[a] The dose of BDEF added to culture gave optimal enhancement in a standard PFC assay against SRBC. LPS was added in 0.1 ml volumes to achieve the required concentrations.

[b] These values were not significant when compared to the control with medium only. All other values were significant ($P<.005$) as compared to the respective control with only medium added.

to augment antigen driven responses and cannot act as a polyclonal activator of several Ag-specific clones. This was supported further by the evidence presented in Table 4.3. Spleen cells were cultured in the presence of medium, BDEF, or a known polyclonal B-cell activator, LPS, and after 4 days the cells were assayed for polyclonal antibody production against 3 antigens: Ox RBC, SRBC, and TNP-SRBC. BDEF was totally ineffective at inducing polyclonal PFC, in contrast to LPS, which was a potent activator of the polyclonal PFC response.

4.3.2 Effect of BDEF on Thymocytes

Duwe and Singhal (1978) had previously shown that enhancing factor from BM cultures could induce proliferation of both spleen cells and thymocytes. This was supported by the results in Table 4.4 that BDEF induced thymocyte proliferation in a dose-dependent manner. In order to elucidate which population of thymocytes was primarily affected by BDEF, thymocytes were separated according to their ability to bind to the lectin peanut agglutinin (PNA). As described by Caplan and Rothenberg (1984) using a panning technique, PNA^+ thymocytes represented an immature (cortical) phenotype, while PNA^- thymocytes most resembled mature (medullary) cells. When either population was cultured in the presence of BDEF, only the PNA^- thymocytes proliferated significantly above background (Table 4.5). The purity of the PNA^+ and PNA^- populations was confirmed by the T-cell mitogen PHA, which only induced proliferation of the PNA^- (mature) thymocytes.

The ability of PNA^- thymocytes to proliferate in the presence of BDEF suggested that enhancement may be mediated via expansion of T-cell help for the Ab response. This is supported by the findings in Figure 4.2 that thymocytes pre-incubated with BDEF, washed, and

TABLE 4.4

Induction of Thymocyte Proliferation by BDEF[a]

[BDEF] in culture	CPM ± S.D.	P Value
--	487 ± 150	--
1/4	1,360 ± 360	<.005
1/8	1,524 ± 254	<.0005
1/16	1,697 ± 407	<.001
1/32	2,051 ± 257	<.0005
1/64	1,569 ± 270	<.0005
1/128	1,560 ± 537	<.005
1/256	907 ± 319	<.05
1/512	505 ± 155	N.S.[b]

[a] For measurement of thymocyte proliferation, 5 X 10^5 cells were cultured in 96 well flat-bottomed plates for a total of 48 hr. During the final 24 hr, wells were pulsed with 2 uCi of ^{3}H-TdR, after which the plates were harvested and thymidine incorporation was determined by liquid scintillation counting.

b N.S. refers to not significant.

TABLE 4.5

Effect of BDEF on Peanut Agglutinin Separated Thymocytes

Preparation added to culture	Thymocyte Proliferation (CPM ± S.D.)[a]		
	Unseparated	PNA^+	PNA^-
Medium	504 ± 137	360 ± 102	802 ± 70
PHA (100 ug/ml)	3,869 ± 430	820 ± 168	21,758 ± 2,403
BDEF (1/8)	798 ± 177	312 ± 155[b]	1,506 ± 338
BDEF (1/16)	718 ± 168	821 ± 421[b]	2,603 ± 509
BDEF (1/32)	1,114 ± 351	471 ± 266[b]	2,725 ± 222
BDEF (1/64)	991 ± 168	401 ± 174[b]	2,423 ± 92

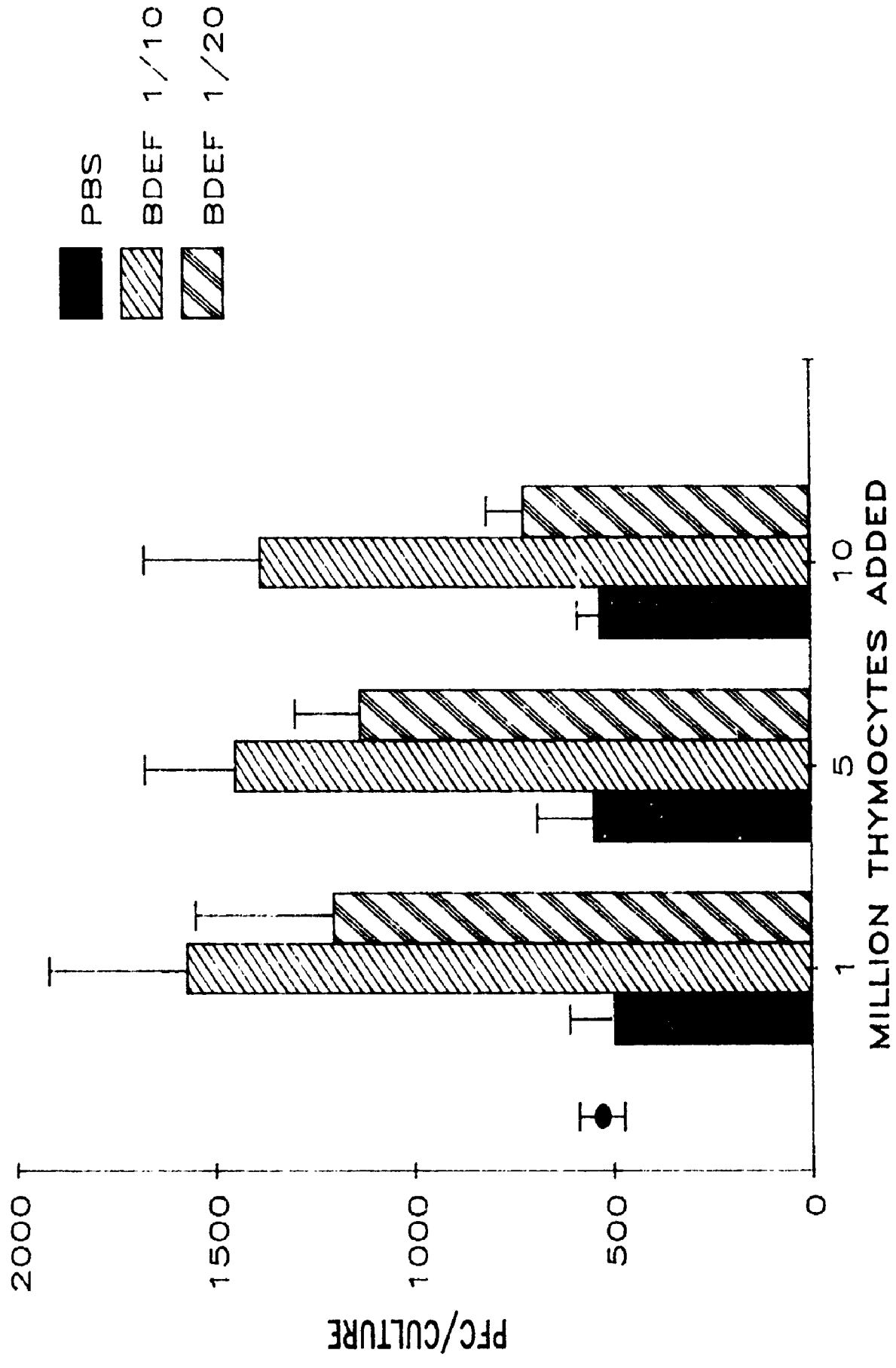
[a] For cell proliferation, the individual populations were cultured in 96 well flat-bottomed plates for a total of 48 hr. During the final 24 hr of culture, wells were pulsed with 2 uCi of ^{3}H-TdR, after which the plates were harvested and thymidine incorporation was determined by liquid scintillation counting.

b All of these values were not significant compared to the medium control. All other values were significant by $P < .05$ as compared to their respective medium controls.

