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# Characterization Of Immunoregulatory Cells And Factors Derived From Normal Human Bone Marrow

Franco Mortari

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# CHARACTERIZATION OF IMMUNOREGULATORY CELLS AND FACTORS DERIVED FROM NORMAL HUMAN BONE MARROW

by

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## ABSTRACT

The bone marrow is a primary lymphoid organ whose main function is to provide a continuous source of hematopoietic cells to the peripheral immune system. This study was undertaken to elucidate the roles of bone marrow cells and their mediators in immunoregulation.

Fractionation of bone marrow cells resolved two distinct immunoregulatory activities. A population of bone marrow cells of the phenotype HNK-$1^+$ (human natural killer cell antigen, CD57), SSEA-$1^+$ (stage specific embryonic antigen) and OKM$1^+$ (human monocyte antigen, CD11B) was found to be highly suppressive for primary antibody responses. Conversely, a lymphocyte enriched bone marrow cells fraction could enhance the same antibody response. Neither population expressed surface markers for mature T nor B cells. Noteworthy was the finding that bone marrow depleted of natural suppressor cells was capable of mounting a primary antibody response. In addition, the two bone marrow cell populations acted antagonistically in the regulation of IgM antibody responses.

Both activities are mediated by soluble factors. Bone marrow Derived Suppressor Factor (BDSF) is a phospholipid that is synthesized de novo by HNK-$1^+$ bone marrow cells during a 24 hour culture. BDSF appears to mediate its suppressive effects by inhibiting interleukin-1 synthesis. On the other hand, Bone marrow Derived Enhancing Factor (BDEF) is a glycoprotein of MW 60,000 daltons and has the additional property of being directly mitogenic for both bone marrow cells and thymocytes. Both molecules act during the inductive phase of the generation of antibody responses and their action is not restricted by major histocompatibility antigens.

The fact that both activities are constitutively present within the bone marrow, in conjunction with their antagonistic action, make them ideally situated mediators to regulate bone marrow immunoglobulin levels. Furthermore, their potential effects on bone marrow stem cells suggest that the two activities contribute to the maintenance of bone marrow homeostasis.

## DEDICATION

To my parents, Umberto and Marisa, and to my sister Lucia for being a constant source of encouragement, especially during the most difficult times. Their love and financial support were instrumental in the completion of this thesis.

I would also like to express my admiration for Lisa who had the fortitude to "put-up" with me during my studies, under less than ideal conditions. Lisa you are a great daughter but most of all you are my best friend.

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| | |
|---|---|
| Ab - | antibody |
| Ag - | antigen |
| BDSF - | bone marrow derived suppressor factor |
| BDEF - | bone marrow derived enhancing factor |
| BFU - | burst forming unit |
| BM - | bone marrow |
| BSA - | bovine serum albumin |
| BSS - | balanced salt solution |
| C' - | complement |
| CD - | cluster of differentiation |
| CFA - | complete Freund's adjuvant |
| CFU - | colony forming unit |
| CNBr - | cyanogen bromide |
| Con A - | concanavalin A |
| CSF - | colony stimulating factor |
| CTL - | cytotoxic T lymphocytes |
| Da - | daltons |
| DEAE - | diethylaminoethyl |
| DTH - | delayed type hypersensitivity |
| ELISA - | enzyme linked immunosorbent assay |
| $E_R$ - | erythrocyte rosetting |
| $E_N$ - | neuraminidase treated erythrocytes |
| FACS - | fluorescence activated cell sorter |
| Fc - | receptor for the Fc portion of immunoglobulin |
| FCS - | fetal calf serum |
| GA - | glutamic acid-tyrosine |
| GAT - | glutamic acid-alanine-tyrosine |
| GT - | glutamic acid-tyrosine |
| HBSS - | Hanks'balanced salt solution |
| HLA - | human major histocompatibility complex |
| IBF - | immunoglobulin binding factor |
| Ig - | immunoglobulin |
| I-J - | I-J determinant of the murine MHC complex |
| IL-1 to 7 - | interleukin 1 to 7 |
| IFN - | interferon |
| KLH - | keyhole limpet hemocyanin |
| LT - | lymphotoxin |
| MHC - | major histocompatibility complex |
| MLR - | mixed lymphocyte response |
| MW - | molecular weight |
| NRS - | normal rabbit serum |
| NS - | natural suppressor |
| NK - | natural killer |
| NP - | 4-hydroxy-3-nitrophenyl |
| ORBC - | ox red blood cells |
| PAGE - | polyacrylamide gel electrophoresis |
| PBS - | phosphate buffered saline |

| | |
|---|---|
| PFC - | plaque forming cell |
| PHA - | phytohemagglutinin |
| PLL - | poly-L-lysine |
| PMA - | phorbol-12-myristate-13-acetate |
| RP-HPLC - | reverse phase-high pressure liquid chromatography |
| RT - | room temperature |
| SRBC - | sheep red blood cells |
| TdT - | terminal deoxyribonucleotidyl transferase |
| TGF-beta - | transforming growth factor-beta |
| TLC - | thin layer chromatography |
| TLI - | total lymphoid irradiation |
| TNF-alpha - | tumour necrosis factor-alpha |
| TRF - | T cell replacing factor |
| TsF - | T suppressor factor |
| TsiF - | T suppressor inducing factor |

# Chapter 1

# INTRODUCTION AND HISTORICAL REVIEW

### 1.1.1 Bone Marrow Architecture

The adult bone marrow (BM) is a primary site of hematopoiesis whose main function is to provide a continuous source of stem cells that replenish the peripheral pool of hematopoietic cells. From various reports (De Bruyn, 1981; Weiss and Chen, 1975) it is clear that the BM consists of two functionally distinct compartments: a vascular and a hematopoietic. The vascular compartment is comprised largely of venous blood vessels which are the recipients of blood cells that have matured within the environment of the BM and are ready for transport to the periphery (De Bruyn, 1981). The site of BM hematopoiesis is thought to be the extravascular compartment and specifically the areas between the numerous marrow sinuses (Lichtman, 1981).

Although the BM is a densely packed and heterogeneous tissue, there is a highly structured order of the cells that reside within this organ. The general architecture of the BM has been reviewed in detail by various investigators (Lichtman, 1981; De Bruyn, 1981; Weiss and Chen, 1975). As a brief review, the BM receives arterial blood from two sources: the nutrient artery and the periosteal capillary network. A series of sinuses is found radially at the periphery of the BM. These sinuses eventually drain into a central longitudinal venous vessel called the central sinus. This structure eventually becomes the central vein, which in turn serves as the delivery system for BM derived cells destined for transport to the peripheral organs. The central sinus wall which envelops the central vein consists of a complete layer of endothelial cells and a less dense lining of adventitial reticular cells. Mature BM cells destined for the periphery must traverse the central sinus wall to gain entry into the

central vein. The bone marrow sinuses have been shown in the mouse to range in diameter from 5 to 30 μm (Lichtman, 1981).

Human BM, although having the same general structure as rodent BM, also possesses trabecular bone and a large number of fat cells which are derived from adipocytes that engorge themselves with lipids (Lichtman, 1981).

The mechanism by which BM is able to discriminate between which hematopoietic cells are ready for export to the blood remains an issue of intense investigation. One study advocates the accumulation of an anionic compound at the advancing margin of the diapedesing blood cells as part of the recognition event that allows mature BM cells to migrate through the central sinus wall (De Bruyn and Michelson, 1981).

### 1.1.2 Bone Marrow as a Source of Hematopoietic Cells

Mammalian BM is a continuous source of rapidly renewing B lymphocytes (Osmond and Nossal, 1974), as well as a source of multipotential precursor cells with the ability to give rise to cells of myeloid and erythroid lineage (Wu et al., 1967). B-cell ontogeny is thought to begin in the embryonic yolk sac (Stutman, 1973) and then stem cells migrate to the fetal liver which becomes the primary hematopoietic site during gestation (Berridge et al., 1984). Maturation of blood cells continues within the BM during postnatal life and concludes in peripheral organs, such as the spleen and lymph nodes. It is this entire repertoire of developing hematopoietic cells that provides a defence mechanism for vertebrates against pathogens that invade the host.

The interaction between different cell types is a requirement for the generation of an effective immune response. This was initially

demonstrated by Claman et al. (1966) and then supported by the experiments of Mitchell and Miller (1968), Rajewski et al. (1969), Mitchinson (1971), and Rosenthal and Shevach (1973). B cells are the essential elements of the immune system responsible for the development of humoral immunity. The differentiation of B cells has been proposed to occur in two distinct stages (Calvert et al., 1984). In the initial antigen independent stage, immunoglobulin (Ig) genes are activated to facilitate their expression. This involves complex genetic rearrangement events that bring different genes that encode the various segments of complete Ig molecules in close proximity. The second stage encompasses the division and differentiation of surface Ig expressing B cells following exposure to Ag. The initial maturation events for B cells occur within the BM. The residence of small lymphocytes (which includes B cells) within the BM has been elegantly shown to average 3 days although cells have been shown to emigrate from the BM as early as 12 hours (Brahim and Osmond, 1970).

Although lymphocytes make up a substantial fraction of all BM cells, 40 to 50% in young humans (Rosse, 1976) and around 30% in mice (Miller and Osmond, 1974), other cell types have also been shown to originate from the same site; for example, macrophages (Metcalf and Moore, 1971), dendritic cells (Steinman et al., 1974), intrathymic cells (Barclay and Mayrhofer, 1981), and Langerhans cells (Katz et al., 1979).

More evidence for the existence of pluripotent hematopoietic stem cells has come from experimentation in animal models where BM cells were transferred to irradiated hosts (Wu et al., 1968; Nowell et al., 1970; Abramson et al., 1977). Through the use of cytological markers, the above studies clearly demonstrated that B cells, T cells and cells of myeloid lineage were all generated from a common precursor cell.

Cells of the B lineage comprise between 56 and 76% of small lymphocytes within the human BM compartment (Kamps and Cooper, 1982). B cells at different stages of maturation have been found within the BM compartment (Campana et al., 1985). The recognizable stages of B cell maturation are thought to progress from a $TdT^+$ B-cell precursor stage to the next subset of cells that have begun Ig gene rearrangement. Loss of TdT activity is soon accompanied by the expression of cytoplasmic IgM chains in large pre-B cell blasts. Surface $IgM^+$ and $IgD^-$ cells follow this progression until one observes the eventual expression of both IgM and IgD on the surface of mature B cells (Campana et al., 1985; Kincade, 1987).

BM accessory cells, like stromal reticular cells and myeloid cells, have been found in close association with developing B cells in selective areas of the BM (Bofill et al., 1985; Kamps and Cooper, 1982). This could imply that the former cells may be providing a favourable microenvironment for the maturation of B cells.

### 1.1.3 Bone Marrow as a Source of Immunoglobulin

Human marrow contains B cells at all stages of maturation, from the TdT blast to the mature plasma cell (DeGast and Platts-Mills, 1979a; DeGast and Platts-Mills, 1979b). The BM is the major site of Ig production during secondary responses (Benner and Van Oudenaren, 1977; Ryser and Dutton, 1977; Benner et al, 1977; Koch et al., 1981), as well as being able to spontaneously produce IgA (Alley et al., 1982). However, this organ is clearly unable to sustain Ab synthesis in response to a primary Ag challenge (DeGast and Platts-Mills, 1977a; Duwe and Singhal, 1979; Claman, 1974; Benner and Van Oudenaren, 1977; Ryser and Dutton, 1977; Benner et al., 1977; Koch et al., 1981).

In 1961, van Furth et al. described the inability of human BM to mount a primary Ab response, while producing substantial levels of IgG and IgA. These observations have been substantiated by other reports showing that while human BM is the source of B-cells and contains cells at different stages of maturation (Gathings et al., 1977) it is still unable to respond to primary Ag challenge and to synthesize IgM Ab (De Gast and Platts-Mills, 1979a; Alley et al., 1982).

Attempts to facilitate Ab synthesis from human BM by the addition of mature T cells proved much less successful when compared to peripheral lymphocytes (De Gast and Platts-Mills, 1979b). Quantitatively, human BM produced 10 fold more IgG than PBL but very low levels of IgM, which could not be elevated by primary immunization (Kodo et al., 1984).

The paucity of IgM Ab in human BM were paralleled by similar findings in animal models. Initial studies on murine BM have consistently found a clear inability of BM to mount a primary Ab response, in spite of the high frequency of PFC precursors present within this organ (Benner et al., 1974a; Ryser and Dutton. 1977). On the other hand, BM has been shown to synthesize more IgG and IgA than any other organs (Hill, 1976; Benner et al., 1977; Benner et al., 1974a).

This lack of primary IgM synthesis by BM contrasts with the ability of this organ to synthesize IgM during secondary responses (Benner et al., 1974a; Benner et al., 1974b; Benner et al., 1977) or in pathological conditions like rheumatoid arthritis (Panush et al., 1985; Vaughn et al., 1976; McMillan et al., 1976; Cohen, 1980) where the bone marrow becomes a major source of autoantibodies that are pathogenic.

These observations suggest that primary IgM Ab synthesis is incompatible with normal BM functions and therefore might be under the

control of one or a combination of regulatory mechanisms. The idea that this might be the result of the presence of suppressor elements within the BM compartment has been proposed by various investigators (Singhal et al., 1972; Drury and Singhal, 1974; Duwe et al., 1979; Roder et al., 1979; McGarry and Singhal,1982; Dauphinee and Talal, 1979; Corvese et al., 1980; Levy et al., 1981; Dorshkind et al., 1980; Muraoka and Miller, 1980; Layton et al., 1983).

A multitude of immune response regulators have been described. A brief review of the more intensively investigated suppressor mechanisms for Ab production will follow.

### 1.1.4 Idiotype Networks

Since Neils Jerne initially proposed the existence of a network of Ab reactivities that have potent regulatory activities (Jerne, 1974), the controversy over its existence and its in vivo regulatory function has continued. There is considerable disagreement with regards to the role of idiotype networks in immune regulation (Urbain, 1986; Cohn, 1986; Kearney and Vakil, 1986). In spite of this, there are observations to support the notion of a regulatory idiotypic Ab network. The tenets of this hypothesis are that during an Ab response, the Ig produced, referred to as Ab1, are themselves immunogenic by virtue of the unique structure of their Ag receptor site, also referred to as the paratope. These newly synthesized Abs are thought to elicit an Ab response, Ab2, that is directed against the paratope. In turn Ab2 induces Ab3 and so on. The hypothesis further predicts with some experimental evidence that complementary idiotype-paratope interactions are regulatory towards one another and that antigen elicits an Ab response by its ability to disturb

the dynamic equilibrium of the network of existing idiotype-anti idiotype interactions (Jerne, 1974; Richter, 1975; Paul and Bona, 1982; Nisonoff and Bengasser, 1975; Eichmann and Rajewsky, 1975; Rowley et al., 1981; Takemori et al., 1982, Kim et al., 1983). Of interest are the findings of Hayglass et al. (1986) which demonstrate the extension of the network of regulatory idiotypic circuits to T suppressor cells whose action is restricted by idiotypic determinants present on Ig heavy chains. The physiological relevance of the idiotype network theory continues to be an area of intense research and controversy.

### 1.1.5 Fc Mediated End Product Feedback

The observation that B-cell activity can be suppressed by secreted Ab led Sinclair and Panoskaltsis (1987,1988) to propose and experimentally demonstrate that the end product of an Ab response is suppressive for the generation of an immune response. The latter mechanism relies on the Fc portion of secreted IgG to deliver a negative signal to the responding B cell via the triggering of its Fc receptor. A further requirement of this model is that the secreted IgG binds to Ag which is bound to the Ag receptor and crosslinks the B cell Fc receptor with Ag bound surface Ig in order to deliver a suppressive signal. This inhibitory Fc signal can be reversed by the action of helper T cells and their products (Lees and Sinclair, 1975; Hoffman and Kappler, 1978; O'Garra et al., 1987). End product feedback and the anti-idiotype network may have common intermediates in their mode of action, however neither mechanism seems to be directly involved in BM mediated immunosuppression as addressed in this thesis.

### 1.1.6 Immunoglobulin Binding Factors

Another family of molecules that have been implicated in down regulating Ab responses are the Immunoglobulin Binding Factors (IBF). In spite of some difficulties encountered in initially identifying the active ingredient in IBF containing supernatants, active preparations can clearly suppress IgM, IgG (Gisler and Fridman, 1976), IgE (Hirashima et al., 1980), and IgA (Yodoi et al., 1983) synthesis. IBFs are generally derived from activated T cells and in some instances have been shown to both enhance as well as suppress Ig synthesis (Ishizaka, 1984; Kiyono et al., 1985). Daley et al. (1986) have also described an IBF that is antigen specific, possesses I-J determinants and binds to the Fc portion of Ig molecules. The factor can also suppress both primary and secondary PFC responses. Of interest are reports demonstrating that some IBFs demonstrate Ig isotype specificity (Daeron and Fridman, 1985) while others do not (Daley et al., 1986). Regarding their mechanism of action, all available evidence dismisses the possibility that IBF binding results in cytolysis of target cells (Daeron and Fridman, 1985; Darby and Hoover, 1986). The general role of IBFs is to regulate Ab responses in an isotype-specific manner, however there is no reported evidence of their presence within the BM.

### 1.1.7 Suppressor T Cells

The Ab response is the end result of a number of interactions between different subsets of hematopoietic cells and factors. Since the initial observation by Gershon and Kondo (1970) of suppressor T cells, a large body of evidence has accumulated that describe the details of T suppressor networks. Reports of suppressor T cells in the human system

have also been abundant (Lanier et al., 1983; Meuer et al., 1983; Nishimura and Sasazuki, 1983).