FIGURE 4.2

Thymocytes Pre-Incubated with BDEF Augment the PFC Response

Thymocytes were pre-incubated with either PBS or BDEF for 3 hr at 37°C. Cells were then washed three times to remove BDEF, resuspended in tissue culture medium, and added to Mishell-Dutton cultures. The control response was 522 ± 57 PFC/culture as represented by the closed circle. The background response of unstimulated cells was 63 ± 37 PFC/culture. As a control, the 1/10 and 1/20 dilutions of BDEF added directly to culture resulted in 1,600 ± 350 and 1,484 ± 454 PFC/culture, respectively. Vertical bars represent standard deviation.


PBS
BDEF 1/10
BDEF 1/20
PFC/CULTURE
2000
1500
1000
500
0
1
5
10
MILLION THYMOCYTES ADDED

transferred to Mishell-Dutton cultures could enhance the Ab response compared to thymocytes incubated with PBS, which had no effect. Furthermore, cellular absorption of BDEF by both whole thymocytes and more effectively by splenic T-cells (Table 4.6) suggest further that BDEF functions via the T-cell arm of the response.

4.3.3 Failure of BDEF to act as a Costimulator of Thymocyte Proliferation

The ability of BDEF to cause proliferation of thymocytes promoted a direct comparison to IL-1 and IL-2, which are mitogenic for thymocytes and can synergize effectively with sub-optimal doses of T-cell mitogens such as Con A and PHA. BDEF was tested for the ability to synergize with Con A to induce thymocyte proliferation in the costimulator assay as described by Shaw et al. (1978). Table 4.7 demonstrates that BDEF could not synergize with Con A, however by itself was mitogenic for thymocytes. This is in contrast to IL-1 and IL-2, which both displayed costimulator activity. As expected, IL-1 was not as efficacious as IL-2 in this assay.

To further support the evidence that BDEF lacked IL-2 activity, BDEF was compared with IL-2 for the ability to promote growth of the IL-2 dependent cell line CTLL-2. As shown in Table 4.8, BDEF could not induce CTLL-2 proliferation above background at all doses tested, while IL-2 induced a vigorous proliferative response.

4.3.4 Physical Properties of BDEF

The production of BDEF from cultured BM cells was shown to require active protein synthesis. In the presence of 10 ug/ml of cycloheximide, which resulted in an 85% decrease in protein synthesis, there was a lack of BDEF production by BM cells as compared to cells cultured in the absence of cycloheximide (Table 4.9). The decrease in

TABLE 4.6

Removal of BDEF Activity by Cellular Absorption

Preparation added to culture[a]	Absorbing Population[b]	PFC/culture ± S.D.	P Value
—	—	5,000 ± 1,290	—
BDEF	—	10,250 ± 2,270	<.005[c]
BDEF	Splenocytes	9,750 ± 2,210	N.S.[d]
BDEF	Bone Marrow	8,500 ± 1,690	N.S.[d]
BDEF	Thymocytes	7,330 ± 1,570	<.05[d]
BDEF	Splenic B-cells[e]	9,830 ± 1,810	N.S.[d]
BDEF	Splenic T-cells[f]	4,690 ± 1,250	<.005[d]

[a] BDEF was added at a predetermined optimal dilution in 0.2 ml.

[b] BDEF was absorbed with 1 X 10^7 cells in a 0.5 ml volume for 60 min at 37°C.

[c] P value compared to medium control.

[d] P value compared to BDEF control.

[e] Splenic B-cells were prepared by treatment of whole spleen cells with anti-Thy 1.2 Ab plus complement to remove T-cells. Adherent cells were removed by plastic adherence for 1 hr at 37°C.

[f] Splenic T-cells were prepared by passage of whole spleen cells through nylon wool columns.

TABLE 4.7

Failure of BDEF to Synergize with Mitogen in the Costimulator Assay

Preparation added to culture[a]	CPM ± S.D.	P Value
Medium	135 ± 42	—
Con A	383 ± 66	<.005[b]
IL-1	225 ± 15	<.005[b]
IL-2	356 ± 35	<.005[b]
BDEF	306 ± 68	<.005[b]
Con A + IL-1	1,119 ± 144	<.005[c]
Con A + IL-2	3,835 ± 918	<.005[c]
Con A + BDEF	380 ± 24	N.S.[c]

[a] 50 ul of each preparation was added to culture in quadruplicate. The final concentration of Con A in culture was 5 ug/ml. Each CPM value represents the optimum responses of several doses tested for each preparation or combination of preparations.

[b] P values compared to control cells cultured with medium.

[c] P values compared to thymocytes cultured with Con A only (N.S. refers to not significant).

TABLE 4.8

Failure of BDEF to Support Growth of the IL-2 Dependent Cell Line, CTLL-2[a]

Preparation added to culture[b]	CPM ± S.D.	P Value
Medium	600 ± 347	--
BDEF (1/4)	637 ± 182	N.S.[c]
BDEF (1/8)	323 ± 195	N.S.[c]
BDEF (1/16)	541 ± 229	N.S.[c]
IL-2 (1/128)	188,730 ± 2,195	<.0005

[a] The CTLL-2 assay was performed as described in Materials and Methods section 3.2.2.

b 50 ul of each preparation was added to culture in quadruplicate. The BDEF preparation used was pretested for its ability to augment thymocyte proliferation.

[c] N.S. refers to not significant.

the Ab response could not be attributed to contaminating cycloheximide in the Mishell-Dutton cultures since: a) Ab synthesis did not drop below the positive control value; and b) cycloheximide (MW = 281) would be removed during dialysis.

The ability of cycloheximide to inhibit production of BDEF (Table 4.9) suggested that it was a protein, or at the very least that protein synthesis was required for its production. Puchalski (1981) had previously demonstrated that murine BDEF was sensitive to the proteolytic enzyme trypsin, suggesting that it was a protein. A common feature of proteins is their ability to be precipitated out of solution by ammonium sulfate. To confirm the protein nature of BDEF, equal volumes (3 ml) were precipitated with either 45 or 90% saturated ammonium sulfate (SAS), and then tested in a PFC assay for enhancing activity. Figure 4.3 demonstrates that BDEF was effectively precipitated using 90% SAS, while 45% SAS did not precipitate any activity. Mortari et al. (1989) had also shown that human BDEF could be precipitated by 90% SAS, and were able to derive an Ab in rabbits directed against human BDEF, called anti-p60 Ab. However, while this Ab could effectively interact with human protein, there was no binding of anti-p60 Ab to either murine BDEF or whole murine BM supernatants using an ELISA system (Table 4.10).

In order to separate the enhancing activity from the other activities in BM supernatants, HPLC and anion exchange chromatography were employed. Using HPLC gel filtration, BM proteins were resolved into 5 distinct fractions based on the profile obtained at 280 nm. These individual fractions were collected and tested in a PFC assay for enhancing activity. Figure 4.4 demonstrates that all of the activity

TABLE 4.9

Inhibition of BDEF Production by Cycloheximide

Pretreatment with Cycloheximide (ug/ml)	[Supernatant] in Culture	PFC/culture ± S.D.	P Value
—	—	925 ± 133[a]	—
0	1/10	1,017 ± 216	N.S.[b]
0	1/50	1,606 ± 180	<.0005
0	1/250	1,844 ± 331	<.005
0	1/1250	1,356 ± 299	<.025
0	1/6250	1,019 ± 239	N.S.[b]
10	1/10	1,038 ± 207	N.S.[b]
10	1/50	1,100 ± 128	N.S.[b]
10	1/250	1,175 ± 189	<.05
10	1/1250	1,088 ± 183	N.S.[b]
10	1/6250	1,158 ± 183	<.05

[a] The background response of unstimulated cultures was 88 ± 64 PFC/culture.

b N.S. refers to not significant.

FIGURE 4.3

Ammonium Sulfate Precipitation of BDEF

BDEF prepared from Balb/c mice was precipitated with either 45 or 90% saturated ammonium sulfate and tested in a PFC response using syngeneic Balb/c spleen cells. All 3 samples were tested at a 1/40 final concentration in culture. The control response, represented by the closed circle, was 2,166 ± 218 PFC/culture. The background response of spleen cells in the absence of SRBC was 131 ± 39 PFC/culture. Vertical bars represent standard deviation.