Regulatory T-cell circuits are thought to operate by the antagonistic action of a suppressor T-cell population on helper T cells. The activation of the suppressor population has been shown to occur either through an intrinsic property of the immunizing Ag (Yowel et al., 1979, Sorensen et al., 1982) or through the action of inducing factors (Asherson et al., 1986). In the murine system, several suppressor T-cell subsets interact sequentially to yield the inhibitory effect (Dorf and Benacerraf, 1984). The phenotypic differences between the various T-suppressor populations were extensively reviewed by Green et al. (1983). The earliest acting population in the T-suppressor circuitry are $Ly1^+2^-$, $Qa1^+$ and $I\text{-}J^+$ T cells, known as Ts1 cells, which induce an intermediate subset of T cells, referred to as Ts2. This latter transducer population of T cells is phenotypically $Ly1^+2^+$, $Qa1^+$ and $I\text{-}J^+$ and has an effect on the generation of the third stage cell known as Ts3 or the T-suppressor effector cell. Ts3 cells are distinguished phenotypically from the other two subsets by their surface expression of $Ly1^{-2}$ and $I\text{-}J^-$. The above network of T-cell interactions is further complicated by the description of a contrasuppressor T-cell circuit which acts to abrogate suppressor activity. Contrasuppressors also act through three phenotypically distinct cellular intermediates to yield effector cells that can reverse the suppressive action of T-suppressor cells.

In humans, a similar network of T-suppressor cells has been described (Ballieux and Heijnen, 1983; Damle et al., 1987). The phenotypes of the human T-suppressor inducer, transducer and effector populations are $CD4^+$ $CD45R^-$(Leu18) $p80^+$ (Leu8), $CD4^+$ $p80^-$, and $CD8^+$ $CD28^-$

(Tp44/9.3) respectively. Normal human BM does contain a resident population of $CD8^+$ T-suppressor cells, and they have been estimated to comprise between 5% and 9% of total nucleated BM cells (De Gast and Platts-Mills, 1979b; Janossy et al., 1981). $CD8^+$ cells have been shown to outnumber $CD^{4+}$ cells in the BM, ranging between a ratio of 2:1 to 6:1 (Janossy et al., 1981). It is well accepted that T cells that reside within the BM compartment have migrated from peripheral lymphoid organs. This idea is further supported by observations indicating a complete absence of transitional forms of cells expressing T cell-lineage specific differentiation surface markers on precursor cells identified by their cytoplasmic terminal transferase (TdT) activity (Janossy et al., 1981). BM resident $CD8^+$ T cells have been documented to have suppressive activity on Ig synthesis (De Gast and Platts-Mills, 1979b; Mills et al., 1985). The above reports further support the notion that BM contains regulatory activities and that these regulatory mechanisms may have profound influences on normal hematopoietic events.

Although both BM T-suppressor cells and the BM derived natural suppressor (NS) cells addressed in this thesis have similar suppressive effects for humoral responses, whether they act via the same mechanism remains obscure. This issue will be dealt in more detail in section 2.4 of this thesis.

### 1.1.8 Suppressor Factors

Soluble factors produced by cells of the immune system are one means by which the different components of the system can communicate. The biochemistry of many antigen-specific suppressor factors has been

extensively reviewed by Webb et al. (1983). The key distinguishing features of various T-suppressor factors are whether their action is MHC restricted and Ag specific or non-specific.

Better characterized Ag-specific suppressor factors have been shown to regulate responses to: the synthetic copolymers of amino acids, like GAT, GA and GT (Webb et al., 1983b), SRBC (Fresno et al., 1982), KLH (Taniguchi et al., 1981) and NP (Minami et al., 1981). These factors have been shown to suppress both primary and secondary humoral immune responses as well as DTH reactions. A large number of T-suppressor factors are protein in nature, ranging in MW from 19,000 to 70,000 daltons. All of these factors are derived from T cells of immunized animals or from T-cell hybridomas. The properties of two factors that are part of the T cell suppressor circuitry were reviewed by Green et al. (1983). For example, T suppressor inducer factor (TsiF) is produced by T cells treated with the anti-Ly-2 (anti-CD8) monoclonal. The factor behaves much like the cells synthesizing it, that is its action is I-J and Igh restricted. Furthermore, it requires the presence in culture of the Ly-1,2+ transducer cell to eventuate its effects. TsiF seems to be composed of two protein chains, produced by different cells, with one chain acting as the I-J restriction element and the other chain binding to Ag. Another Ag specific factor is T suppressor factor (TsF), which is produced by I-J-, CD8+ T cells and can suppress T cell helper activity. TsF appears to be also a protein of 68,000 Da, with the ability to bind Ag. The factor requires the presence of I-J+, CD4+ T cells to exhibit suppressor activity on the target T cell population which bears the phenotype I-J- and CD4+ (Flood et al., 1982). The target cell and TsF must be Igh compatible in order to observe suppression. How both of the above suppressor factors

operate is not clear but the internalization of the molecules by the cells binding them is thought to be a likely event.

The protein nature of T-suppressor factors and the requirement for immunization are two of the key distinguishing features between the bone marrow derived suppressor factor (BDSF) described in this thesis and other factors. Furthermore BDSF is a naturally occurring molecule produced by cells in the BM. A detailed discussion of the physico-chemical properties of BDSF is presented in chapter 3.

### 1.1.9 Other Suppressor Cells

Various other suppressor cells have been described that affect immune functions. Of note are natural killer (NK) cells that have been demonstrated to suppress Ig synthesis (Abruzzo and Rowley, 1983; Targan et al., 1985; Arai et al., 1983), as well as the generation of multipotent myeloid stem cells (CFU-S) (Holmberg et al., 1984; O'Brien et al., 1983). Suppressive NK cells bear the NK lineage specific marker HNK-1 as well as Fc receptors for IgG. The mechanism by which they inhibit antibody responses is not clear, although the idea that cytolytic activity may be involved has been advanced by various investigators (Targan et al., 1985; James and Ritchie, 1984). NK-like cells generated by mixed lymphocyte culture (MLC) have also been shown to have suppressive activity (D'Amore and Golub, 1985). These cells are of the phenotype HNK-$1^+$ but express Fc receptors for IgM rather than IgG. They also have the ability to down regulate NK cytolytic activity.

Another subset of cells described to have inhibitory activity for Ab responses and T-cell proliferation are L-cells (Horowitz and Bakke, 1984). They represent a heterogeneous population of cells that comprise

about 14% of circulating PBL. L-cells express Fc receptors for IgG (CD16) as well as markers recognized by the monoclonals OKM1 (CD11B) and HNK-1 (CD57). The assignment of these cells to either the lymphoid or myeloid lineage has been difficult. Horowitz and Bakke (1984) in fact suggested that they may belong to a distinct population of cells.

Even B cells have been implicated to influence various arms of the immune system. Their suppressive effects range from the inhibition of Ab production (Gilbert and Hoffman, 1982) to the suppression of DTH reactions (Asherson et al., 1979, Katz et al., 1974). The mechanism by which B cells inhibit immune functions has not been fully elucidated. Reports vary from the activation of suppressor T cells (Tsukuda et al., 1981), to the elaboration of suppressive mediators (Gilbert and Hoffman, 1983) or to their direct action on effector cells (Miyama-Inaba et al., 1982).

Another suppressor population of cells that has received considerable attention are the macrophages. Bacille Calmette-Guerin (BCG) or Corynebacterium parvum have been used as agents to stimulate suppressor activity from macrophages (Young et al., 1983; Mizushima et al., 1984). Activated macrophages have been shown to suppress Ig synthesis (Doft et al., 1976; Brown et al., 1980), mitogen activated cellular proliferation of B and T cells (Allen and Moore, 1979), graft-versus-host responses (Wren et al., 1983), DTH reactions (Lamoureux and Poisson, 1974) and the generation of cytotoxic T cells (Klimpel and Henney, 1978). Suppressor macrophages have been observed to develop in both animals and humans that bear tumours. Their presence has supported the notion that they may be the primary causative agents for the general immunosuppressive status observed in tumour bearing hosts (Kirchner et al., 1975; Herberman et al., 1979). Two general mechanisms proposed to explain how BCG-activated

macrophages exert their suppressive influence on the immune system are: by the production of soluble factors, or by cell to cell contact. In general prostaglandins of the E series, polyamine oxidases and cyclic AMP have all been identified as potential mediators of macrophage suppressor activity (Allison, 1978).

Certainly a large volume of evidence exists to support the idea that macrophages are important regulators of the immune system (reviewed by Druker and Wepsic, 1983), however their Ag non-specific action requires their initial activation by bacterial products. The natural suppressor activity that is normally present within the BM compartment appears to be unrelated to the BCG-induced suppressor activity of macrophages.

### 1.1.10 Bone Marrow Derived Suppressor Cells and NS Cells

The initial report by Singhal et al. (1972) was the first to demonstrate a suppressor population of BM cells that could inhibit primary IgM synthesis. This observation was soon followed by reports from other investigators demonstrating that resident BM cells have the inherent ability to inhibit the generation of Abs (Okumura and Kern, 1975; Adler et al., 1976; Duwe and Singhal, 1979a; Corvese et al., 1980; Levy et al., 1981; McGarry and Singhal, 1982; Bains et al., 1982; Layton et al., 1983; Garzelli et al., 1983; Matsumoto and Shibata, 1985). The suppressive activity of these BM derived cells was not solely restricted to Ig production. Subsequent investigations have shown the ability of BM suppressor cells to inhibit the generation of cytotoxic T cells (Muraoka and Miller, 1980), DNA synthesis of BM cells (Soderberg, 1984), MLR ( Dorshkind and Rosse, 1982; Inamura et al., 1987), LPS and Con A activated DNA synthesis (Levy et al., 1981) and NK activity (Punjabi et al., 1983).

The common element in the majority of reports on BM suppressor activity is that the cells involved were naturally occurring in the normal hematopoietic environment. Alternative methods of elevating BM suppressor activity involved the initial destruction of BM tissue by the bone seeking radioisotope strontium ($^{89}Sr$), or by total lymphoid irradiation (TLI) which resulted in an induction of BM-derived suppressor cells during the wave of reconstitution of immune cells (Levy et al., 1981; Strober, 1982). This has led to the designation of these cells as natural suppressor (NS) cells. NS activity has been succinctly defined as "the ability of unprimed null cells to suppress the response of lymphocytes to immunogenic and mitogenic stimuli" (Maier et al., 1986). NS cells have been found in mice (Duwe and Singhal, 1979b; Oseroff et al., 1984), rats (Noga et al., 1988), rabbits (Soderberg, 1984) and humans (Bains et al., 1982).

The phenotype of BM NS cells has been elusive due to the absence of well-defined surface antigens of mature hematopoietic cells. The controversy over their identity is substantiated by the number of differing reports in the literature describing them to be B cells (Fuchs et al., 1978), pre-T cells (Dauphinee and Talal, 1979) or immature myeloid cells (Corvese et al., 1980). The general properties of NS cells that investigators have consistently found are that BM suppressor cells seem to belong to the null phenotype subset of immune cells, and have a large granular lymphocyte morphology reminiscent of NK cells. To date there is no evidence of either class II product expression or Ig molecules on the surface of these cells. NS cells are insensitive to anti-Thy-1, anti-L3T4 (anti-CD4) or anti-Ly2 (anti-CD8) plus complement treatments and are not firmly adherent to glass or plastic. They do however express Fc receptors for IgG. For review see: Maier et al. (1986).

An important aspect of NS cells is that their action is antigen non-specific. This may account for the wide spectrum of action of NS cells in various test systems. This property allows NS cells to down regulate different immune responses without the need to develop new Ag-specific clones of suppressor cells. Another noteworthy fact about NS cells is that they appear to be exclusively found in areas of active hematopoiesis. NS activity has also been observed in secondary lymphoid organs, like the spleen, during a brief period of neonatal life when this organ functions as an active hematopoietic site. Splenic NS activity is also found during the period of tissue regeneration following treatments that damage secondary lymphoid organs. For example, TLI involves subjecting the host to fractionated doses of radiation ranging from 2,000 rads to 4,000 rads (Strober et al., 1983). The following lymphoid organs are directly targeted for destruction with this treatment: cervical, mediastinal, axillary, hilar, para-aortic, pelvic and inguinal-femoral lymph nodes as well as the spleen and thymus. The shielded diaphragm and long bones act as a source of stem cells or alternatively the hosts receive an infusion of marrow cells. During the wave of repopulation that ensues, the spleen becomes a site of intense hematopoiesis for a short period of time. It is during this period that NS activity in the spleen parallels that found in the BM. Furthermore, the suppressive activity is present during the same time frame when the spleen is extremely susceptible to tolerance induction (Strober, 1984). The increased frequency of splenic NS cells during neonatal life has allowed investigators to successfully establish in vitro cloned cells with NS activity from the spleens of young mice (Hertel-Wulff et al., 1984).

NS activity is neither MHC nor HLA restricted. This feature of NS cells and their reported presence in various animal species may support the idea that NS cells represent a primordial suppressive mechanism that has been preserved through evolution. The species non-specific action of NS cells is documented in a report using murine NS cells to regulate mixed lymphocyte reactions of human, rat and monkey cells (Knann-Shanzer and Van Bekkum, 1987). The localization of such cells to organs which are sites not only of intense hematopoiesis but also tolerance induction further argues for an important role of NS cells in normal BM functions.

Of major clinical importance are the number of reports where the augmentation NS activity has successfully been used for the treatment of Hodgkin's disease (Kaplan, 1980), intractable rheumatoid arthritis (Strober et al., 1983), the prolongation of skin allografts survival (Slavin et al., 1976) and the prevention of graft-versus-host disease (Strober et al., 1987).

The mechanism of action of this poorly-defined "null" subset of BM derived suppressor cells is still unknown. The lack of natural killer lysis or natural cytotoxic activity of NS cells, excludes the lyses of targets as a potential mode of action. Another proposed mechanism is through the activation of T suppressor cells (Okada and Strober, 1982; Peeler et al., 1983). A third alternative mode of action of NS cells is through the release of factors that can mediate suppressive activity. Evidence for such a mechanism has come from various laboratories. Duwe and Singhal (1978) as well as McGarry et al. (1982) demonstrated the presence of an inhibitor of Ig synthesis in supernatants of normal BM cell cultures. Further evidence for a soluble factor mediating NS suppressive activity came from studies using neonatal mouse spleen, which is enriched

for NS activity, as a source of the inhibitory activity (Argyris, 1981; Jadus and Peck, 1986). Definitive evidence for the mechanism(s) of action of NS cells is presently lacking and remains speculative.

Claims that prostaglandins of the E series contribute to the suppressive effect of NS cells were demonstrated by Choi et al. (1988). The above authors however also show that indomethacin partially reverses the inhibitory effects of NS cells, therefore prostaglandins may not fully account for the observed suppressive effects. The question still remains whether any of the above described factors are direct products of the BM derived suppressor cells.

### 1.1.11 Scope of Thesis

The general scope of this thesis is to explore the immunoregulatory functions of bone marrow. More specifically, this thesis will address the role of human BM cells and their BM derived factors in the regulation of immune responses, such as Ab production and DNA synthesis.

Furthermore this thesis will provide evidence to support the presence of NS cells in human BM and describe the phenotype of these cells. This study will also deal with the mechanism of action of NS cells. This will be approached from two aspects. One set of studies will address the characterization of the mediator (BDSF) responsible for the inhibitory activity of NS cells. A second set of studies on the target cell for BDSF activity will deal with the mechanism of action of this novel mediator. These experiments will form the basis on which an hypothesis will be proposed to explain how the observed suppressive action of BM-derived NS cells may be responsible for the inability of BM to respond to primary antigenic challenge.

This thesis will also describe a second immunostimulatory activity of human BM cells and the strategies used to characterize the mediator responsible for this immunoenhancing activity.

The characterization of these BM-derived immunoregulatory activities may further the understanding of how internal regulatory controls can influence cells destined for peripheral immune functions.

# Chapter 2

# CHARACTERIZATION OF HUMAN BONE MARROW DERIVED NATURAL SUPPRESSOR CELLS

## 2.1 Introduction

The bone marrow, long considered primarily a site of hematopoiesis, is now being recognized also as a source of immunoregulatory activities. The literature is inundated with reports on the ability of BM to down regulate Ab responses (Singhal et al., 1972; Adler et al., 1976; Corvese et al., 1980; Levy et al., 1981; Bains et al., 1982). Reports have also described the enhancement of Ig synthesis by BM derived factors (Saffran et al., 1986; Petrov et al., 1979). Of relevance to this study is the fundamental observation that normal BM is unable to mount a primary Ab response. This apparent anergy for IgM production has been postulated to be either the result of BM not having the necessary machinery to respond to primary Ag challenge (Benner et al., 1974a; DeGast and Platts-Mills, 1979b) or due to the presence of an in situ suppressor activity (Benner et al., 1974b; Singhal et al., 1972; Layton et al., 1983).

Although both sides of this argument are backed by experimental evidence, the preponderance of this evidence supports the idea advocating the presence of a suppressor population within the BM compartment. Various animal models have proved useful in the initial attempts to identify a suppressor population of BM cells (Duwe and Singhal, 1979b; Oseroff et al., 1984; Soderberg, 1984; Noga et al., 1988). However the number of studies on human BM have been limited (Bains et al., 1982; Bains et al., 1986).

One aspect that has contributed to the uncertainty for the existence of BM-derived NS cells is that they have remained poorly defined. This is largely due to the unavailability of monoclonal Abs that recognize BM cells at different stages of differentiation. The phenotype

of murine BM derived NS cells agreed upon by a majority of investigators in the field is as follows: Fc-gamma$^+$, Ia-, sIg$^-$, non-adherent, large morphology and non-cytolytic (Maier et al., 1986). The phenotype of human BM-derived NS cells remains obscure.

One of the objectives of the present study was to investigate further the immunoregulatory roles of human BM. Through the use of both physical separation and negative selection techniques, attempts were made at segregating the two major regulatory populations of human BM. The other focus of this study was to address the question of BM unresponsiveness to primary Ag challenge. The ability to deplete BM of the suppressive population of cells allowed direct analysis of the ability of BM to respond to Ag challenge.

In the following chapter, evidence will be presented to support the notion that normal human BM contains a population of naturally occurring suppressor cells; furthermore the data suggest that the inability of BM to respond to primary Ag challenge is partially due to the presence of BM suppressor cells. The phenotypic identification of human NS cells was also intended to elucidate the lineage to which these cells may belong to. Only through the characterization of such cells can we begin to understand the functional relevance of suppressor cells within the BM. These cells may have important clinical relevance in view of reports indicating the beneficial effects of regimens that elevate NS activity and are presently being included in new treatment modalities (Slavin, 1987).

# MATERIALS AND METHODS

## 2.2.1 Source of Human Tissues

Tonsillar cells were derived from tonsillar tissue obtained from young individuals undergoing routine tonsillectomies at either St. Joseph's Hospital or Victoria Hospital (London, Canada). The tissue was cut into small fragments and then dispersed into a single cell suspension by the use of a glass homogenizer (Canlab, Mississauga, Canada).

Bone marrow (BM) cells were obtained from ribs of haematologically normal individuals undergoing thoracic surgery. The ribs were extensively flushed by means of a syringe with an 18 gauge needle containing Hanks' balanced salt solution (HBSS) (Gibco, Burlington, Canada).

Peripheral blood lymphocytes (PBL) from normal volunteers was collected in heparinized Vacutainer tubes (Becton Dickinson, Mississauga, Canada). This blood was diluted with an equal volume of HBSS before being loaded onto a density gradient.

## 2.2.2 Ficoll-Paque Gradients

Single cell suspensions from the above human tissues were loaded onto equal volumes of Ficoll-Paque (Pharmacia, Dorval, Canada) and then centrifuged at 400 X g for 30 minutes at 20$^{0}$C. Mononuclear cells residing at the interface were collected and washed two times in large volumes of HBSS by spinning the cells at 100 X g for 10 minutes. Pelleted cells were resuspended in appropriate culture medium.

### 2.2.3 Media for Human Tissue Culture

Hanks' balanced salt solution was supplemented with 100 μg/ml streptomycin and 100 U/ml of penicillin (Gibco), pH 7.2. Complete culture medium consisted of RPMI-1640 (Gibco) supplemented with 10% human AB+ serum (Red Cross, London, Canada), 100 μg/ml streptomycin, 100 U/ml penicillin, and 5 X $10^{-5}$M 2-mercaptoethanol, pH 7.2. Phosphate buffered saline (PBS) 0.15 M, was made up lacking Ca++ and Mg++, pH 7.2.

### 2.2.4 Human Plaque Forming Cell (PFC) Assay

Normal tonsillar cells or PBL were plated at a concentration of 1 X $10^6$/well in Nunc 24-well plates (Gibco). The cells were cultured in a total volume of 0.5 ml of RPMI complete medium for 7 days at 37°C in a humidified 93% air and 7% $CO_2$ atmosphere. Sheep red blood cells (SRBC) (Woodlyn Laboratories, Guelph, Canada) were used as antigen (Ag) at a dose of 25 μl/well of a 0.25% SRBC suspension. Each culture was harvested individually, washed 2 times with HBSS, and then assayed for hemolytic plaque formation on a poly-L-lysine (PLL) coupled SRBC monolayer as described by Dosch and Gelfand, (1977). Briefly, microwells from flat bottom 96-well Nunc plates (Gibco) were coated with 100 μl of PLL (25 μg/ml) for 1.5 hr. The PLL was suctioned off and 100 μl of a 2% SRBC suspension in PBS was added to each well. The plate was centrifuged at 1000 rpm for 5 min and let stand at RT for 1 hr. Excess SRBC were removed by gently using a Pasteur pipette such that a one cell thick monolayer would be left in each microwell. SRBC monolayers were left with 50 μl of HBSS to avoid drying, and 30 μl of cells from each PFC tissue culture were added to two separate wells. These wells subsequently received 30 μl of 1:5 diluted Hemo-Low guinea pig complement (Cedarlane, Hornby, Canada).

The plate was incubated at 37°C for 1 hr and then each well was scored for the number of plaques that had developed on an inverted microscope.

### 2.2.5 Separation of BM Cells by Fc Rosetting

Fc mediated rosetting was carried out as follows. Ox red blood cells (ORBC) were initially coated with a subagglutinating dose of rabbit anti-ORBC IgG antibodies by incubating 1 ml of IgG diluted in HBSS with 1 ml of 10% (v/v) ORBC at RT for 2 hr. The coated ORBC were washed twice in PBS before being resuspended at a concentration of 5% (v/v) in RPMI-1640 supplemented with 10% FCS. Rosettes were formed by incubating equal volumes of Ab coated ORBC and BM cells at 2 X $10^7$/ml at 37°C for 15 min. Fc rosettes were gently resuspended and overlaid on a Ficoll gradient and separated as described in section 2.2.2. Rosetted cells that were contained in the cell pellet, were treated with Tris-ammonium chloride (pH 7.2) to lyse ORBC. Non-rosetting cells were recovered from the Ficoll interface. Both rosetting and non-rosetting cells were washed 2 more times in HBSS prior to being used in tissue culture.

### 2.2.6 Monoclonal Ab Treatments

The monoclonal Abs used to deplete cells of specific populations were as follows: anti-HNK-1 (CD57) (Dr. J. Roder, Mt. Sinai Hospital, Toronto), anti-SSEA-1 (Dr. J. Harris, London Cancer Clinic, London), OKM1 (CD11B), OKT3 (CD3), OKT8 (CD8) (Ortho Diagnostic Systems, Raritan NJ), B1 (CD20) (Coulter Immunology, Hialeah, FL) and anti-Leu 11b (CD16) (Becton Dickinson, Mountain View, CA).

For the cytolytic treatment, BM cells or tonsillar cells resuspended in 2 ml of cold RPMI-1640, were incubated with an optimal dose

of Ab for 1 hr at 4°C. The cells were washed 2 times with HBSS before exposing them to a 1:12 dilution of Low-Tox-H-rabbit complement (Cedarlane) for 1 hr at 37°C. These cells were further washed in HBSS and then resuspended in complete culture media.

### 2.2.7 Elutriation of BM Cells

Elutriation of human BM cells was carried out on a Beckman J2-21M centrifuge fitted with a JE-6B rotor (Beckman). A total of 4 X $10^8$ BM cells were loaded into the rotor using a Piper 31 peristaltic pump (Watson-Marlow, England) at a flow rate of 28 ml/min while the rotor was spinning at 4200 rpm. By progressively lowering the rotor speed by 100 rpm, 100 ml fractions of eluted cells were collected. Cell size and number of each 100 ml fraction was confirmed using a Coulter Counter (model ZBI) and Channelizer (Coulter). Cells were pooled into four arbitrary fractions according to their cell-size profile. The entire procedure was carried out at 4°C using 0.15 M PBS buffer that had been supplemented with 2% FCS. The general morphology of cells in each fraction was assessed by analyzing cytospins of each pooled fraction following treatment with Wright's stain.

### 2.2.8 Wright's Staining

Cytospins of cells of interest were prepared using a Shandon Elliott Cytospin (Fisher). Briefly, 1 X $10^6$ cells in a volume of 200 $\mu$l and 50 $\mu$l of FCS were placed in a cytospin holding cup positioned in front of a glass microscope slide. The unit was centrifuged at 700 rpm for 5 min to force the cells onto the glass slide. The cells were air dried and then fixed with methanol for 5 min. The slides were covered with Wright's

stain (2.5 g/l of methanol) for 7 min at RT, and then flooded with PBS (pH 6.8) and left in this buffer for 9 minutes. Slides were finally washed in water and used for morphological determinations under a microscope. Routinely, a minimum of 200 cells were counted on each slide to arrive at percentage values.

### 2.2.9 Statistical Analysis

A one-tailed Student's t test was used to determine the significant differences between control and experimental groups. Differences with a p value $< 0.05$ were considered statitically significant. The statistical analysis applied to the data tested whether two sample means were statistically different according to the methods described by Castle (1972). The normal t distribution table was from Bahn (1973). For a detailed explanation of the formulas used and an example of the statistical analysis refer to Appendix 1.

# RESULTS

## 2.3.1 Suppression of Ab Responses by BM Natural Suppressor (NS) Cells

The addition of human BM cells to normal human peripheral cells responding to Ag challenge with SRBC routinely resulted in a significant suppression of the PFC response when compared to control values. The suppressive effects of BM became evident when the ratio of BM cells to responding cells was 0.6 or greater (Figure 2.1). The suppressive effects of BM were not HLA restricted since BM cells suppressed the Ab response of histoincompatible peripheral cells. Addition of low numbers of BM cells, in the range of $2 \times 10^5$ to $5 \times 10^5$, repeatedly yielded an enhanced primary Ab response.

## 2.3.2 Enrichment of BM NS cells by Fc Rosetting

To determine whether the BM derived suppressor cells of interest expressed Fc receptors for IgG, BM cells were subjected to an Fc-dependent rosetting procedure. Table 2.1 demonstrates that BM derived NS activity is enriched in the Fc+ cell fraction. Fc+ BM cells had a two-fold greater capacity to suppress the Ab response when compared to the effect of an equal number of unfractionated BM cells. A comparison to Fc- BM cell fraction further demonstrates the enrichment of suppressor activity in the Fc+ population. These data suggest that human BM-derived NS cells bear the Fc-gamma receptor.

FIGURE 2.1

Regulation of PFC Response by BM Cells

One million tonsillar cells were cultured with SRBC for 7 days prior to assessing the in vitro Ab response of the cultures ( ● ). Parallel cultures were set up that received varying doses of normal human BM cells at the initiation of culture and the anti-SRBC specific PFC response of these cultures is indicated ( ■ ). The values reported represent the mean PFC count of triplicate cultures assayed in duplicate.

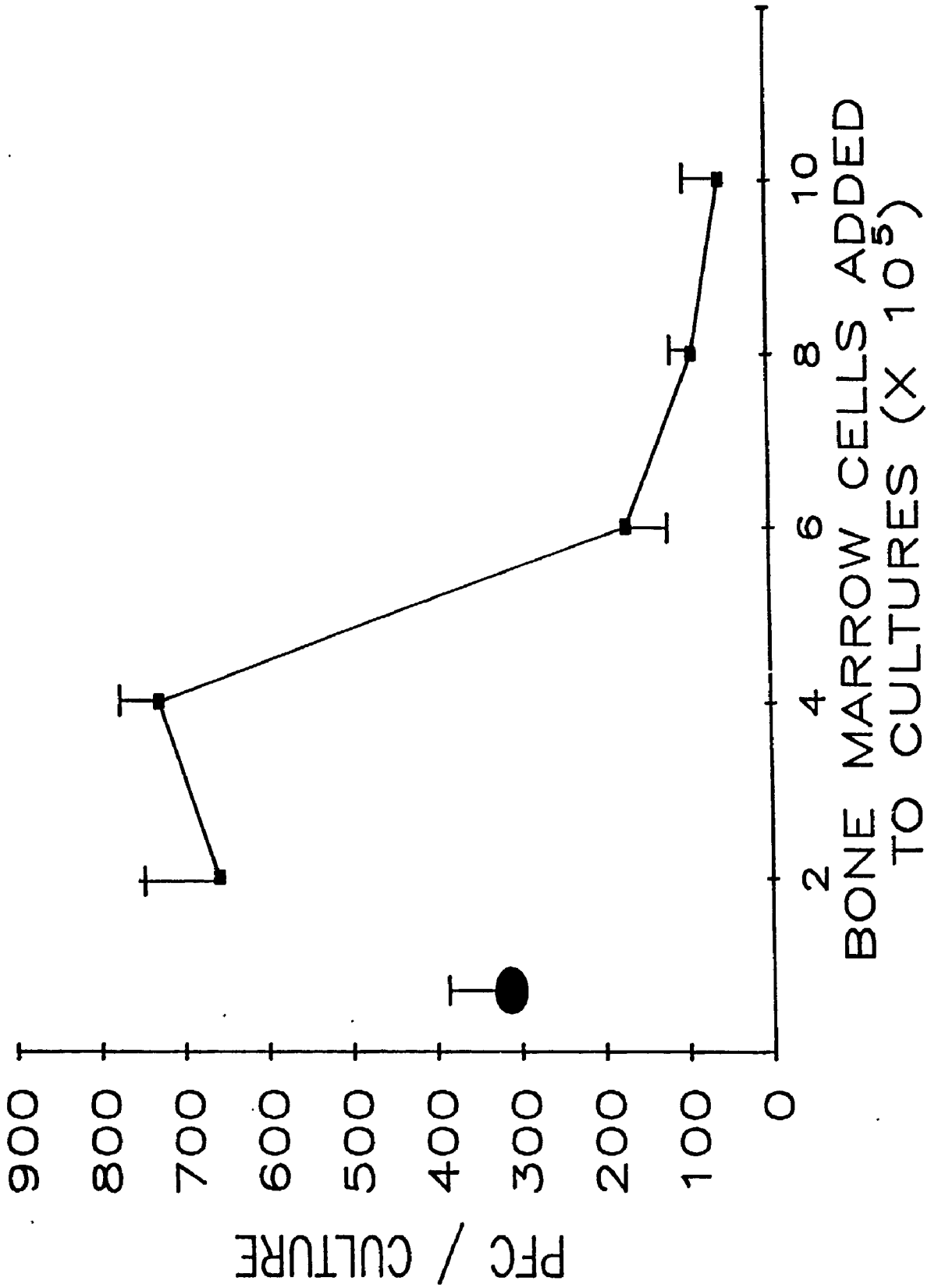

PFC / CULTURE
900
800
700
600
500
400
300
200
100
0
2
4
6
8
10
BONE MARROW CELLS ADDED
TO CULTURES (X $10^5$)

TABLE 2.1

Separation of BM NS Activity by Fc Rosetting

| Responder Cells | BM Cells | FcR Status of BM Cells | PFC/Culture ($\bar{X} \pm$ S.D.) | Percent Suppression |
|---|---|---|---|---|
| $1 \times 10^6$ | --- | ---- | $49 \pm 8$[a] | --- |
| $1 \times 10^6$ | --- | ---- | $4140 \pm 102$ | --- |
| $1 \times 10^6$ | $6 \times 10^5$ | Unfract | $2795 \pm 63$[b] | 32 |
| $1 \times 10^6$ | $6 \times 10^5$ | + | $1500 \pm 250$[b] | 64 |
| $1 \times 10^6$ | $6 \times 10^5$ | - | $3775 \pm 70$[c] | 9 |

Tonsillar cells were cultured in vitro with SRBC for 7 days in the presence of unfractionated BM cells, FcR+ BM cells, or FcR- BM cells and then tested for the anti-SRBC specific PFC response. The data is derived from one experiment representative of 3 separate experiments.

[a] The PFC response of tonsillar cells cultured in the absence of SRBC.

[b] Statistical difference from the control response $p < 0.0005$.

[c] Statistical difference from control response $p < 0.001$.

### 2.3.3 Enrichment of BM NS Activity by Elutriation

In an effort to better characterize the BM cells responsible for NS activity, elutriation was used to fractionate cells into populations of varying size and density. Four arbitrary fractions (F) were individually collected following such a procedure, F1-F4. The size distribution profile of the unfractionated BM cells and of the elutriated fractions is shown in Figures 2.2a and 2.2b respectively. A comprehensive summary of the morphology of each elutriated BM cell fraction and its effect on the *in vitro* Ab production is shown in Table 2.2. It is evident that $6 \times 10^5$ unfractionated BM cells significantly suppress the Ab response by 55%; however, only $2 \times 10^5$ BM cells from F4 are required to suppress the same Ab response by 57%. Fractions 1-3 did not demonstrate the capacity to significantly suppress the PFC response. In spite of the heterogeneous morphology of F4, which included cells largely of myeloid lineage, there was a greater than three-fold enrichment for suppressor activity in this fraction. The recovery distribution of total nucleated BM cells after elutriation was as follows: F1, 6%; F2, 25%; F3, 27%; and F4, 42%.

### 2.3.4 Phenotype of Human BM NS Cells

#### 2.3.4.1 Effect of anti-HNK-1 + C′ Treatment on BM NS Activity

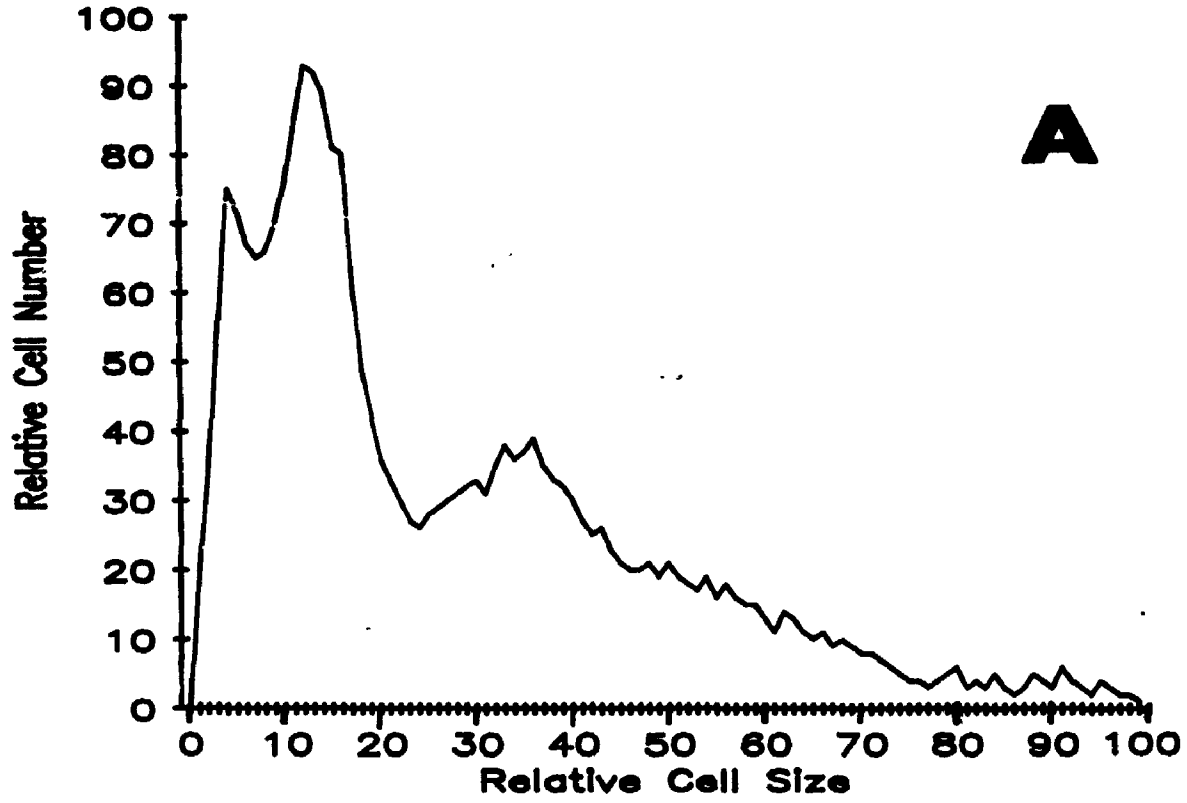
In order to assess the phenotype of BM NS cells, Ab plus complement (C′) mediated cytolysis was used to deplete BM of specific cell populations. These cells were subsequently used to assess whether the normally resident Ab suppressive activity had been affected by the cell depletion. Treatment of BM with the anti-HNK-1 (CD57) monoclonal results in a loss of 3% of the total nucleated cells, when compared to C′ only treated BM cells. More importantly, anti-HNK-1 depletion of BM cells

FIGURE 2.2

Relative Cell Size Profile of Elutriated BM Cells

A. Unfractionated human BM cells were sized on a Coulter Channelizer prior to being loaded onto an elutriator for fractionation. The relative cell size versus the relative cell number is shown in panel A.