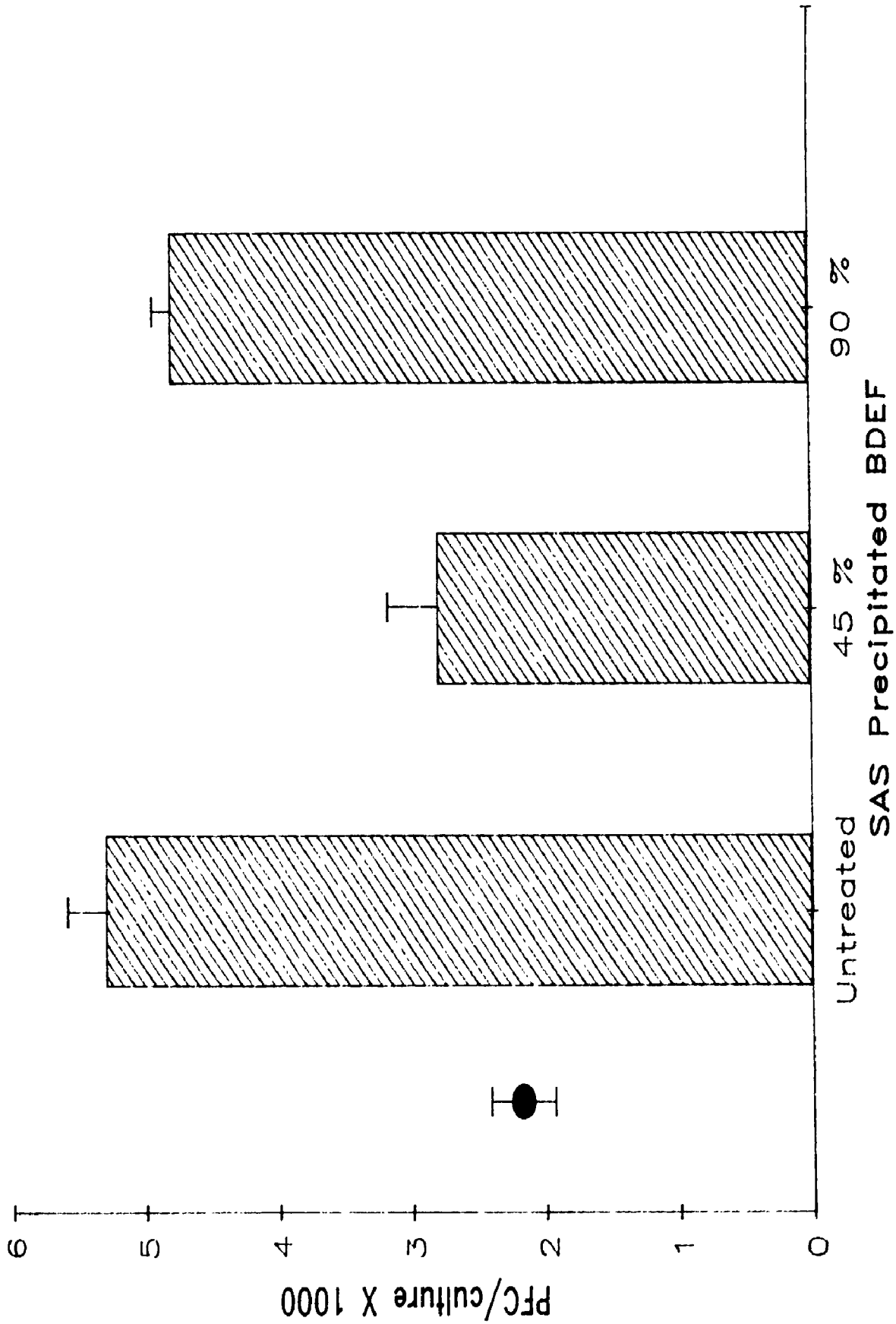

PFC/culture X 1000
0
1
2
3
4
5
6
Untreated
45 %
90 %
SAS Precipitated BDEF

TABLE 4.10

Failure of Murine BDEF to be Recognized by Specific Antibody Against Human BDEF in an ELISA

Substrate	OD Reading
Buffer only	0.064
Human BM supernatant (2.5 ug/well)[a]	2.887
Murine BM supernatant (2.5 ug/well)	0.056
Murine BM supernatant (1.25 ug/well)	0.053
Murine BM supernatant (.625 ug/well)	0.058
Murine BDEF (2.5 ug/well)	0.056
Murine BDEF (1.25 ug/well)	0.061
Murine BDEF (.625 ug/well)	0.057

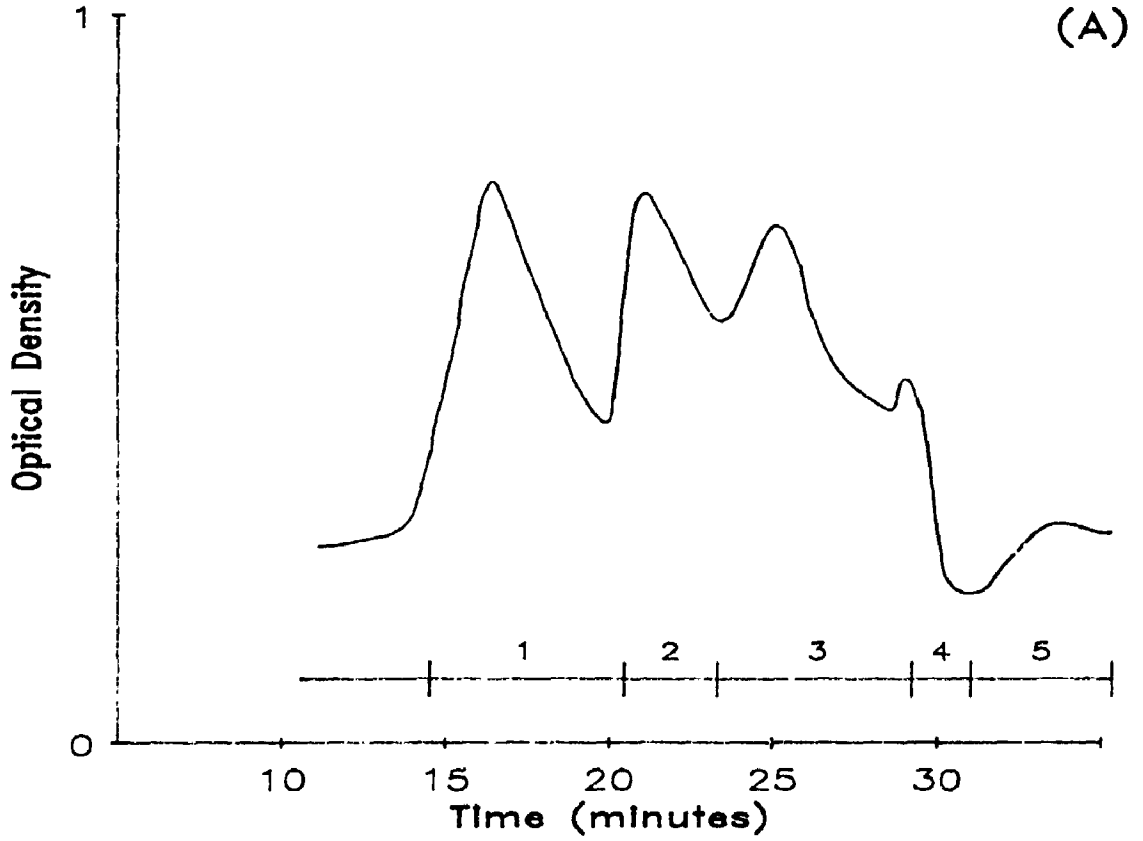
[a] Human BM supernatant was prepared as already described for murine BM supernatants.