B. BM cells collected following elutriation, were sized and the relative cell size versus the relative cell number for each of the four elutriated fractions is plotted in panel B.


A
Relative Cell Number
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Relative Cell Size

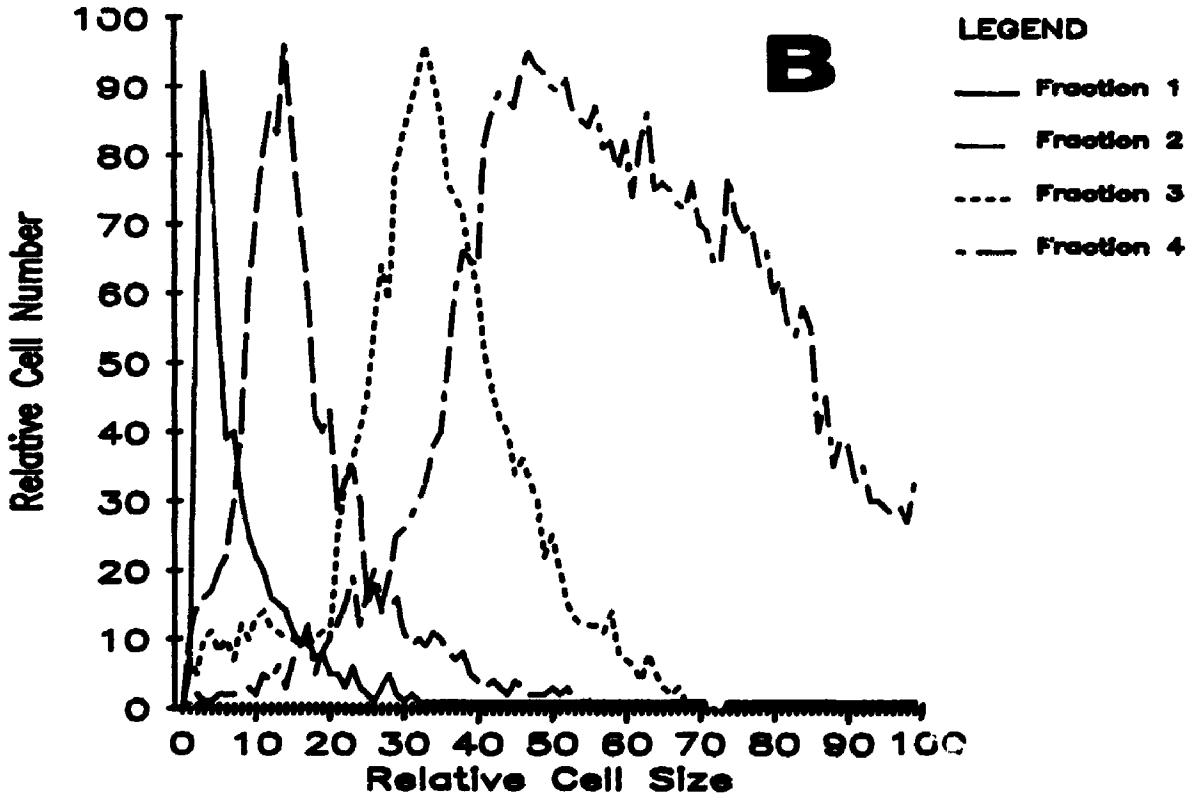

B
LEGEND
Fraction 1
Fraction 2
Fraction 3
Fraction 4
Relative Cell Number
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Relative Cell Size

TABLE 2.2

Effect of Elutriated Human BM Cells on Ab Synthesis

| Responding cells | Addition to Culture | Morphology of BM cells | PFC/Culture ($\bar{X} \pm$ S.D.) | % Shift in Response |
|---|---|---|---|---|
| PBL | --- | --- | 815 ± 65 | --- |
| PBL | [a]UNF BM | --- | 365 ± 31 | - 55[b] |
| PBL | [c]Elutr F1 | RBC 96%<br>Lymphocytes 4% | 800 ± 46 | - 2 |
| PBL | Elutr F2 | Lymphocytes 92%<br>Normoblasts 8% | 1475 ± 176 | + 81 |
| PBL | Elutr F3 | Normoblasts 41%<br>Bands-Segs 40%<br>Monocytes 16%<br>Lymphocytes 3% | 715 ± 52 | - 12 |
| PBL | Elutr F4 | Bands-Segs 54%<br>Monocytes 30%<br>Normoblasts 10%<br>M-Myelocytes 3%<br>Lymphoblasts 3% | 353 ± 37 | - 57[b] |

[a] $6 \times 10^5$ unfractionated human BM cells were added to tonsillar cultures and on day 7 the anti-SRBC PFC response was enumerated.

[b] Statistical difference from control $p < 0.005$

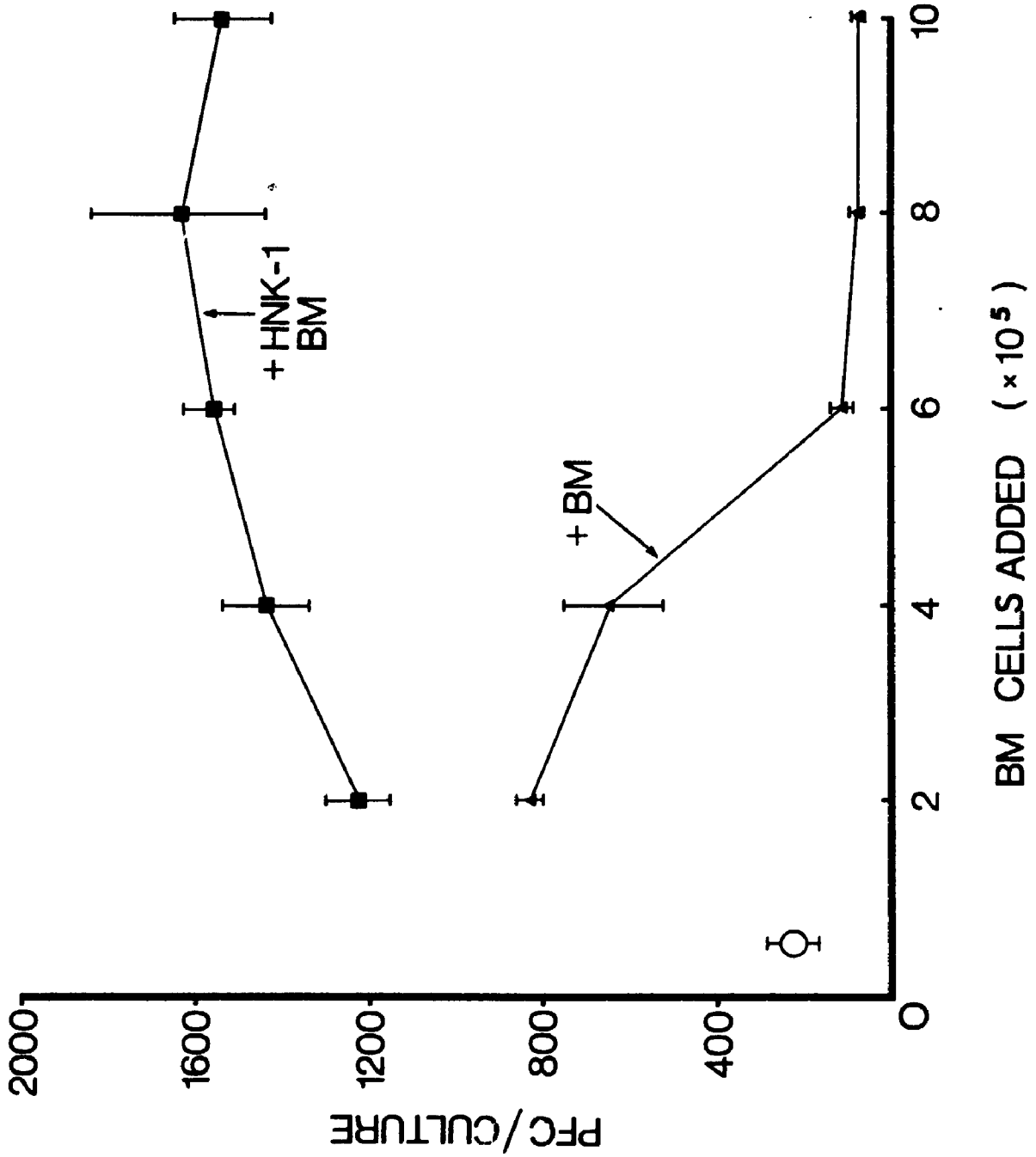
[c] Elutriated fractions of BM cell were added to cultures at a concentration of $2 \times 10^5$/well.

This experiment was repeated 3 times.

FIGURE 2.3

Expression of HNK-1 Antigen on BM Suppressor Cells

Tonsillar cells were cultured *in vitro* with SRBC alone (○), or with varying doses of untreated BM cells (■) or with BM cells previously treated with anti-HNK-1 plus complement (▲). The PFC response against SRBC was enumerated on day 7. Cytolytic treatment of BM cells with the above monoclonal antibody resulted in a net loss of 3% of total nucleated BM cells.


2000
1600
1200
800
400
0
PFC/CULTURE
+ HNK-1
BM
+ BM
0
2
4
6
8
10
BM CELLS ADDED (×10⁵)

resulted in complete abrogation of the normal suppressive activity in BM (Figure 2.3). All doses of anti-HNK-1 treated BM cells added to cultures enhanced the PFC response to varying degrees. Noteworthy are two findings of this study. The addition of a high number of treated BM cells, which normally results in suppression of the PFC response, now yielded only enhancement. Secondly, the addition of a relatively small number of anti-HNK-1 treated BM cells to cultures resulted in an augmentation of the Ab response above the levels of the normally enhanced response (Fig. 2.3). This increased PFC response reached a plateau, likely due to the limitations of the culture system in being able to support a limited number of Ab forming cells.

2.3.4.2 Effect of anti-SSEA-1 + C′ Treatment on BM NS Activity

Treatments of BM cells with the monoclonal Ab anti-SSEA-1 plus C′ resulted in a loss of the resident Ab suppressive activity; similar to that observed following anti-HNK-1 monoclonal treatment (section 2.3.4.1). Furthermore addition of the anti-SSEA-1 treated BM cells to Ab cultures resulted in a significant enhancement of the PFC responses at all doses (Figure 2.4). Data is also presented to demonstrate that C′ only treatment of BM does not detrimentally affect these cells, as they exhibit the typical bifunctional activities of untreated BM cells on an *in vitro* Ab response.

2.3.4.3 Effect of Other Monoclonal Ab Treatments on BM NS Activity

In a continuing effort to more precisely define the surface markers present on human BM NS cells, various monoclonals were used to deplete BM of specific populations. Table 2.3 summarizes the Abs used and

FIGURE 2.4

Expression of SSEA-1 Antigen on BM NS Cells

Tonsillar cells were cultured *in vitro* with SRBC alone (△), or with varying doses of untreated BM cells (●), or with BM cells previously treated with complement only (▲), or with BM cells treated with anti-SSEA-1 plus complement (■). The PFC response against SRBC was enumerated on day 7. Cytolytic treatment of BM cells with the anti-SSEA-1 monoclonal antibody resulted in a net loss of 37% of total nucleated BM cells.

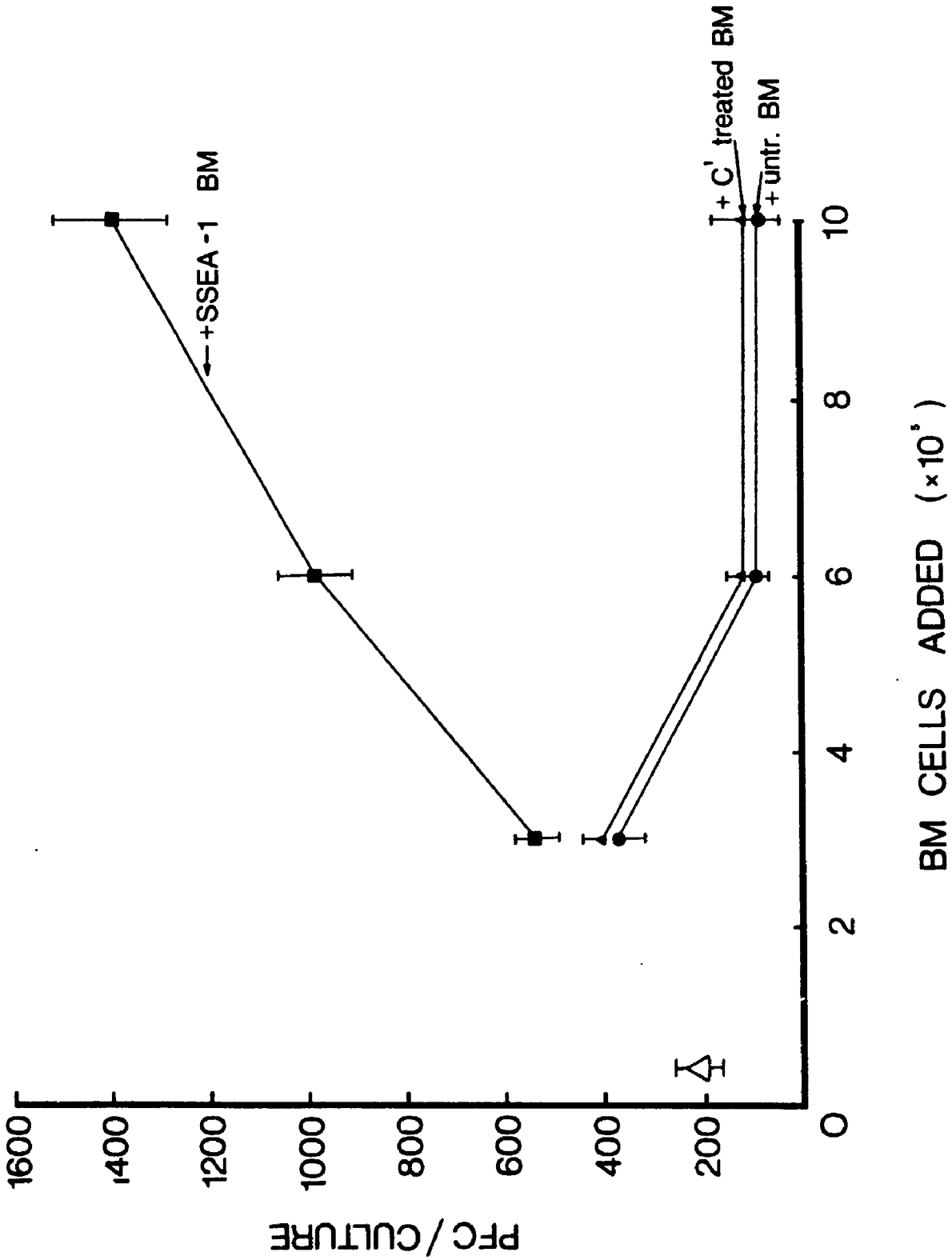

+SSEA-1 BM
+C' treated BM
+untr. BM
PFC / CULTURE
BM CELLS ADDED (×10⁵)
1600
1400
1200
1000
800
600
400
200
0
2
4
6
8
10

TABLE 2.3

Effect of Ab + C′ Cytolysis of Human BM on Suppressor Activity

| Treatment of BM Cells Used in Ab Cultures | PFC/Culture ($\bar{X} \pm$ S.D.) | Percent Suppression |
|---|---|---|
| --- | 354 ± 22[a] | --- |
| BM[b] | 139 ± 19[c] | 61 |
| BM + OKT3 + C′ | 152 ± 20[d] | 57 |
| BM + OKT8 + C′ | 144 ± 11[d] | 59 |
| BM + B1 + C′ | 151 ± 27[d] | 55 |
| BM + OKM1 + C′ | 187 ± 23[e] | 47 |
| BM + Leu11b + C′ | 141 ± 25[d] | 60 |

[a] Tonsillar cells were cultured for 7 days with SRBC and the PFC response specific for the immunizing Ag was enumerated.

[b] Untreated BM cells were added to PFC cultures at a 1:1 with responding cells and the anti-SRBC specific PFC is reported.

[c] Statistical difference from control cultures, $p < 0.0005$.

[d] Statistical difference from cultures that received untreated BM cells, $p > 0.1$.

[e] Statistical difference from cultures that received untreated BM cells, $p < 0.005$.

These experiments were carried out in duplicate.

the effect that this had on the resident suppressor activity of BM. BM cells were treated with the following monoclonals: OKT3 (CD3), OKT8 (CD8), LEU 11b, OKM1 (CD11B), and B1 (CD20). All of these Abs, except OKM1, proved ineffective in their ability to deplete BM of NS regulatory activity. The small but significant loss of inhibitory activity resulting from OKM1 plus C' treatment suggests that this myeloid Ag may be present on the cell surface of human BM NS cells, possibly at a reduced density that may render the cells less sensitive to Ab mediated cytolysis. These data suggest that BM NS cells do not bear the surface markers of mature B cells, T cells, T-suppressor cells, nor the high affinity Fc receptor found on highly cytolytic NK cells and recognized by the Leu11 monoclonal.