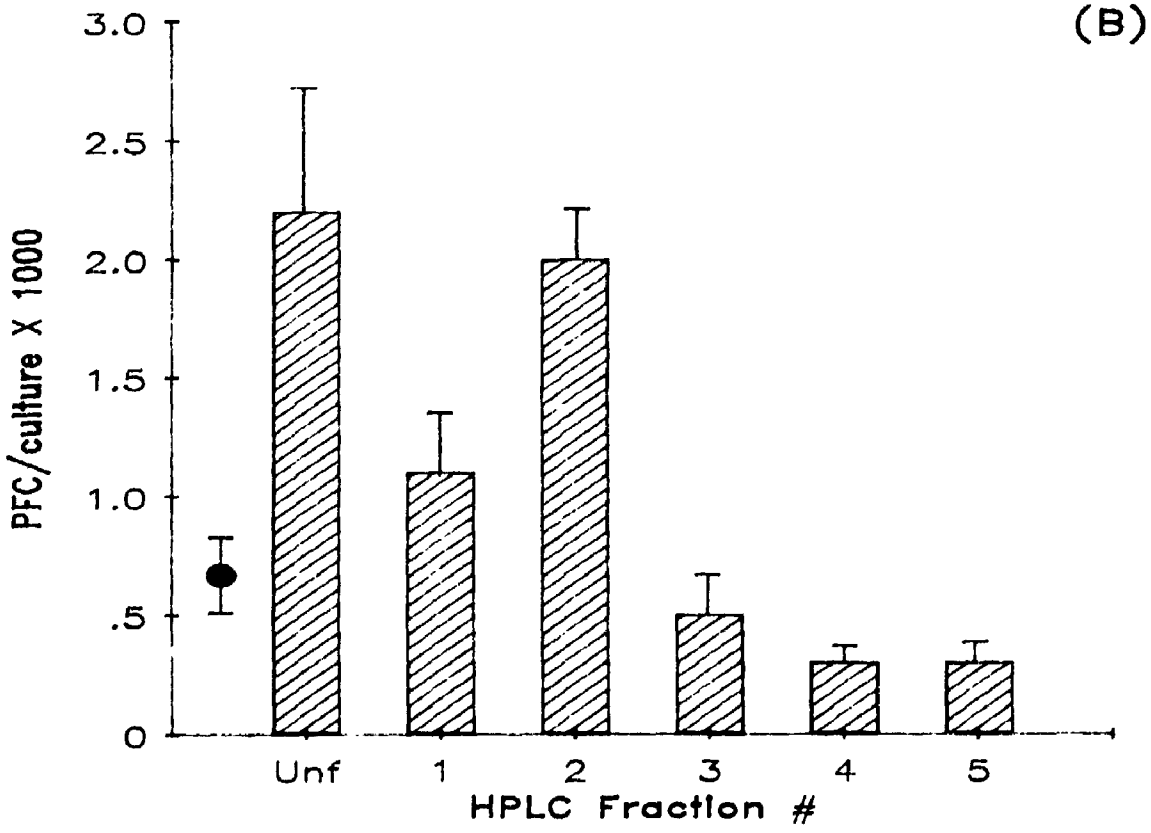
FIGURE 4.4

Fractionation of BM Supernatants by HPLC

(A) HPLC fractionation was performed as described in Materials and Methods section 4.2.8. The curve represents the elution profile of BM proteins scannned at 280 nm. The numbers 1-5 indicate the fractions which were collected according to the peaks on the curve. The MW of the standards were as follows: Thyroglobulin, 670 kDa (15′); IgG, 158 kDa (20′); Ovalbumin, 44 kDa (22′); Myoglobin, 17 kDa (26′); Vitamin B-12, 1.35 kDa (32′).

(B) The fraction 1-5 were tested for their ability to enhance the PFC response of Balb/c spleen cells. Unfractionated BM supernatant was also tested. In each case material was added to a final concentration of 1/100 in culture. The closed circle represents the control PFC response, which was 654 ± 158 PFC/culture. The background response of unstimulated cells was 58 ± 44 PFC/culture. Veritcal bars represent standard deviation.


(A)
1
0
Optical Density
1
2
3
4
5
10
15
20
25
30
Time (minutes)


(B)
3.0
2.5
2.0
1.5
1.0
.5
0
PFC/culture X 1000
Unf
1
2
3
4
5
HPLC Fraction #

was present in fraction 2. Unfractionated material also augmented the response, as was previously reported by Duwe and Singhal (1978). The peak MW of fraction 2 was 85 kDa, but its range was from 100 to 45 kDa.

To further understand the physical nature of BDEF, a preparation obtained by Amicon ultrafiltration was applied to a DEAE-cellulose anion exchange column and fractions were collected by eluting bound material with a linear gradient of buffer of increasing ionic strength. The fractions, which were collected in 2 ml volumes, were filter sterilized and tested in either a PFC response or for the ability to augment thymocyte proliferation. As shown in Figures 4.5 and 4.6 the majority of the activity eluted at a fairly high ionic strength of approximately 0.3-0.6 M NaCl, suggesting a fairly strong negative charge on the molecule. There was also a small peak which eluted at approximately 0.8-0.9 M NaCl, which could either represent a distinct entity or material that was strongly bound to the column.

Lastly, to compare BDEF with other lymphokines such as IFN-gamma, which is sensitive to low pH, BDEF was dialyzed overnight at 4°C against either PBS (pH 7.4) or glycine-HCl buffer (pH 2.2) and subsequently tested for activity after equilibration dialysis in PBS. The results in Figure 4.6 clearly demonstrate that BDEF was not affected by low pH, similar to both murine and human IL-2. Furthermore, BDEF was unable to inhibit proliferation of the IFN-gamma sensitive cell line WEHI-279 (Table 4.11). This was in contrast to the decrease of proliferation in the presence of 20 U/ml of rIFN-gamma, which could be reversed by the addition of anti-IFN-gamma Ab (XMG-1.2).

FIGURE 4.5

Enhancement of the PFC Response by DEAE-Cellulose Separated BDEF

BDEF was applied to a DEAE-Cellulose column as described in Materials and Methods section 4.2.9. Fractions were collected in 2 ml volumes and tested individually in a PFC response of syngeneic Balb/c spleen cells. The solid line represents the number of PFC/culture of each fraction, while the dotted line represents the approximate molarity at which each fraction eluted from the column. The positive control response of spleen cells and SRBC, represented by the closed triangle, was 4,425 ± 951 PFC/culture. The background response in the absence of antigen was 388 ± 230 PFC/culture. Vertical bars represent standard deviation.