### 2.3.5 Antagonistic Action of BM Immunoregulatory Cells

To investigate whether the two immunoregulatory activities of BM had an antagonistic action on Ab synthesis, experiments were designed to test whether the immunoenhancing activity of BM, which appeared after HNK-1 depletion of BM cells, could be reversed by the addition of untreated BM, which contains suppressor activity. Figure 2.5 demonstrates that Ab cultures receiving 6 X $10^5$ HNK-1 depleted BM cells yielded an enhanced PFC response (1588 $\pm$ 149) significantly greater than the control PFC response (212 $\pm$ 19). These enhanced cultures were shown to be susceptible to BM suppressor activity, in a dose dependent fashion.

The corollary of the above findings suggested that a suppressed Ab response, induced by the addition of untreated BM cells, could be up-regulated by the addition of suppressor depleted BM cells. The experiments performed to address this issue are summarized in Figure 2.6. In this series of experiments, human tonsillar cells generated a PFC

FIGURE 2.5

Suppression of Enhanced Ab Responses by BM Cells

Tonsillar cells were cultured in vitro with SRBC alone (■), or with $6 \times 10^5$ anti-HNK-1 + complement-treated BM cells (●). To the latter cultures, that displayed an enhanced Ab response, varying doses of untreated BM cells were added at culture initiation (▲). The anti-SRBC specific PFC response was enumerated day 7. The results are derived from one experiment that was representative of two separate experiments.

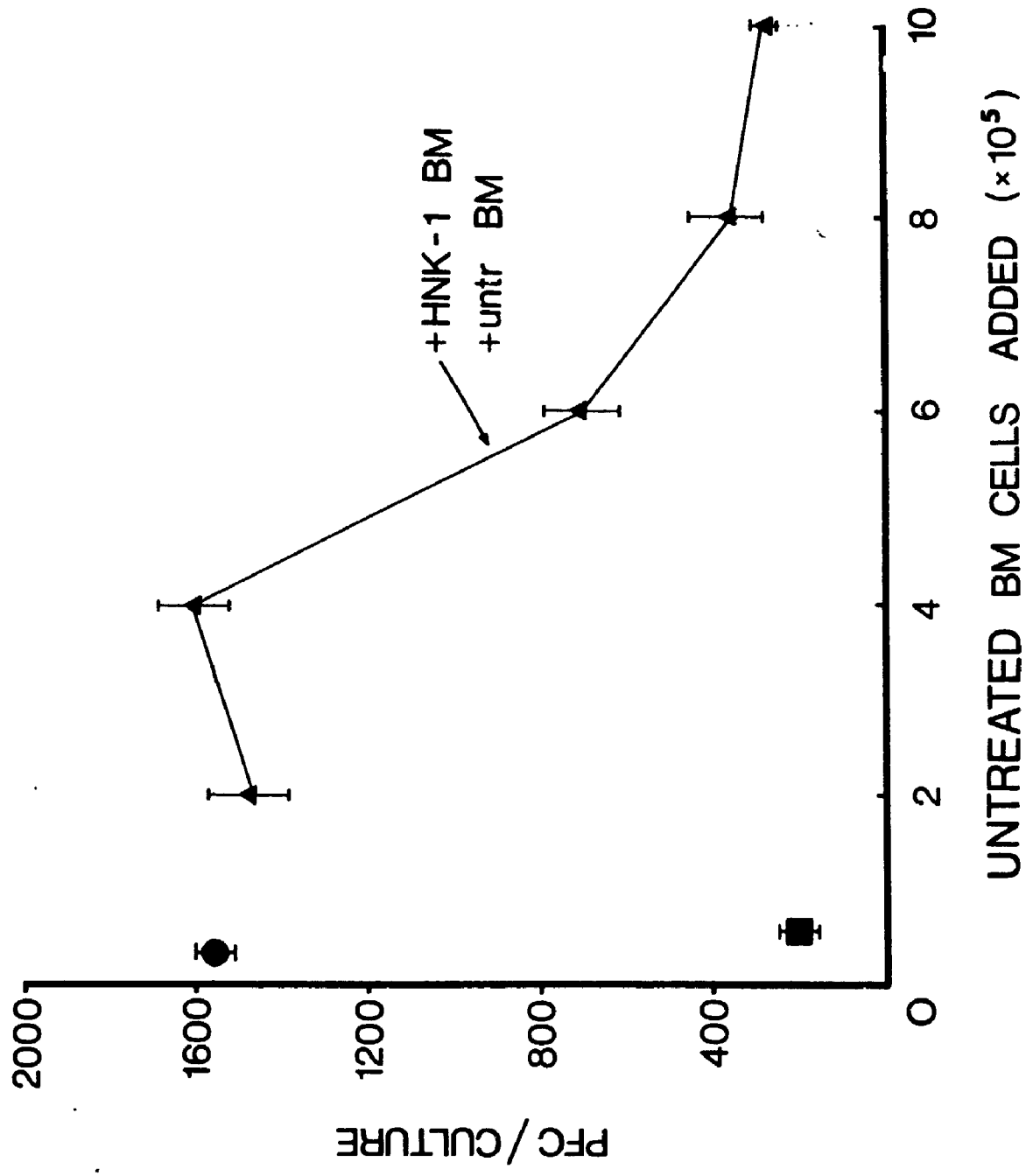
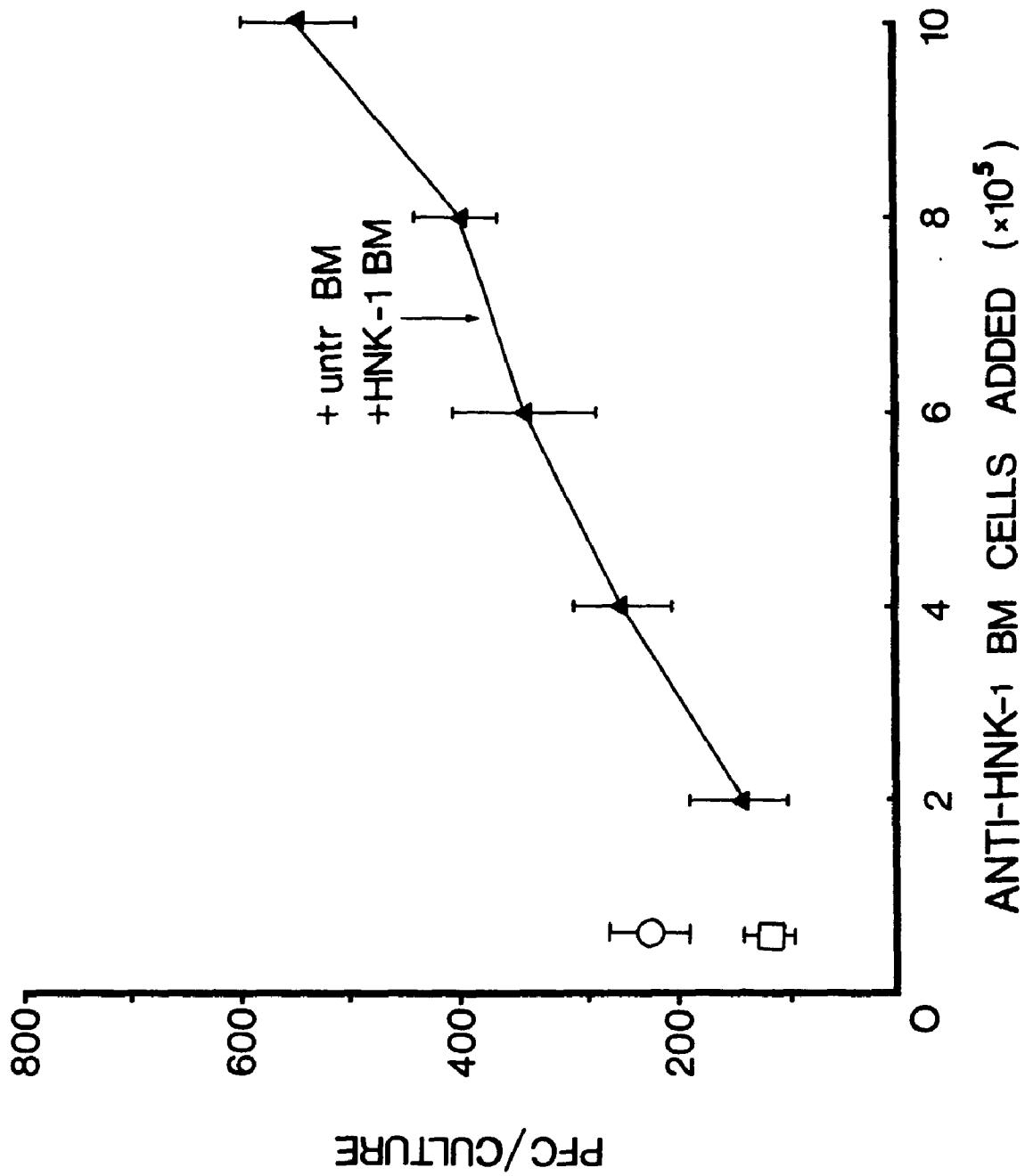

PFC / CULTURE
2000
1600
1200
800
400
0
2
4
6
8
10
UNTREATED BM CELLS ADDED (×10⁵)
+HNK-1 BM
+untr BM

FIGURE 2.6

The Ability of NS Depleted BM cells to Enhance a Suppressed Ab Response

Tonsillar cells were cultured *in vitro* with SRBC alone (○), or with a predetermined suppressive dose ($6 \times 10^5$) of untreated BM cells (□). The suppressed cultures were titrated with varying doses of anti-HNK-1 plus complement treated BM cells (▲). The PFC response against SRBC was enumerated on day 7.


PFC/CULTURE
800
600
400
200
0
2
4
6
8
10
ANTI-HNK-1 BM CELLS ADDED (×10⁵)
+ untr BM
+HNK-1 BM

response of 234 ± 28. This response could be partially suppressed by the addition of 6 X $10^5$ untreated BM cells to a level of 107 ± 14 PFC per culture. To these latter cultures, increasing doses of HNK-1 depleted BM cells were added. It is clear that the suppressed response could be modulated to a maximal value of 539 ± 55. The above data support the hypothesis that naturally occurring immunoenhancing and immunosuppressive activities of BM act antagonistically in the regulation of primary Ab responses.

### 2.3.6 Ability of Suppressor Depleted BM to Produce Ab

The preceding data indicated that a suppressor population of BM cells could down regulate primary Ab synthesis in peripheral tissues. It was of interest to also address the issue of whether the normally observed inability of BM cells to mount a primary Ab response could be due to the action of the naturally occurring suppressor cells, and not simply due to the immature status of resident BM cells. Experiments designed to test this hypothesis involved generating an *in vitro* Ab response using untreated or HNK-1 depleted BM cells as responders. To circumvent difficulties posed by the limited numbers of mature T cells and macrophages within the BM, all cultures were supplemented with mature accessory cells, in the form of 5 X $10^5$ T cells and 1 X $10^5$ macrophages. The day 7 PFC response of such cultures is demonstrated in Figure 2.7. It is evident that only anti-HNK-1 treated (suppressor cells depleted) BM cells can be induced to respond to the immunizing Ag. The magnitude of these responses is in fact comparable to responses generated with peripheral lymphocytes. In contrast, untreated BM cells, cultured in a

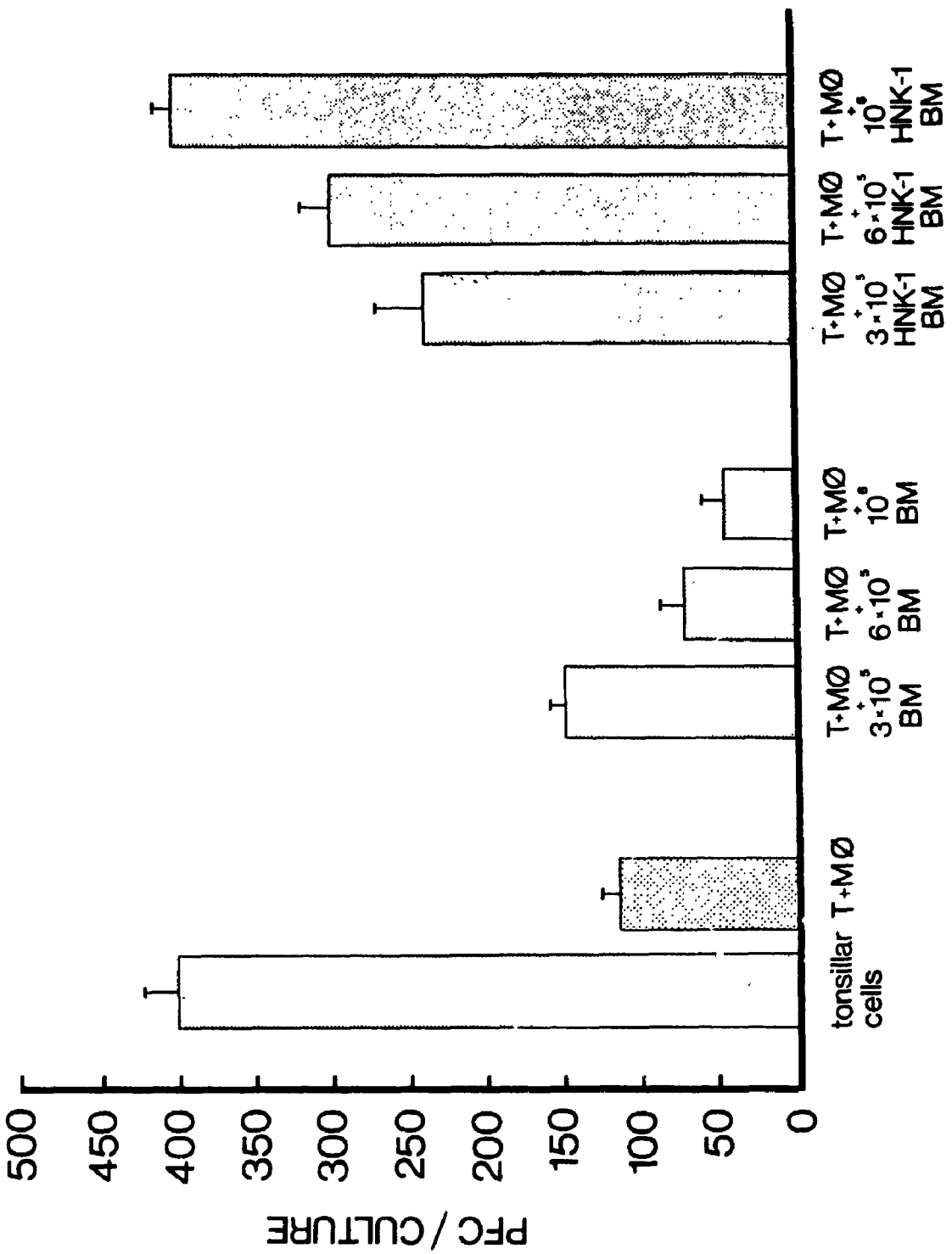
similar manner, are unable to mount Ab responses of similar magnitude as the suppressor depleted cultures.

Noteworthy are the observations that the limited Ab response generated by 3 X $10^5$ untreated BM cells, 144 $\pm$ 18, is further down-regulated in a dose dependent manner by the addition of increasing numbers of untreated BM cells. This is more supportive evidence for a naturally occurring suppressor cell population within the BM compartment.

FIGURE 2.7

The Ability of NS Depleted BM Cells to Mount a Primary Ab Response

Three different doses of untreated or anti-HNK-1 plus complement-treated BM cells were cultured with predetermined doses of accessory T cells (5 X $10^5$) and macrophages (1 X $10^5$) obtained from tonsillar cells. Tonsillar cells or T cells plus macrophages cultured in the presence of Ag (SRBC) served as controls. The day 7 PFC response of one experiment representative of 3 separate experiments is reported.


PFC / CULTURE
500
450
400
350
300
250
200
150
100
50
0
tonsillar cells
T+MØ
T+MØ 3×10⁵ BM
T+MØ 6×10⁵ BM
T+MØ 10⁶ BM
T+MØ 3×10⁵ HNK-1 BM
T+MØ 6×10⁵ HNK-1 BM
T+MØ 10⁶ HNK-1 BM

## 2.4 Discussion

In spite of the number of reports on BM associated suppressive activity, few investigators have formally described the phenotype of the BM-derived suppressor cells or whether such cells are present in human BM. This study was intended to address these issues.

The observations that human BM is able to suppress the generation of an *in vitro* Ab response supports previous observations from this laboratory of BM suppressor activity in both humans (Bains *et al.*, 1982) and mice (Duwe and Singhal, 1978; Duwe and Singhal, 1979b; McGarry and Singhal, 1982). Furthermore, the results substantiate the non-HLA restricted action of the naturally occurring BM cells on PFC response, as had been initially described by Bains *et al.* (1982). The enhanced PFC response observed by the addition of limited numbers of BM cells to Ab cultures appears to be the result of the action of a distinct immunoenhancing activity also residing within the BM cell population. This activity is more fully dealt with in chapter 4.

The accumulated evidence in this chapter suggests that within the heterogeneous BM compartment exists a population of cells that have inherent suppressive effects on primary IgM responses. In previous reports from this laboratory it was demonstrated that BM NS cells act during the inductive phase of primary Ab responses and require Ag to direct their suppressive signal (Bains *et al.*, 1982; Duwe and Singhal, 1979a). BM suppressor cells seem ideally situated to potentially control early differentiation and/or activation events of newly emerging antigen reactive B cells from the BM compartment. Because the cells responsible for this suppressive activity are natural constituents of BM and do not

require stimulation, they have been designated as Natural Suppressor (NS) cells (Strober, 1984; Maier et al., 1986; Jadus and Peck, 1986).

This study also provides formal evidence for a specific lineage to which NS cells may belong. In addition to possessing Fc receptors, the suppressor population of BM cells was found to bear the HNK-1, SSEA-1 and OKM1 surface antigens. These findings, in conjunction with the lack of reactivity with monoclonals specific for mature T cells, B cells or cytolytic NK cells, suggest that BM NS cells are unlikely to belong to the lymphoid lineage and may in fact be of myeloid nature. This is consistent with the observed reactivity of NS cells with the monoclonal anti-SSEA-1 (Fig. 2.4). This Ab was defined by Harris et al. (1984) to recognize the same stage-specific embryonic antigen-1 that Strauss et al. (1983) found to be exclusively expressed on polymorphonuclear leukocytes and macrophages but not lymphocytes.