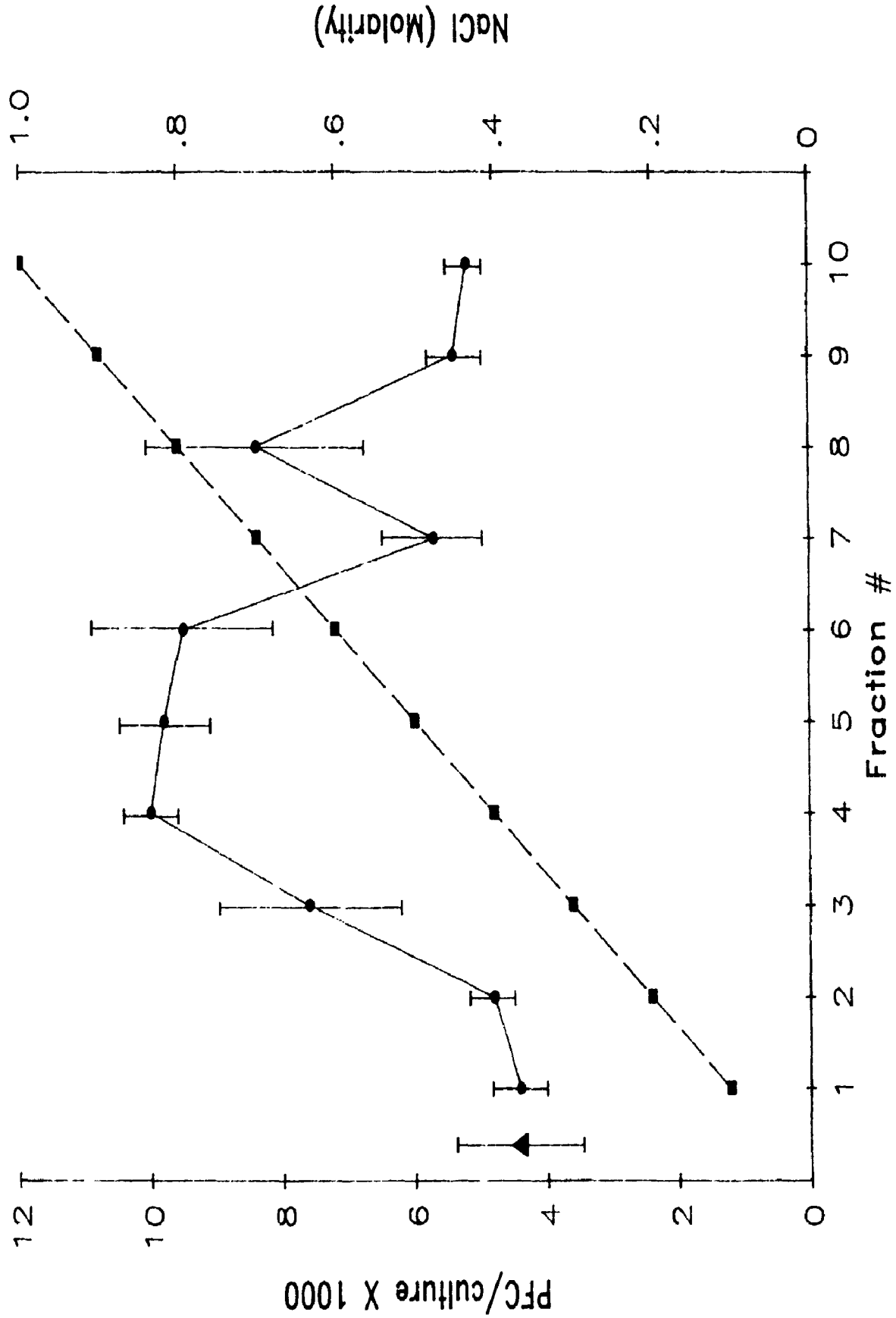

NaCl (Molarity)
1.0
.8
.6
.4
.2
0
PFC/culture X 1000
12
10
8
6
4
2
0
Fraction #
1
2
3
4
5
6
7
8
9
10

FIGURE 4.6

Induction of Thymocyte Proliferation by DEAE-Cellulose Separated BDEF

Fractions were collected in 2 ml volumes and tested individually for the ability to induce proliferation of Balb/c thymocytes. Thymocytes were cultured at a density of 5 X 10^5 cells/well in 96 well flat-bottomed plates for 24 hr, and pulsed for a further 24 hr with ^{3}H-TdR. CPM were determined by liquid scintillation counting. The solid line represents the CPM of each fraction, while the dotted line represents the approximate molarity at which each fraction eluted from the column. The background response of unstimulated thymocytes was 185 ± 19, represented by the closed triangle. Vertical bars represent standard deviation.

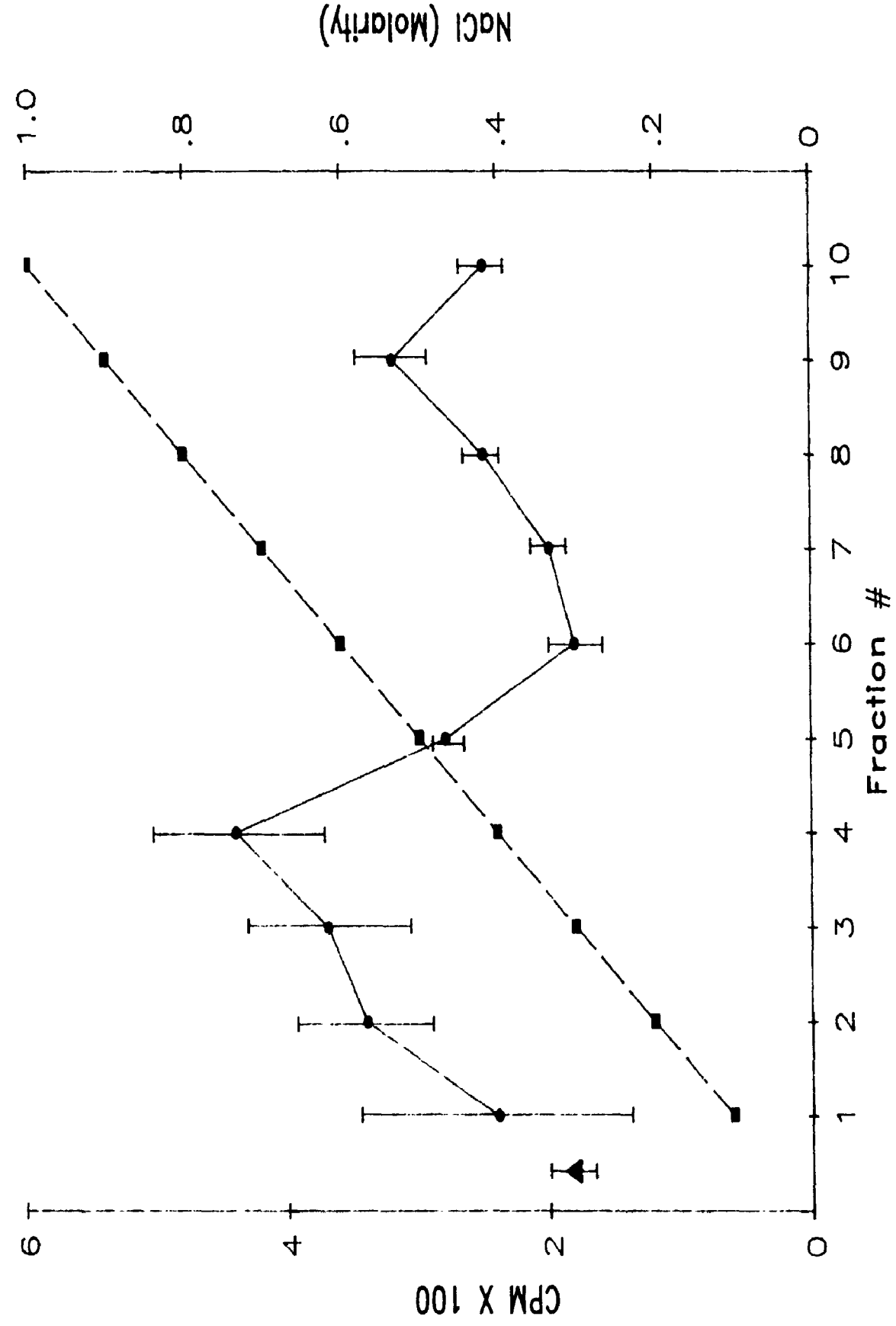

NaCl (Molarity)
1.0
.8
.6
.4
.2
0
Fraction #
1
2
3
4
5
6
7
8
9
10
CPM X 100
6
4
2
0

FIGURE 4.7

Insensitivity of BDEF to low pH

BDEF prepared from Balb/c mice was treated as described and tested for enhancing activity in a PFC assay using syngeneic Balb/c spleen cells as responders. The closed circle represents the positive control response in the absence of BDEF, which was 2,100 ± 178 PFC/culture. The background response in the absence of SRBC was 178 ± 64 PFC/culture. Vertical bars represent standard deviation.

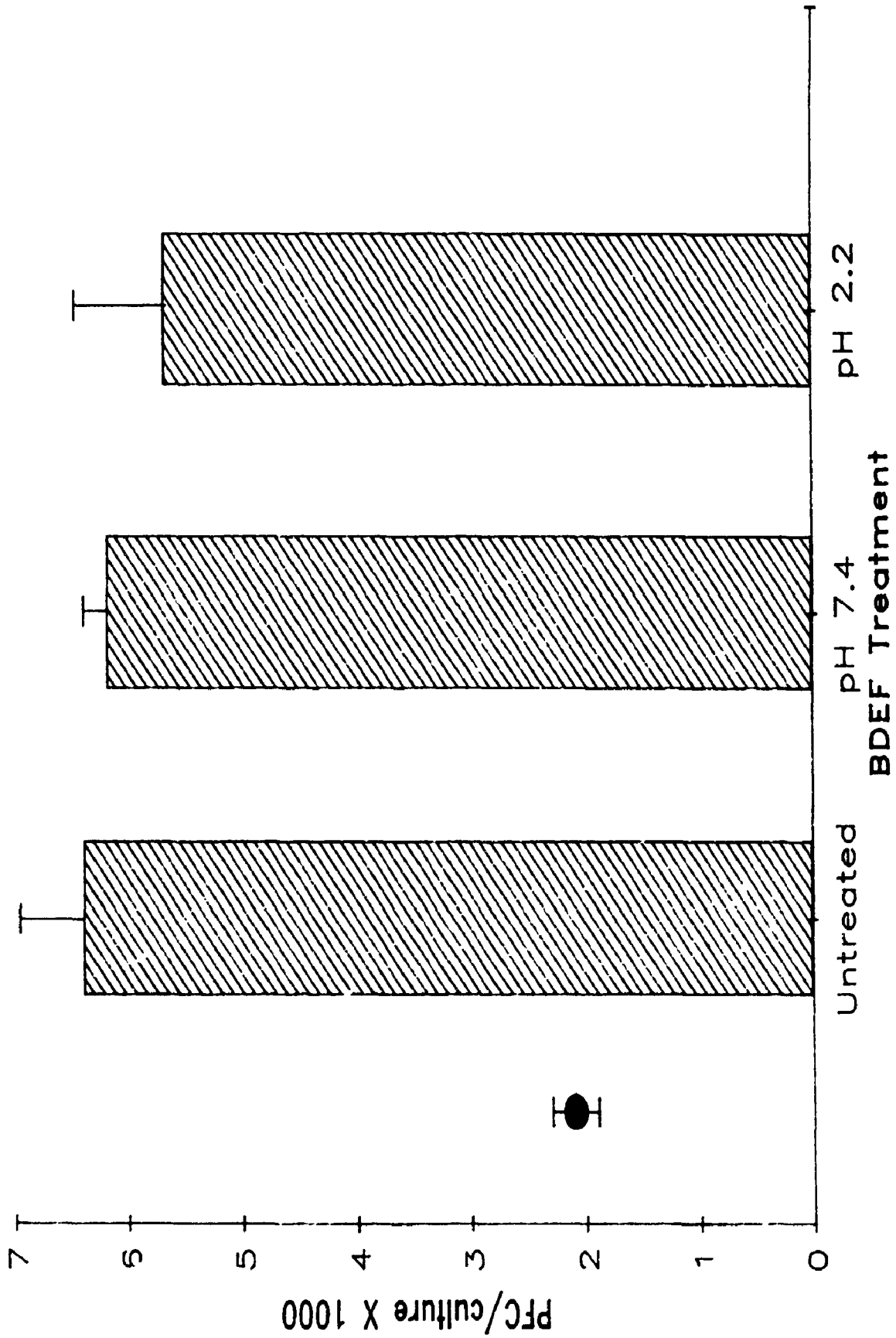

PFC/culture X 1000
0
1
2
3
4
5
6
7
Untreated
pH 7.4
pH 2.2
BDEF Treatment

TABLE 4.11

BDEF Does Not Inhibit Proliferation of the Cell Line Wehi-279

Preparation added to culture[a]	DPM ± S.D.
Medium	299,922 ± 29,830
BDEF (1/2)	333,603 ± 456
BDEF (1/4)	358,294 ± 13,030
BDEF (1/8)	366,603 ± 28,860
BDEF (1/16)	379,789 ± 3,482
IFN-gamma (20 U/ml)	219,441 ± 14,159
IFN-gamma (20 U/ml) + 200 ng/ml XMG-1.2	359,253 ± 19,874
200 ng/ml XMG-1.2	361,068 ± 1,755

[a] The assay for WEHI-279 cells is described in Materials and Methods section 3.2.X. The dose of BDEF indicated represents the final dilution in culture. IFN-gamma and XMG-1.2 (anti-IFN-gamma Ab) were both kindly provided by Dr. G. Strejan.

4.4 DISCUSSION

The results presented in this chapter describe the activities of a naturally occurring mediator, BDEF, which is the constitutive product of cultured BM cells. Upon crude fractionation by Amicon ultrafiltration, BDEF is contained in a preparation of molecules > 10 kDa in size. The production of the factor requires culture of BM cells and is not simply an elaboration of intracellular components after death in culture, since Drury and Singhal (1974) have previously shown that BM sonicates possess no immunoregulatory activity. To support this further is the observation that introduction of the protein synthesis inhibitor cycloheximide to BM cultures resulted in a loss of enhancing activity from BM culture supernatants. The constitutive production of BDEF by cultured BM cells appears to be a unique feature of this molecule.

As previously reviewed in section 4.1, many biological mediators, or cytokines, are produced by activated cells usually located in peripheral sites of immune function. The signal to induce cytokine production is typically activation by antigen, mitogen, phorbol esters, or even other cytokines. The cell types which synthesize and secrete many of the commonly described cytokines are either T_H cells or macrophages (Coffman et al., 1988; Dinarello, 1988; Beutler and Cerami, 1989). Factors which are necessary for hematopoietic cell development are also commonly produced by BM derived cells, called stromal cells, which comprise many types of adherent cells and fibroblasts derived from long term BM culture (Kincade, 1987). Stromal cells can produce colony stimulating factors (CSFs)

after stimulation with IL-1 or TNF (Miyajima et al., 1988; Zucali et al., 1988) and transformed stromal cell lines secrete IL-7 (Namen et al., 1988). Other examples of CSF production by transformed cell lines include production of M-CSF by L-cells (Metcalf and Moore, 1971), and production of IL-3 by the myelomonocytic leukemia WEHI-3 (Ihle, 1989). The production of BDEF from BM cells is unique in that activation events are not required, and production is not associated with a transformed phenotype. Constitutive production of BDEF may relate to its role as a potential growth regulator in the BM.

Inhibition of BDEF production by treatment of BM cultures with cycloheximide confirmed previous findings that murine BDEF was protein in nature. It was demonstrated by Puchalski (1981) that BDEF activity was heat sensitive and sensitive to enzymatic digestion with trypsin. Further evidence presented here on the protein nature of BDEF was its ability to be precipitated by 90% SAS. This was similar to human BDEF, which could also be precipitated by 90% SAS (Mortari, 1989). The relative size of murine BDEF appears to be similar to human BDEF, purifying in a broad peak between 45-100 kDa. Due to this similarity and the ability of human BDEF to act on murine cells, murine BDEF was tested for reactivity in an ELISA using a rabbit Ab derived against human BDEF, called anti-p60 (Mortari et al., 1989). However there was no cross-reactivity against murine BDEF, suggesting that although the mediators possess similar biological activity, they may differ structurally.

The ability of BDEF to augment Ab responses to SRBC in vitro and to induce proliferation of thymocytes confirmed previous findings in this laboratory (Duwe and Singhal, 1978; Puchalski, 1981; Mortari et

al., 1989). Enhancement of the Ab response was directed toward the Ag present in culture, and in the absence of Ag, BDEF could not induce polyclonal B-cell activation in comparison to LPS. In addition to its ability to augment the Ab response, BDEF was also able to augment proliferation against alloantigen in the MLR. Many of the well characterized cytokines, specifically the interleukins, possess many of the activities ascribed to BDEF. Both IL-1 and IL-2 are essential components of T-cell activation which can augment Ab responses in vitro (Wood and Gaul, 1974; Farrar et al., 1982) and induce thymocyte proliferation, especially in synergy with mitogens (Gery and Waksman, 1972; Paetkau et al., 1976). However, while BDEF is directly mitogenic for thymocytes, it cannot synergize with Con A to induce thymocyte proliferation. In addition, BDEF can further be distinguished from IL-2 by its inability to support proliferation of the IL-2 dependent cell line CTLL-2.