It is also conceivable that NS cells may represent an immature form of NK cells with potent suppressive activity in the absence of cytolytic activity. The described phenotype of human NS cells, HNK-$1^+$ OKM$1^+$ OKT$3^-$, easily fits into the maturation scheme for NK cells suggested by Abo et al. (1983). The above authors proposed that HNK-$1^+$ OKT$3^-$OKM$1^-$ NK precursors first appear in very low frequencies in fetal tissues and then in the BM. While in this organ, precursor NK cells acquire and then subsequently lose the OKT3 Ag, they maintain expression of the HNK-1 Ag and acquire the OKM1 Ag. It is plausible that BM NS cells expressing HNK-$1^+$ OKM$1^+$ may represent a late stage of NK cell maturation within the BM, prior to their acquirement of cytolytic function. This is fully consistent with the reported low levels of NK activity associated with BM cells, compared to the high NK activity in peripheral tissues (Abo et al.,

1984; Abo et al., 1983). The suggestion that HNK-1$^+$ OKM1$^+$ BM derived cells represent a population of cells with potent Ab suppressive activity is fully consistent with observations by Tilden et al., (1983) demonstrating a phenotypically similar subset of peripheral cells with the ability to suppress both T cell proliferation and B cell Ig production. In addition, the authors show that HNK-1$^+$ OKM1$^+$ suppressor cells are functionally distinct as suppressor cells when compared to CD8$^+$ T cells.

The possibility that NS cells may be lineage related to NK cells is additionally supported by claims from other investigators that described BM-derived suppressor cells found in spleens of mice as NK related cells (Strober, 1984; Levy et al., 1981). The lack of reactivity of BM NS cells with the NK lineage specific marker, the Leu11 monoclonal, is an indicator of the non-cytolytic nature of these cells. This is in full agreement with reports demonstrating that HNK-1$^+$ Leu11$^-$ BM cells represent NK precursors with minimal cytolytic activity (Abo et al., 1984; Lanier et al., 1983). Furthermore, that cells of this phenotype may be of clinical significance is supported by the studies of Leroy et al. (1986) who demonstrated the emergence of HNK-1$^+$ Leu11$^-$ cells in the peripheral blood of patients who had recently undergone BM transplantation. Noteworthy is that the presence of these cells was highly correlated with the suppressed humoral responses of these patients. The provisional assignment of HNK-1$^+$ NK precursor cells to the myeloid lineage is consistent with all of the findings of this report. In addition, the findings of NK cells in immunodeficient humans and mice that lack the lymphoid component of the immune system (Peter et al., 1983; Dorshkind et al., 1985), supports the interpretation that NK cells may belong to the myeloid lineage.

The physical fractionation of BM cells clearly resolved the two opposing regulatory activities of BM. The morphological analysis of elutriated BM fraction IV, which is enriched for suppressor activity also argues that NS cells may be of myeloid lineage.

The consistent finding of a bifunctional regulatory activity of unfractionated BM cells on Ab responses, that is enhancement at low doses and suppression at high doses, does not appear to be a culture induced phenomenon. In fact the ability to isolate Ab enhancing activity following elutriation or by Ab depletion of BM of NS cells, argues for a distinct population of BM cells with Ab enhancing activity. The need for a large number of BM cells in order to observe suppression of PFC responses could be explained by postulating that NS cells may require interactions with other BM cells for expression of their activity; thus low doses of BM cells would reduce the probability of this interaction and result in the expression of only enhancing activity.

The immunoenhancing activity of suppressor depleted BM cells suggests that there is an active stimulatory effect on the responding PBL population. This enhanced response may be explained by three possible mechanisms: 1) suppressor cells-depleted BM cells may contain immunocompetent B cells that are able to respond to Ag challenge during the <u>in vitro</u> culture, 2) suppressor cells-depleted BM cells can directly enhance PFC responses, or 3) both of the above mechanisms may be operative. The NS-depleted BM cells are capable of responding to Ag challenge is supported by the presented evidence demonstrating that HNK-1 depleted BM cells can give rise to a primary IgM response when provided with accessory cells, as may be the case in PFC cultures. What is important to consider is the magnitude of the Ab responses by BM cells

alone and by PFC cultures that have been augmented by the addition of NS-depleted BM cells. The finding that the maximal PFC response of the latter cultures cannot be numerically accounted for by the additive effect of responding BM cells and the PBL response, suggests that a true enhancement of the PFC response is occurring. Therefore, both of the previously suggested mechanisms to explain the increased Ab response appear to be operative. This does not detract from the crucial finding of an Ab enhancing activity within the BM compartment.

That the inherent suppressor and enhancing activities of BM cells are antagonistic in nature is clearly argued for by the experiments demonstrating the ability of BM cells enriched for either activity to regulate PFC responses in the opposite direction when added to cultures that had previously received BM cells with the complementary activity. This suggests that the factor that may determine which of the two BM immunoregulatory effects is displayed may simply be the quantitative difference between the two antagonistic signals.

Of importance and interest is the finding that BM cells can potentially respond to Ag challenge when provided with accessory cells, in the form of mature T cells and macrophages. This observation demonstrates that the BM contains part of the machinery necessary for Ab synthesis. Furthermore, that removal of NS cells from BM is a requirement for the generation of a primary Ab response, is indicative that the normally observed unresponsiveness of BM Ag challenge is attributable in part to the presence of suppressor cells within the BM compartment. This is a key finding of this study and allows the speculation that primary Ab responses are incompatible with normal BM functions.

# Chapter 3

# CHARACTERIZATION OF HUMAN BONE MARROW DERIVED SUPPRESSOR FACTOR

## 3.1 Introduction

The evidence for the existence of NS cells within BM and neonatal spleen is abundant (Duwe and Singhal, 1979b; Strober, 1982; Oseroff et al., 1984; Maier et al., 1986), and so are reports demonstrating the ability of NS cells to inhibit various immune functions (Muraoka and Miller, 1980; Soderberg, 1984; Inamura et al., 1987; Levy et al., 1981). However the mechanism by which BM derived NS cells inhibit immune responses remains poorly defined.

Of the limited attempts made to address the likely mode of action of NS cells, a number of reports have advocated the use of soluble mediators (Duwe and Singhal, 1978; McGarry et al., 1982; Argyris, 1981; Jadus and Peck, 1986). Definitive proof that soluble mediators released from NS cells exhibit suppressive activities is lacking. Furthermore, these inhibitory factors have not been fully characterized with respect to their biochemical properties. More importantly, there has been a failure to demonstrate that the above implicated mediators of inhibitory activities are direct products of NS cells.

In view of the lack of research on the identity of human NS cells and their mechanism of action, the studies outlined in the following chapter were undertaken to address these issues. In this section of this thesis, evidence is presented to support the argument that human NS cells exert their inhibitory activity via the release of a mediator that has been named Bone marrow Derived Suppressor Factor (BDSF). The data will demonstrate that BDSF has a similar spectrum of action and kinetics as NS cells. Results from biochemical analyses of BDSF will be presented to support the suggestion that BDSF is of lipid nature and is a direct product of BM NS cells. Finally, the focus will be shifted to the

mechanism of action of BDSF. The results of target studies will be discussed in the context of how BDSF may mediate its inhibitory activity. Evidence will be presented that suggests that macrophages may be the preferential target cells of BDSF, and that the observed suppressive effects may be the result of interference by BDSF with normal macrophage functions and in particular, IL-1 synthesis. This will be one of the first studies to address the mechanism of action of human NS cells.

### 3.2.1 Cell Cultures

Human cells for tissue culture were prepared as described in section 2.2.1. The human PFC assay was set up as detailed in section 2.2.4.

### 3.2.2 E-rosetting of Human Cells

To prepare neuraminidase treated SRBC (En) 9 ml of 10% SRBC, were mixed with 1 ml of neuraminidase (1 U/ml) (Sigma) and incubated at 37°C for 30 min. The SRBC were washed 2 times in PBS and resuspended in PBS to a concentration of 10% (v/v).

Rosetting of human lymphocytes was carried out by mixing the following : 1) lymphoid cells resuspended in HBSS and at $10^7$/ml, 2) En were added at a ratio of 30% of the volume of added lymphoid cells, and 3) 30% (v/v) of FCS. This mixture was incubated at 37°C for 5 min prior to the cells being pelleted by centrifugation at 1000 rpm for 5 min. The cells were incubated at 4°C for 2 hrs and then gently resuspended with a Pasteur pipette and layered onto Ficoll-Paque gradients. Tubes of Ficoll-Paque plus cells were centrifuged at 400 Xg for 20 min. The resulting interfaces, containing non SRBC-rosetting cells ($E_R$-), were harvested and washed 2 more times in HBSS prior to being used in an assay. The cell pellets, containing SRBC-rosetting cells ($E_R$+), was first treated with warm lysing buffer at 37°C for 5 min to remove contaminating SRBC. Lysing buffer was prepared by mixing 90 ml of 0.83% ammonium chloride with 10 ml of 2.1% tris-hydroxymethyl-aminomethane (pH 7.2) (Fisher). $E_R$+ cells were washed 3 times in HBSS prior to usage. In some instances, this fraction was rosetted a second time to ensure that all cells in this population

were indeed $E_R$+, and also to minimize any contamination from non rosetting cells.

### 3.2.3 Con A Proliferation Assay

Human tonsillar cells or PBL were plated in flat bottom 96-well Nunc plates (Gibco) at a concentration of 2 X $10^5$/well, in 200 µl total volume of RPMI-1640 supplemented with 10% FCS. Where indicated Con A (Calbiochem, La Jolla, CA) was added to cultures at a concentration of 5 µg/ml. After 24 hrs, cultures were pulsed with 1 uCi of $^3$H-thymidine (New England Nuclear) for a further 24 hrs. Cultures were harvested onto glass fiber filters using a Titertek Cell Harvester (Flow Labs.) and counted on a Beckman LS-3801 beta-counter (Beckman Instruments, Toronto, Canada).

### 3.2.4 Mice

Balb/c mice (Jackson Laboratories, Bar Harbor, Maine) used for *in vitro* antibody (Ab) synthesis were 6 to 8 weeks of age, while animals used as a source of thymocytes were 4 to 6 weeks old.

### 3.2.5 Media for Murine Cultures

Complete culture medium consisted of minimal essential medium (MEM) (Gibco) supplemented with 1% glutamine (200 mM), 1% nonessential amino acids (100X), 1% sodium pyruvate (100 mM), 3% sodium bicarbonate (7.5% solution), 0.5% gentamicin (Schering Pharmaceutical Co., Kenilworth, N.J.), and 10% fetal calf serum (FCS) (Gibco), pH 7.2.

Nutritional cocktail was prepared by mixing 35 ml of MEM, 5 ml of essential amino acids (50X), 2.5 ml of nonessential amino acids, 2.5 ml

L-glutamine, 500 mg of glucose, 5.6 ml of 8.8% $NaHCO_3$, and 25.3 ml FCS, at a pH of 5.0.

Balanced salt solution (BSS) consisted of mixing the following reagents into 1 litre of water: 0.67 g dextrose, 5.3 g NaCl, 0.27 g KCl, 0.04 g $KH_2PO_4$, 0.24 g $Na_2HPO_4 \cdot 7H_2O$, 0.12 g $CaCl_2 \cdot 2H_2O$, 0.13 g $MgCl_2 \cdot 6H_2O$, 0.13 g $MgSO_4 \cdot 7H_2O$, and 6 mg phenol red, pH 7.2.

### 3.2.6 Murine PFC

For the generation of an *in vitro* PFC culture, quadruplicate splenocyte cultures, containing 1.5 X $10^7$ cells were set up in a total volume of 1 ml of complete medium, in 24-well Nunc plates (Gibco) at 37°C for 5 days in a humidified 90% air and 10% $CO_2$ atmosphere. SRBC were used as Ag by adding 30 µl/well of a 3% (v/v) suspension. Cultures were fed daily with 90 µl of nutritional cocktail. After the incubation period, cultures were individually harvested, washed in BSS, and then assayed in duplicate for direct hemolytic plaque formation according to the method of Cunningham and Szenberg (1968).

### 3.2.7 Interleukin-1 (IL-1) Assay

The thymocyte proliferation assay was performed essentially as described by Wood *et al.*, (1985) with the following modifications. 1.5 X $10^6$ Balb/c thymocytes were cultured in 96-well flat bottom plates using RPMI supplemented with 5% FCS and 2.5 X $10^{-5}$M 2-mercaptoethanol. Cultures also received 2.5 µg/ml of phytohemagglutinin (PHA-P) (Difco, Detroit, MI) and where required IL-1. Plates were incubated for 68 hours and [illegible] each well pulsed for 4 hours with 1 uCi of tritiated thymidine ($^3$H-thymidine) (New England Nuclear, Boston, MA). Cultures were harvested individually

onto glass fiber filter paper using a Titertek Cell Harvester (Flow Laboratories, Mississauga, Canada). $^{3}$H-thymidine incorporation was determined by liquid scintillation counting on a Beckman LS-3801 beta-counter (Beckman Instruments).

3.2.8 Preparation of Human Bone marrow Derived Suppressor Factor

Bone marrow cells recovered from density gradient separations were cultured in Nunc tissue culture dishes (Gibco) at a concentration of 1 X $10^{7}$/ml for 24 hours in serum free RPMI-1640 (Gibco). The supernatants from such cultures were collected and the cells reincubated for a further 24 hours incubation in fresh RPMI-1640 medium. Supernatants from these cultures were pooled and used as a source of both Bone marrow Derived Enhancing Factor (BDEF) and Bone marrow Derived Suppressor Factor (BDSF).

3.2.9 Enrichment for BDSF by Lipid Extraction

BDSF activity was enriched for by taking lipid extracts of the above BM cell supernatants. Crude BDSF supernatants were mixed with 20 volumes of a chloroform-methanol (2:1) mixture. This was shaken and then allowed to stand for 5 hrs at room temperature (RT). PBS was subsequently added to the mixture to a final concentration of 20% (v/v). This was shaken again and let stand overnight at RT. The organic phase was collected and reduced to 0.5 ml by evaporating the solvents on a Buchi Rotavapor "R" (Canlab, Mississauga, Canada). For long term storage the lipids were transferred to glass vials and the air replaced with $N_2$ before sealing them. These vials were stored at -20$^{o}$C. On the days of the assays, the lipid extracts were diluted (at least 1:100) in complete culture medium

by continuous vortexing and then letting this stand at 37°C for 5 min so that volatile solvents could evaporate.

### 3.2.10 Isotopic Labeling of Lipids

To label BDSF with a radioactive precursor, $^{3}$H-acetate was added to the BM cultures being used to generate the BM factors at a concentration of 1 μCi/ml. After 24 hrs, culture supernatants were harvested as usual and cells set up for a further 24 hr culture in the presence of fresh $^{3}$H-acetate. BDSF was prepared by lipid extraction as described above in section 3.2.9.

### 3.2.11 Culture of Elutriated BM Fractions for BDSF Production

BM cells recovered following elutriation were cultured at a concentration of 1 X $10^{7}$/ml in RPMI-1640 (Gibco) for 24 hrs. The cell free supernatants of all four elutriated BM cell fractions were used directly in tissue cultures without further processing.

### 3.2.12 Silicic Acid Chromatography

Silicic acid chromatography of human BM derived lipids was performed as follows. One gram of Bio-Sil-A (100-200 mesh) (Bio-Rad, Mississauga, Canada) was resuspended in chloroform. The slurry was poured into a thin glass column and packed by washing the column with 10 ml of chloroform. The lipid sample to be fractionated was loaded onto the column and neutral lipids that did not interact with the column matrix were eluted with 10 ml of chloroform and labelled as fraction 1. Next, 10 ml of 90% chloroform and 10% methanol were passed through the column to elute slightly polar lipids. These lipids were collected in fraction 2.

Finally, the most polar lipids were eluted from the column using 10 ml of a solvent mixture containing 50% chloroform and 50% methanol. These lipids were labelled as fraction 3. All three fractions were concentrated by evaporating a portion of the solvents under a stream of $N_2$ and then diluting the lipids in complete medium prior to usage in tissue cultures.

### 3.2.13 Reverse Phase HPLC (RP-HPLC) of BM Derived Lipids

Fractionation of BM derived lipids by RP-HPLC was performed using a Hewlett-Packard HP-1084-B HPLC unit equipped with a $C_{18}$ reverse phase column (HP part # 799150D-174) (Hewlett-Packard, Mississauga, Canada). The solvent system consisted of a 15 minute gradient using 90% methanol and 10% isopropanol as the starting solvent and ending with 20% methanol and 80% isopropanol at a flow rate of 2.0 ml/min. Lipid samples to be fractionated were injected in volumes ranging between 5 and 25 $\mu$l. The UV monitor was set up to scan absorbance at 210 nm, so as to detect carbon-carbon double bonds. Thirty fractions were generated by collecting the eluent at 0.5 min intervals during the 15 minutes run. Using the chromatograph as a guide, four larger fractions were created by pooling the following original fractions: FI = 1-4, FII = 5-10, FIII = 11-18, and FIV = 19-30. The volume of each fraction was reduced to 0.25 ml under a stream of $N_2$ before each fraction was tested for biological activity.

### 3.2.14 Radioactivity Profile of RP-HPLC Fractionated Lipids

$^3$H-acetate labeled lipids were fractionated by RP-HPLC as described in section 3.2.13. Each of the 30 fractions, of 1 ml volume, generated from the RP-HPLC separation, was directly collected into a scintillation vial containing 10 ml of Scinti-Verse II (Fisher). The radioactivity of

each vial was quantified using a Beckman LS-3801 beta-counter (Beckman Instruments).

### 3.2.15 Thin Layer Chromatography (TLC) of Lipids

Plates (20 X 20 cm) pre-coated with Kieselgel 60 (BDH, Toronto, Canada) were first activated by placing them in an oven at 70°C for 1 hr. Once the plates had cooled, lipids extracted from BM cells culture supernatants were spotted onto the plates. These were then transferred to a developing tank that had been saturated with the following solvent mixture: chloroform, isopropanol, ethanol and 1 M acetic acid (20:20:60:10). The run was allowed to proceed until the solvent front had migrated the entire length of the plate. Unsaturated lipids could be temporarily visualized by exposing the TLC plate to iodine vapours.