IL-3 was initially characterized by its ability to induce Thy-1 and 20 alpha-SDH expression in spleen cells from nu/nu mice, suggestive of early T-cell differentiation events (Ihle et al., 1981). However, unlike BDEF, neither IL-3 nor GM-CSF was able to induce proliferation of thymocytes, even when assayed on PNA^+ (cortical) and PNA^- (medullary) populations (Keller et al., 1985). By contrast, BDEF induces proliferation of both unfractionated and PNA^- thymocytes, while having no effect on PNA^+ cells. To date there have been no reports indicating that any of the other CSFs can induce proliferation of thymocytes, even in the presence of mitogen.

Both IL-4 and IL-5 are the products of activated T_{H2} cells which enhance Ab responses and influence isotype expression (Coffman et

al., 1988). IL-5 acts late in the Ab response by providing a differentiation signal to activated B-cells, resulting in Ig secretion (Takatsu et al., 1988). However, Puchalski (1981) has previously shown that murine BDEF is maximally active when added at culture initiation. More recently, Mortari et al. (1989) demonstrated that human BDEF lacks detectable IL-5 activity by its inability to restore the PFC response of T-depleted PBLs as compared to human IL-5. IL-4 can induce thymocyte proliferation, but unlike BDEF, only in the presence of a costimulatory signal (Miyajima et al., 1988). Although IL-5 can induce differentiation of CTL (Takatsu et al., 1988), it cannot stimulate proliferation of thymocytes (Aarden et al., 1979). In addition, IL-6, IL-7, and TNF-alpha can all induce thymocyte proliferation, again only in the presence of a costimulatory signal (Wong and Clark, 1988; Chantry et al., 1989; Ranges et al., 1988).

Although BDEF can induce proliferation of both unseparated and PNA^- thymocytes, it is not known whether this is a direct or indirect effect on the target populations. For example, Wong and Clark (1988) have cautioned that interpretation of many activities to IL-1 may be indirect, and activities may be mediated by elaboration of IL-6. In fact, Helle et al. (1989) demonstrated that IL-1 induced proliferation of lectin-stimulated thymocytes is dependent upon endogenous IL-6 production. The function of IL-6 in this pathway is to induce IL-2 production and IL-2R expression, although IL-1 may also increase the sensitivity of the target cells to IL-6 (Helle et al., 1989). Similarly, Le et al. (1988) demonstrated that IL-6 can induce both unseparated and PNA^- thymocytes to proliferate in the presence of lectin via an IL-2 dependent pathway. However they also report that

IL-6 can induce thymocyte proliferation via an IL-2 independent pathway.

More relevant to thymocyte proliferation induced by BDEF are recent findings that several combinations of cytokines can induce proliferation of whole thymocytes and thymocyte subsets in the absence of a mitogenic signal (Suda et al., 1990). They have shown that IL-1 (alpha), IL-6, and TNF-alpha can enhance proliferation of unseparated thymocytes in the presence of IL-2, and that under these conditions IL-1 induced proliferation was not dependent upon endogenous IL-6 or TNF-alpha production. None of the cytokines was able to induce proliferation by itself. Upon separation of the thymocytes into distinct subsets, IL-1 had the greatest affect on double negative ($CD4^-8^-$) cells, IL-6 on single positives ($CD4^+8^-$ and $CD4^-8^+$), and TNF-alpha on both. No combinations of lymphokines could induce proliferation of double positive ($CD4^+8^+$) cells.

Consistent with the ability of these cytokines to induce proliferation is their endogenous production in the thymus (Suda et al., 1990). For example, $CD4^-8^-$ thymocytes produce IL-2, IL-4, IFN-gamma, and TNF-alpha. Thymic epithelial and stromal cells produce IL-1, IL-6, and IL-7, while thymic MØ produce TNF-alpha. Production of these cytokines typically involves a stimulatory agent, such as phorbol ester and calcium ionophore (Suda et al., 1990). It becomes obvious that proliferation of thymocytes due to BDEF may involve a pathway of one or several cytokines. Interestingly, none of the cytokines was able to induce proliferation of double positive thymocytes (Suda et al., 1990). This is consistent with the findings that BDEF can stimulate proliferation of mature PNA^- cells, while not affecting

immature PNA^+ cells which are comprised mainly of postmitotic $CD4^+8^+$ double positive cells (Boyer et al., 1989). It is possible that BDEF may be able to induce IL-2 production and IL-2R expression by PNA^- thymocytes, analogous to IL-6. Alternatively, BDEF may induce IL-6 production, which in turn influences the IL-2/IL-2R activation pathway. Further evidence is required to determine exactly the mechanism of BDEF mediated thymocyte proliferation.

The mitogenic activity of BDEF toward unseparated and PNA^- thymocytes suggests that T-lineage cells may be a possible target for BDEF. This is supported by several findings. Firstly, BDEF is able to augment the MLR, which monitors the proliferation of T_H cells directed against MHC class II antigens. Secondly, thymocytes pre-incubated with BDEF for 3 hr then transferred to Mishell-Dutton cultures can augment the Ab response, as compared to thymocytes incubated in PBS for the same period of time. This indicates that thymocytes which can provide T-cell help for the Ab response are activated or expanded in the presence of BDEF. Alternatively, thymocytes may simply carry-over enhancing activity to the Mishell-Dutton cultures. In either event, it is likely that PNA^- cells are responsible for transferring the activity. Thirdly, splenic T cells and to a lesser extent whole thymocytes are able to absorb enhancing activity from BDEF preparations. Absorption by splenic T-cells is consistent with BDEF induced proliferation of PNA^- thymocytes, since these cells resemble mature splenic T cells by markers and function (Conlon et al., 1982; Caplan and Rothenberg, 1984). Thus it may be that augmentation of both humoral and cellular responses reflects the ability of BDEF to expand or activate T-helper cells participating in those responses.

While the evidence presented here suggests a function of BDEF at the level of mature thymocytes and T-cells, more primitive populations of cells may also be affected. Bains et al. (1986) had shown that human BDEF could augment T-cell colony generation from human BM precursors. The cell surface expression of CD3 was also increased, suggesting development of mature T cells associated with TcR gene rearrangements. The ability of BDEF to induce thymocyte proliferation and support human T-cell colonies suggests that it may mediate extrathymic differentiation of pre-T cells within the BM compartment. There is experimental evidence to suggest that pre-T cells in BM can express receptors for antigen, prior to passage through the thymus (Chervenak et al., 1985; Hurme and Sihvola, 1985; Benveniste and Miller, 1985). Benveniste and Miller (1985) demonstrated that generation of self-Ag reactive cells in liquid culture of BM cells required IL-2 and an unidentified factor present in supernatants from PMA-stimulated EL-4 cells. It is possible that this unknown factor may be BDEF, or that production of BDEF during BM cell culture is an important step in generating Ag-reactive cells. The ability of BDEF to effect both immature and mature cells is not surprising, and similar to cytokines such as IL-7, and their activities suggest that such cytokines may have been highly conserved throughout mammalian evolution.

CHAPTER 5

CONCLUSIONS

Bone marrow is a complex organ which is well recognized as the primary site of hematopoietic cell generation in all mammals. The cells which make up blood, those of lymphoid and myeloid lineages, are produced in millions of numbers daily as a normal function of the bone marrow. Within this array are found many networks of cells and their products whose role is to regulate the development and function of diverse cell types within the BM. This thesis has addressed two key activities resident in BM which function to enhance or suppress immunity.

The suppression resident in murine BM is referred to as natural suppressor (NS) activity. The work presented here has examined further the physical characteristics of the cells mediating NS activity, and confirms the findings of others that NS cells possess no surface markers characteristic of mature cells. In an attempt to generate cell lines possessing NS activity, BM cells were cultured in medium containing IL-2 as a source of growth factor, since NS cell lines had been derived from both neonatal and TLI-spleen in this way (Strober, 1984). While the cells did possess potent NS activity after 3 months in culture, they were also effective at mediating strong NK activity. It has also been confirmed by others that NK cells generated from BM had NS activity (Azuma and Kaplan, 1988). Thus several possibilities exist concerning the relationship between NS and NK cells. Firstly, NK cells may derive from precursors with NS activity, which retain NS function even after maturation to a competent NK effector cell. Secondly, NS and NK cells may belong to a common family of cells possessing similar immunoregulatory activity as proposed by Maier et al. (1986), or they may be unrelated cells possessing similar activity.