### 3.2.16 Isolation of Lipids from TLC Plates

Larger quantities of lipids were spotted along the origin of activated preparative TLC plates (BDH). The plates were allowed to develop in the same solvent system as described in section 3.2.15. Following the lipid fractionation, a 2 cm vertical strip of the plate was cut and exposed to iodine to reveal lipid bands. This strip was then used as a template to demarcate the areas on the plate where bands had migrated. Five areas of interest were chosen and the silica from each of these regions was gently scraped off the glass plate. The five fractions collected correspond to the following migration distances (cm) from the bottom of the TLC plate: F1 = 16.5-15.0, F2 = 15.0-13.0, F3 = 13.5-11.9, F4 = 8.7-7.0, and F5 = 4.9-3.3. The silica scrapings from each fraction were resuspended in chloroform, methanol and water (10:10:3) to extract

bound lipids. This mixture was vortexed and allowed to stand at RT for 1 hr. Once the silica had settled, the organic solvents were collected and the recovered lipids were concentrated by reducing the volume to 0.25 ml under a stream of $N_2$.

### 3.2.17 Reactivity of Suppressive Lipids with Spray Reagents

Lipids of interest were tested for reactivity with chemical spray reagents following the fractionation of these lipids by TLC. To test the six different reagents, lipid extracts known to contain BDSF activity were run in six separate lanes and then each lane physically cut by scoring the glass plate with a diamond pen. The chromatography spray reagents used were as follows: triphenyl tetrazolium chloride, cis-aconitic anhydride, cupric acetate, molybdenum blue, orcinol and cyclodextrin (Sigma). All reagents were prepared and used as indicated on the instruction forms obtained from the Sigma Chemical Company. Briefly, each of the strips of glass, containing all BM derived lipids, were sprayed with one reagent and then allowed for colour develoment. Lipids that had migrated to the area of the TLC plate that had been shown to contain suppressive activity, were scored for reactivity with each spray reagent using as guidelines the Sigma Chemical Co. information pamphlets for each reagent to interpret the results.

### 3.2.18 Autoradiography of Labeled Lipids

$^3$H-acetate labeled lipids were first run on TLC as described in section 3.2.15. Their position on the TLC plate was determined by first overlaying the plate with Hyperfilm-$^3$H (Amersham, Oakville, Canada). The film was exposed at -70$^0$C, in a light proof box, for 6 days. It was

subsequently developed using a Data-IV X-ray film developer (Picker, Cleveland, OH).

### 3.2.19 Recovery of Labeled Lipids from TLC Plates

To recover the two closely migrating labeled lipids, TLC was used initially to fractionate total BM derived lipids as described in section 3.2.15. An X-ray film of chromatographed lipids was exposed using the same protocol as detailed in section 3.2.18. The developed film was overlaid onto the TLC plate and the area around both labelled lipids was marked off using a syringe needle. The silica from this area of the plate was collected and lipids bound to it were extracted as indicated in section 3.2.16.

To individually recover the two labeled lipid bands, unfractionated BM derived lipids were initially run on TLC plates using a different solvent system comprised of chloroform, methanol, water and 1 N $NH_4OH$ (65:35:4:4). This solvent was designed to increase the separation distance between the two labeled lipid bands of interest. Again, an X-ray film was exposed and then used as a visual indicator of the location of the labeled lipid bands on the TLC plate. In this case, individual bands were marked off and the silica around each lipid was carefully collected. The lipid content of each scraping was extracted following the same procedure described in section 3.2.16.

### 3.2.20 Culture of the U-937 Cell Lines

The human monocytic cell line U-937 was maintained in culture by bi-weekly passage in 25 ml Nunc flasks (Gibco) in RPMI-1640 supplemented with 10% FCS. To induce IL-1 synthesis, 1 X $10^5$ cells were plated in flat

bottom 96-well Nunc plates (Gibco) with 10 ng/ml of PMA (Sigma). Cultures were maintained in RPMI-1640 supplemented with 10% FCS. After 24 hrs, the microwell plates were centrifuged at 1000 rpm for 10 minutes and the cell-free supernatant was collected for testing in the IL-1 assay.

### 3.2.21 Statistical Analysis

The statistical analysis used is described in detailed in section 2.2.9.

# RESULTS

## 3.3.1 Effect of BDSF on Ab Synthesis

To elucidate the mechanism by which human BM cells suppressed the generation of *in vitro* primary Ab responses, BM cells were tested for their ability to release suppressive mediators by culturing them for 24 hr in RPMI-1640 medium, in the absence of serum supplement or lectins. Using protocols originally established in the murine system, and shown to be effective in enriching for suppressor activity, the human BM cell culture supernatants were concentrated using an Amicon ultrafiltration system. Molecules of molecular weight range <10,000 Da and >1,000 Da were recovered and tested for biological activity in a PFC assay using tonsillar lymphocytes as responder cells. In Figure 3.1 evidence is presented that a factor collected from the above BM cultures, named Bone marrow Derived Suppressor Factor (BDSF), could significantly suppress the *in vitro* IgM plaque formation in a dose dependent manner. BDSF was suppressive of Ab responses at doses which had no adverse effects on the viability of the responding cells (Figure 3.1). Having not yet determined the biochemical nature of BDSF, the amount of factor added to cultures was standardized by expressing the concentration of BDSF as volume equivalents of the original BM culture supernatants.

## 3.3.2 Effect of BDSF on Con A Induced Proliferation

To assess whether BDSF could also suppress the concanavalin A (Con A) induced cellular proliferation, tonsillar cells were cultured in the presence of 5 μg/ml of Con A and varying doses of BDSF. As shown in Figure 3.2, BDSF significantly inhibits the lectin induced DNA synthesis

FIGURE 3.1

Effect of BDSF on PFC Responses

Tonsillar cells were cultured in the presence of Ag for 7 days and varying doses of human BDSF were added at culture initiation. The quantity of BDSF added (in 100 $\mu$l volumes) reflects the various concentrations and dilutions performed of BM culture superantants in order to enrich for BDSF activity. The amount of BDSF added represents the volume of original culture supernatant equal to the volume of concentrated BDSF added to the PFC cultures; this is referred to as "culture volume equivalents". The control PFC responses of tonsillar cells in the absence of Ag (-ve C) and with only Ag (+ve C) are indicated. The viabilty of day 7 cultures was assessed by the trypan blue dye exlusion method (●). The data presented are the means and the standard deviations (S.D.) calculated from eight values for each culture of one experiment which was representative of eight separate experiments.

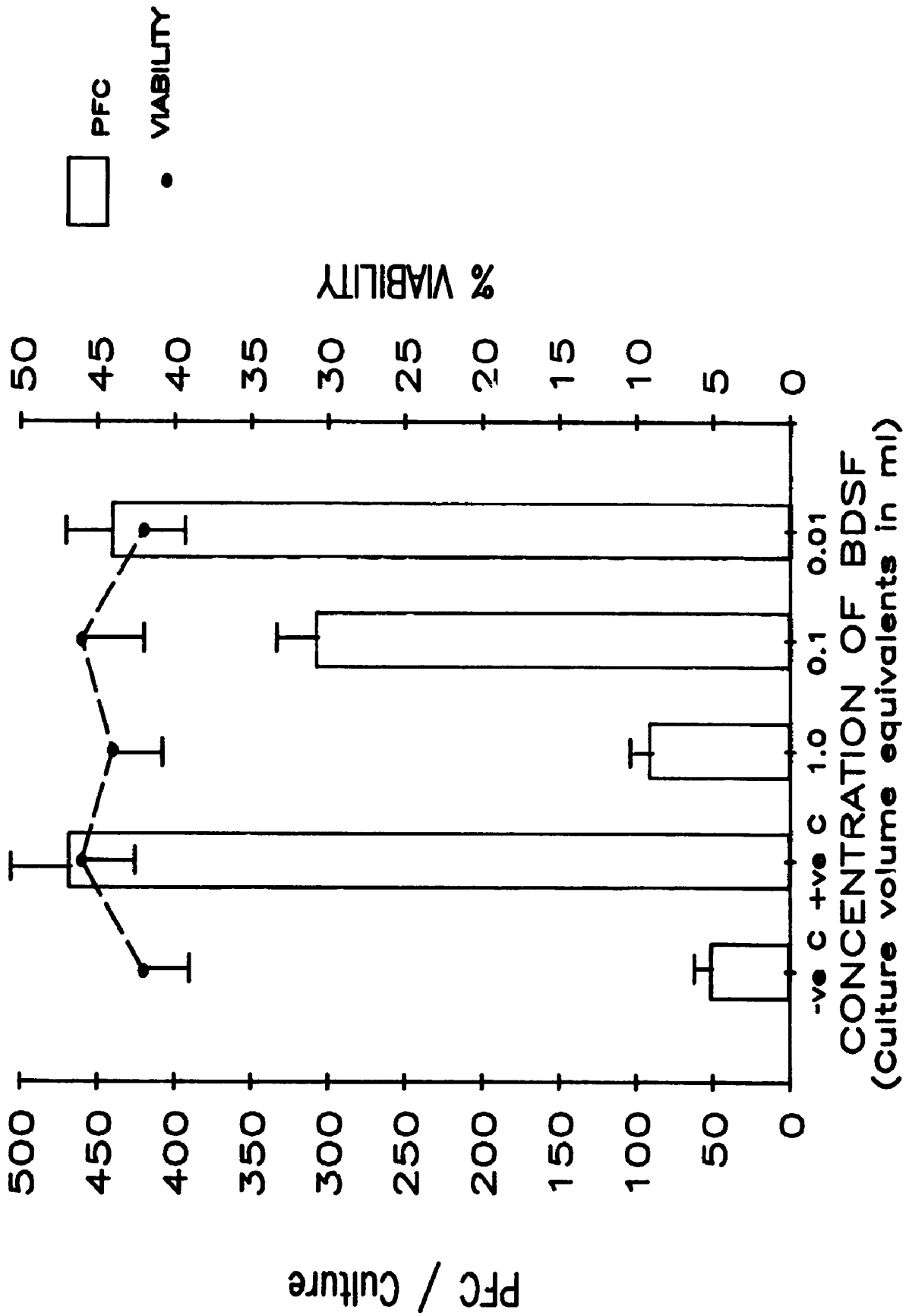
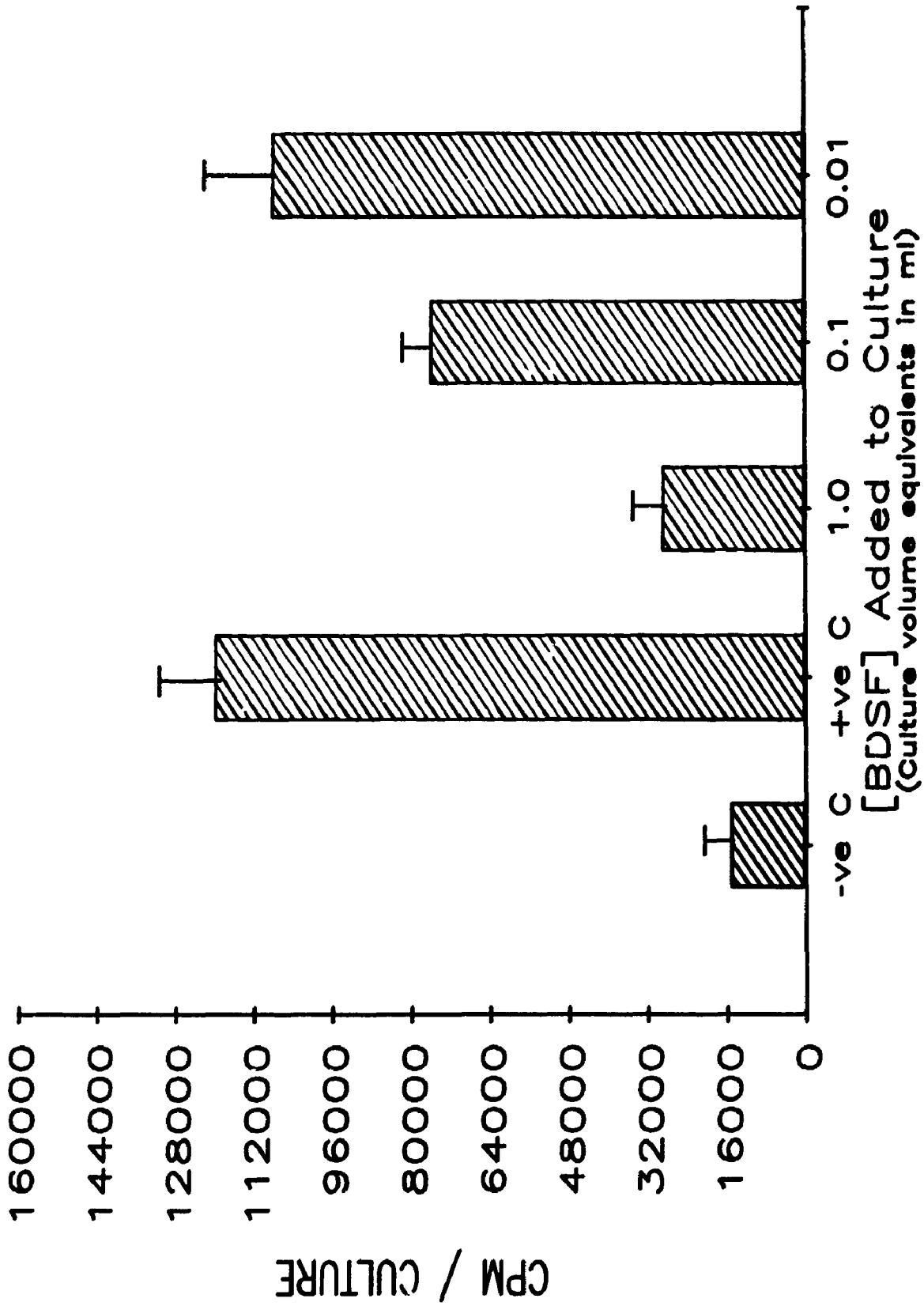

PFC
VIABILITY
% VIABILITY
PFC / Culture
500
450
400
350
300
250
200
150
100
50
0
50
45
40
35
30
25
20
15
10
5
0
-ve c
+ve c
1.0
0.1
0.01
CONCENTRATION OF BDSF
(Culture volume equivalents in ml)

FIGURE 3.2

Effect of BDSF on Con A Proliferative Responses

Tonsillar cells or PBL were plated at a density of 2 X $10^5$/well in 96 well plates and in the absence (-ve C) and presence of 5 $\mu$g/ml of Con A (+ve C). Where indicated BDSF was added in volumes of 20 $\mu$l/well of varying dilutions. The reported values are the mean CPM/culture plus S.D. of one experiment which was representative of six separate experiments.


CPM / CULTURE
160000
144000
128000
112000
96000
80000
64000
48000
32000
16000
0
-ve C
+ve C
1.0
0.1
0.01
[BDSF] Added to Culture
(Culture volume equivalents in ml)

of tonsillar cells and this suppression can be relieved by diluting out the factor.

### 3.3.3 Kinetics of BDSF action

Figure 3.3 illustrates the kinetics of BDSF action on the generation on an *in vitro* human Ab response. The suppressive effects of BDSF are optimal when the mediator is added to the cultures at either day 0 or day 1. Addition of the factor after more than 24 hours from culture initiation does not significantly affect the day 7 PFC response.

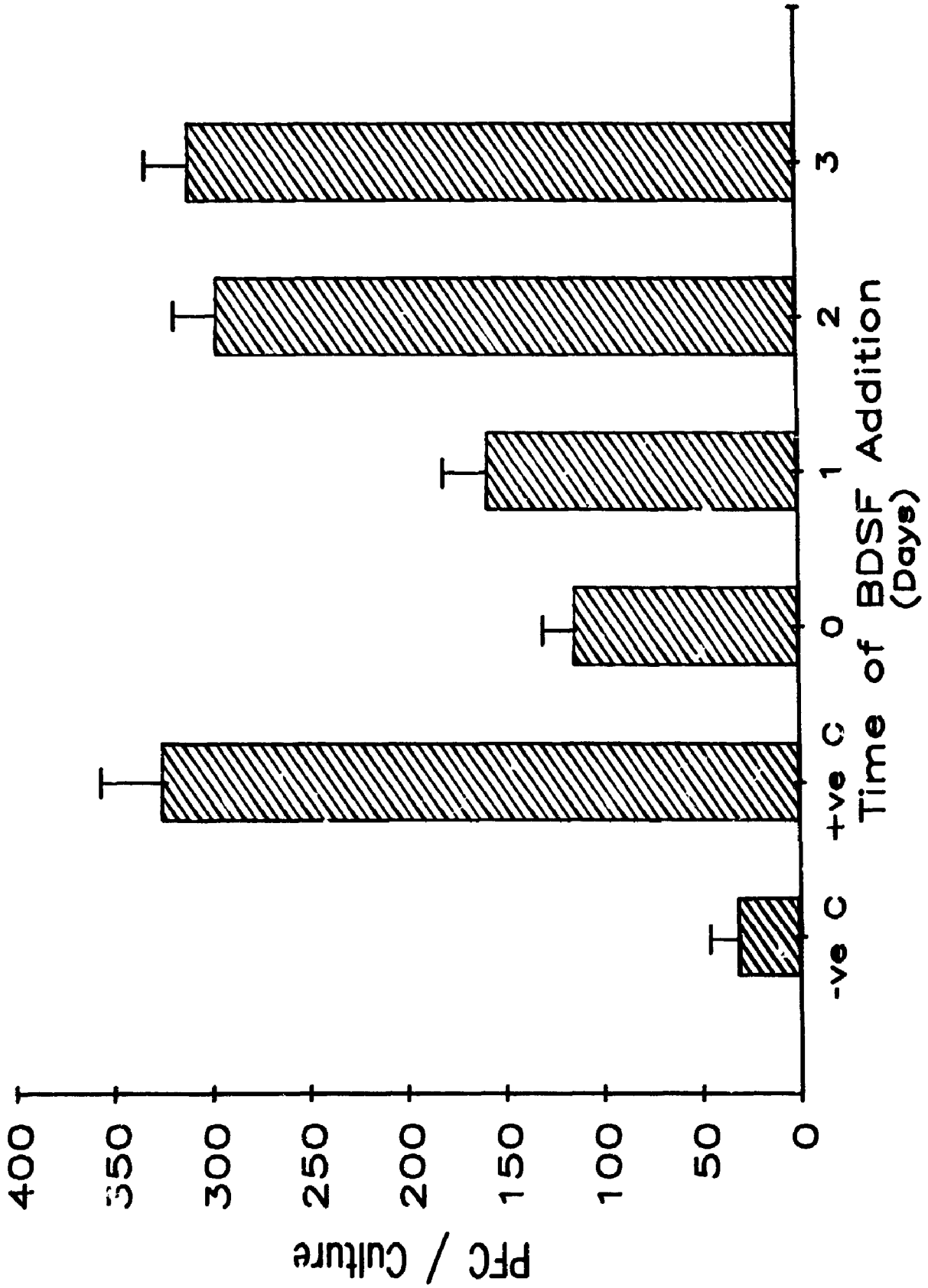
### 3.3.4 Effect of BDSF on BM Cells, Tonsillar Cells and PBL

Having established that BDSF could down regulate both Ab responses and lectin induced proliferation, it became of interest to investigate whether the background levels of DNA synthesis in various human tissues in the absence of stimulatory signals, were sensitive to BDSF suppressive effects. Table 3.1 summarizes data obtained from experiments where BM, tonsils and PBL were used as sources of responding cells. It is evident that a 1 ml volume equivalent dose of BDSF, that is normally highly suppressive of Ab responses, minimally inhibits the background level of cellular proliferation of all three tissues. This suppression ranges from 33% of control values for BM cells to 16% for tonsillar cells. The more striking observations occur when the above three cell populations are first activated by Con A, and then assayed for DNA synthesis in the presence of BDSF. Under these conditions the same dose of BDSF demonstrates an increased ability to suppress DNA synthesis of the same cells. The latter effects now range between 78% and 96% inhibition of control responses.