Lastly, although the end result is suppression, the mechanism by which responses are suppressed are likely different. Most evidence suggests that NK cells function through a cytolytic effect on the responding population (Targan et al., 1985; Azuma et al., 1989), while the lack of NK function of BM cells suggests that BM NS cells function through a non-cytolytic mechanism.

Perhaps under the appropriate culture conditions, such as an MLR where IL-2 and other cytokines are produced, BM cells added to culture may differentiate into NK effectors which mediate immune suppression. Alternatively the cells could also differentiate into Natural Cytotoxic (NC) cells in the presence of IL-3, and mediate NS activity as described by Jadus and Parkman (1986). Whether such differentiation events even take place is highly speculative. This does address the issue "How can NS cells be cloned?" It is obvious that both Strober et al. (1989) and Jadus and Parkman (1986) have derived clones of cells with NS activity, but are these true representatives of the cells as they existed endogenously in the site where they were initially found? Therefore, research must continue in this area to determine how NS cells become activated in situ, and what differentiation events, if any, must occur prior to committment to NS effector function.

The most important discovery in NS cell biology would be the ability to define these cells using antisera against specific markers which they express on their surface. It is evident from the data presented in this thesis and elsewhere that murine NS cells cannot be defined by antibodies recognizing classical cell lineages. The interesting proposal has been put forward by Noga et al. (1988) that NS

activity may be a function of stem cells in the BM compartment. The purpose of this would be to regulate their own proliferation and differentiation, and would endow them with the ability to regulate other events such as immunological activation in close proximity. This hypothesis is supported by the findings of Sugiura et al. (1988) that BM NS activity is associated with a highly enriched WGA^+ population of cells which possess stem cell function. Sykes et al. (1990) have recently shown that NS cells cannot be depleted from BM using an antibody against pluripotent stem cells, anti-Sca-1. However the possibility exists that more committed progenitors which do not express Sca-1 may be the cells which mediate NS activity. Certainly, the derivation of an antibody against a presently uncharacterized surface marker on NS cells would greatly facilitate the further study of NS cell biology.

Suppression by BM is not restricted only to cells but is also mediated by a soluble suppressor factor, BDSF, which is produced in BM cell cultures. BDSF, like NS cells, inhibits Ab and MLR responses, but has no effect on mitogen-driven responses. This is similar to the activity of a factor called NSF, derived from NS cell clones, which can inhibit the MLR but not mitogen-driven responses. By contrast, BM NS cells and NS cell clones can both inhibit mitogen-driven responses. The inability of both BDSF and NSF to suppress the mitogen response may be related to either the magnitude or type of activation signals (pathways) unique to this response. The ability of NS cells to suppress the mitogen response may reflect a requirement for cell-cell contact in order to inactivate the response, which could not be provided by factors alone.

The results in this thesis demonstrate that BDSF functions to inhibit production of IL-2 after stimulation by Ag. This lack of IL-2 production leads to an anergic state in which Ag-stimulated T-cells cannot become fully activated, and hence are not expanded in culture. It is now apparent that interference at the level of IL-2 is an effective way of inducing clonal anergy (Schwartz, 1990). This concept of T-cell clonal anergy had been forwarded by Malkovsky and Medawar (1984) some years earlier, and is reminiscent of the model of B-cell tolerance proposed originally by Bretscher and Cohn (1970). Further work will be required to determine the exact mechanism by which BDSF inhibits IL-2 production; ie. interference with APC-directed events, or direct inhibition at the level of the IL-2 gene. The prevention of IL-2 production would presumably not only affect T-cell responses, but Ab responses dependent upon T-cell help.

The presence of BDSF in the BM may be to inhibit immune activation and allow BM to carry out its primary function of supplying the body with hematological cells. Although immune activation may be a rare event in the BM, it has been shown by Benveniste and Miller (1985) and Hurme and Sihvola (1985) that anti-self reactive T cells can be generated in BM cell cultures. Incidentally, in vitro generation of these cells requires the presence of IL-2. Hence one potential function of BDSF in the BM may be to prevent generation of such self-reactive cells, given that IL-2 and other factors are present in situ. This is consistent with the proposal by Malkovsky and Medawar (1984) that unbridled IL-2 production could potentially induce autoimmunity, especially since self antigen is continually present.

BDSF may also be conveniently located in the BM as a natural

negative regulator of hematopoiesis. The ability of BDSF to inhibit proliferation of the myelomonocytic leukemia WEHI-3 is consistent with this notion. It is interesting to speculate that BDSF may control differentiation events of relatively early, but committed, cells as witnessed by its effect on WEHI-3, and not the more mature monocytic P388D1 tumor cell line. It would be of great interest to examine differentiation events of several cell lineages produced in BM to determine if BDSF is selective at inhibiting, for example, myeloid vs. lymphoid differentiation. The endogenous production of a potent regulatory molecule in the BM would be analogous to TGF-beta production in other immunologically priveleged sites, such as murine decidua (Clark et al., 1990) and the anterior chamber of the eye (Granstein et al., 1990).

Through biochemical characterization, it is important to determine the physical nature of BDSF. Previous work by McGarry et al. (1982) showed that BDSF migrated on TLC plates as a polar lipid molecule. More refined TLC and reverse-phase HPLC methodologies will be required to obtain pure samples of BDSF. Ultimately, the generation of a monoclonal Ab against BDSF would not only facilitate purification, but would also allow for experimental manipulation in vitro to determine the mechanism of action of BDSF.

Bone marrow cells also produce an immunostimulatory agent, called BDEF. As reported here, BDEF augments both Ab and MLR responses, and is directly mitogenic for thymocytes. The specific population of thymocytes affected by BDEF are PNA^- (medullary) cells, which most resemble mature T-cells (Caplan and Rothenberg, 1984). This is supported by the ability of mature splenic T-cells to absorb out

BDEF activity. It is probable that BDEF augments the response by expansion of T-helper cells which are necessary in both cellular and humoral responses. While it is unlikely that specific cells are activated by BDEF, Ag is required to direct the BDEF mediated signal. Perhaps BDEF "primes" T-helper cells to become more efficiently activated by antigen, much like IL-4 treatment of resting B-cells allows more prompt entry into S phase upon subsequent stimulation (Paul, 1987).

It would be interesting to determine the mechanism by which BDEF induces thymocyte proliferation. In light of recent studies that cytokines may induce thymocyte proliferation via a cascade effect (Suda et al., 1990), it is necessary to determine whether BDEF acts by such a pathway. Most important would be to determine whether BDEF could induce either production of IL-2, or IL2R expression, since this is one major pathway by which thymocytes proliferate (Helle et al., 1989; Le et al., 1988). BDEF may also act in synergy with other cytokines to induce proliferation. Previous studies in this lab demonstrated that human BDEF could augment IL-2 driven development of T-cell colonies from immature BM precursors. This interesting finding suggests that BDEF may be able to contribute to extrathymic differentiation. Miller (1980) and Benveniste and Miller (1985) have clearly shown that under the appropriate conditions BM cells have the potential to develop into self-reactive T-cells, in the absence of a thymic influence. This also raises the possibility that BDEF, and NS cells or BDSF, may form a homeostatic network in the BM compartment to influence cell development.

The biochemical characterization of BDEF is necessary to make

an exact comparison to previously described cytokines, although BDEF appears to be biologically unrelated to most well known molecules. The development of an antibody against murine BDEF would greatly facilitate its characterization, and with the rapid expansion of molecular biology techniques, it should be possible to clone the gene coding for murine BDEF.

In conclusion, this thesis has described two immunoregulatory activities present in murine BM. Undoubtedly, the complex nature of biology predicts that additional regulatory influences must also exist. It will be up to investigators of the future to piece together the mechanisms by which these regulatory agents interact to maintain normal physiologic function.

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