FIGURE 3.3

Kinetics of BDSF Action on PFC Responses

Tonsillar cells were cultured without SRBC (-ve C) or with SRBC (+ve C) and the anti-SRBC specific PFC responses were measured on day 7. On days 0, 1, 2, or 3, after culture initiation, a predetermined suppressive dose of BDSF (1.0 ml culture equivalents) was added to cultures in 100 µl volumes.


PFC / Culture
400
350
300
250
200
150
100
50
0
-ve C
+ve C
0
1
2
3
Time of BDSF Addition
(Days)

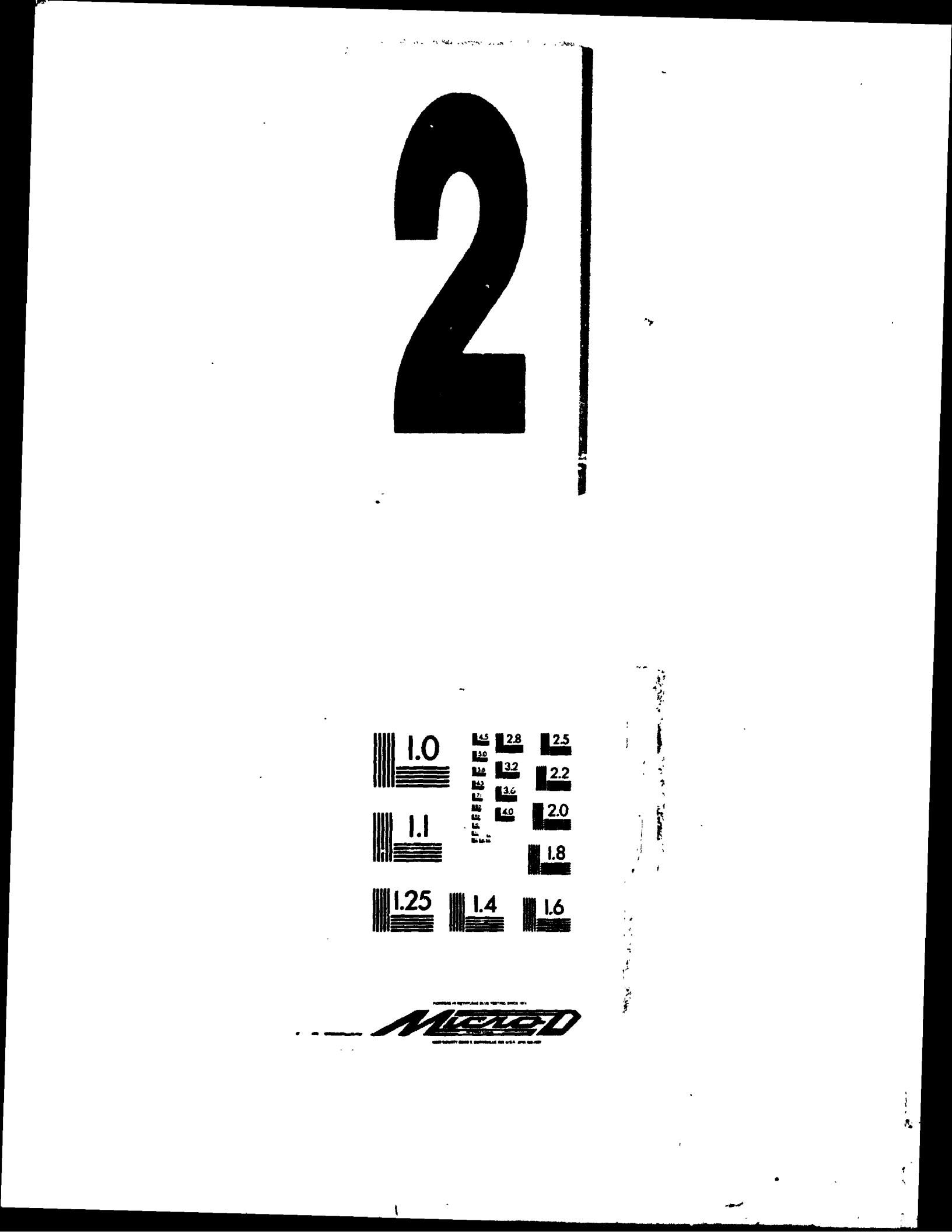

2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8

TABLE 3.1

Effect of BDSF on DNA Synthesis

| Source of Cells | | + BDSF[a] | + Con A[b] | + Con A + BDSF |
|---|---|---|---|---|
| | CPM ± SD | CPM ± SD (c) | CPM ± SD | CPM ± SD (d) |
| BM | 25,571 ± 1,521 | 17,133 ± 2,012 (33) | 39,123 ± 4,312 | 1,565 ± 136 (96) |
| Tonsil | 3,887 ± 409 | 3,265 ± 397 (16) | 145,368 ± 20,315 | 31,771 ± 4,419 (78) |
| PBL | 3,506 ± 511 | 2,887 ± 446 (18) | 24,355 ± 415 | 4,349 ± 612 (82) |

a BDSF prepared by Amicon ultrafiltration was added to cultures as 20 µl/well of a 50X concentrated BM supernatant (equivalent to the content of 1.0 ml of original supernatant).

b Con A was used at a concentration of 10 µg/ml.

(c) % suppression compared to control response.

(d) % suppression compared to the Con A response.

### 3.3.5 Generation of BDSF BM Cells Enriched for NS Activity

To establish a link between the suppressor activity observed with BM cells and that observed with the factor (BDSF), it was necessary to demonstrate that a suppressor enriched BM population could give rise to BDSF activity. Since elutriation proved to be effective method to enrich for NS activity (Table 2.2), an attempt was made to generate BDSF by culturing elutriated BM cell fractions in serum free medium and then determining whether any BDSF-like activity was present in the cell free supernatants. In Table 3.2 a summary of such experiments is presented. The most striking finding is that only those supernatants derived from fraction 4 of elutriated BM cells were capable of significantly suppressing the PFC response. Fraction 4 represents a population of BM cells that was highly enriched for PFC suppressive activity (Table 2.2). The suppressor activity recovered in the culture supernatants of this fraction is enriched by approximately 5 fold; since a dose of only 0.02 ml volume equivalents of supernatant from elutriated BM cells is necessary to suppress the Ab response by 45%, while, 0.1 ml volume equivalents of supernatant from unfractionated BM cells exhibits only 47% suppression of the same response (Table 3.2).

### 3.3.6 Biochemical Nature of BDSF

Initially BDSF was prepared by culturing human BM cells in RPMI-1640 medium without any serum supplement. The collected supernatant was rendered cell free, and then concentrated on an Amicon ultrafiltration system such that molecules of molecular weight range between 10,000 Da and 1,000 Da were collected. These preparations of BDSF when added at a dose of 1 ml volume equivalent of original culture supernatant, could yield a

TABLE 3.2

Generation of BDSF from BM Cell Fractions Enriched for NS Activity

| Responding Cells | Source of BDSF Added to Culture | [a][BDSF] Added | PFC/Culture $\bar{X} \pm$ S.D. | % Shift in Response |
|---|---|---|---|---|
| PBL | ---- | --- | 815 ± 56 | --- |
| PBL | Unfract BM | 0.1 | 431 ± 37 | -47 |
| PBL | Elut F1 Super | 0.02 | 867 ± 58 | + 6 |
| PBL | Elut F2 Super | 0.02 | 1263 ± 193 | +65 |
| PBL | Elut F3 Super | 0.02 | 773 ± 54 | - 5 |
| PBL | Elut F4 Super | 0.02 | 450 ± 49 | -45[b] |

[a] The concentration of BDSF added to culture was standardized by referring to the volume, in ml, of original BM cells culture supernatant that the added amount represented. Experiments were repeated three times.

[b] Statistical difference from cultures without BDSF $p < 0.005$.

suppressive effect on Ab response of the order of 50% (Table 3.3). To determine the biochemical nature of the suppressive molecules, biologically active samples of Amicon prepared BDSF were subjected to ammonium sulphate precipitation or chloroform-methanol lipid extraction. Precipitated proteins from BM cell culture supernatants completely lacked Ab suppressive activity at all doses tested (Table 3.3). On the other hand, total lipid extracts from the same sample were significantly suppressive of the PFC response.

Of further interest were the findings that excluded the possibility that prostaglandins contaminating BDSF preparations may have been responsible for the observed suppressive activity. BDSF generated in the presence of either $10^{-5}$ or $10^{-6}$ M indomethacin was found to be as suppressive as control BDSF preparations.

### 3.3.7 Silicic Acid Chromatography of BM Derived Lipids

In order to determine the relative polarity of lipids that contained BDSF activity, silicic acid chromatography was employed. Figure 3.4 is a photograph of the thin layer chromatograph used to analyze lipids extracted from unfractionated BM cell supernatants and lipids recovered from the three fractions eluted from the silicic acid chromatography column. The sequence of eluted lipids from the column was as follows: fraction 1 represents neutral lipids that were eluted with chloroform; fraction 2 contains slightly polar lipids eluted with a solvent mixture of chloroform-methanol (9:1), and fraction 3 is a pool of the most polar lipids that were eluted with a mixture of 1:1 chloroform and methanol. When these fractions were tested for their ability to suppress *in vitro* Ab responses, the BDSF-like suppressive activity resided largely in

TABLE 3.3

Isolation of Human BDSF[a]

| Culture Condition | Method of Preparation of BDSF | PFC/ Culture $\bar{X}$ + S.D. |
|---|---|---|
| [b]Cells + Ag | ---- | 392 ± 33 |
| Cells + Ag | Amicon (<10Kd->1Kd)[c] | 191 ± 24[d] |
| Cells + Ag | 90% $(NH_4)_2SO_4$ ppt.[e] | 400 ± 47 |
| Cells + Ag | $CHCl_3$-$CH_3OH$ Lipid Extr [f] | 115 ± 22[d] |
| Cells + Ag | Indomethacin Lipid Extr [g] | 126 ± 15[d] |

[a] Data presented are from one experiment which was representative of four separate experiments.

[b] Tonsillar cells were cultured in the absence of BDSF.

[c] BDSF was added to cultures at a concentration of 1 ml of original BM culture volume equivalents.

[d] Statistical difference from control $p < 0.001$

[e] Protein precipitates were added to cultures at doses ranging from 0.1-5.0 μg/ml of culture volume; value reported is the maximal suppressive response obtained.

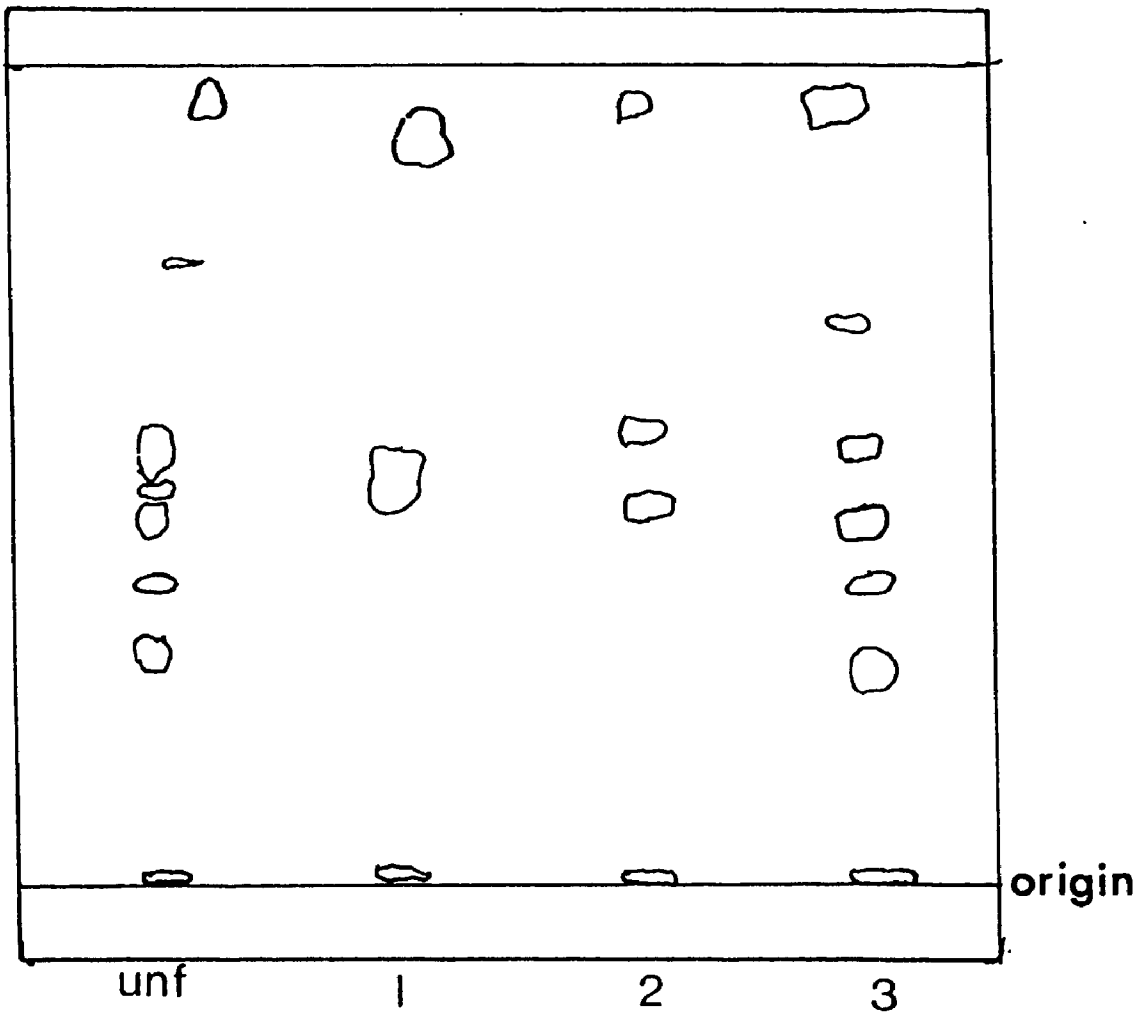
[f] Lipids were added to cultures at a final dilution of 1:500 which was equivalent to the content of 0.02 ml of original supernatant.

[g] BDSF was generated by culturing BM cells in the presence of $10^{-5}$M indomethacin. BDSF was then added to cultures as the equivalent of 0.04 ml of BM culture volume equivalents.

FIGURE 3.4

TLC of BM Lipids Fractionated by
Silicic Acid Chromatography

Lipids extracted from human BM cultures were fractionated by silicic acid chromatography as described in section 3.2.12 of Materials and Methods. The three arbitrary fractions collected and the unfractionated lipids were run on an analytical TLC plate for lipid banding pattern analysis. Unfractionated lipids were run in the lane marked "unf", while lipids eluted with 100% chloroform were run in lane "1", 90% chloroform-10% methanol eluted lipids were in lane "2", and lipids eluted with 50% chloroform-50% methanol were in lane "3". Unsaturated lipids were visualized by exposure to iodine vapours and their location on the TLC plates was traced with a pencil. The migration pattern was then reproduced on a transparency and presented as shown in Figure 3.4.


origin
unf
1
2
3

fraction 2, with some activity in fraction 3 (Figure 3.5). Fraction 1 was completely devoid of PFC suppressor activity. In addition, a comparison of the suppressor activity of unfractionated BM lipids or of those recovered in fraction 2 following silicic acid chromatography, revealed that the latter lipids were much more suppressive than the former. This difference was even more evident at the higher dilutions of lipids used in the cultures. The minimal suppressive activity observed with fraction 3 lipids is likely a result of residual fraction 2 suppressive lipids that were not completely eluted off the column.

### 3.3.8 Fractionation of BM Extracted Lipids by RP-HPLC

Having determined that BDSF activity could be recovered in polar lipid extracts of BM cells culture supernatants, reverse phase HPLC (RP-HPLC) was used to more accurately fractionate the total BM derived lipids. The solvent of choice proved to be a gradient run over a 15 minute period, starting with 90% methanol and 10% isopropanol and finishing with 20% methanol and 80% isopropanol. This created an elution system of decreasing polarity. The chromatograph of separated lipids is shown in Figure 3.6. The biological activity of these lipids was tested by first pooling the original 30 fractions into four arbitrary larger fractions (I to IV) and then adding the latter fractions to PFC responses. Figure 3.6 also demonstrates that all of the BDSF suppressive activity resides in fraction II, which is comprised of original fractions 4 through 10. The elution of suppressor activity in this fraction is consistent with the polar nature of BDSF as shown in section 3.3.7. Fractions I, III and IV were unable to significantly alter the PFC response from control values.

FIGURE 3.5

Effect of Lipids Fractionated by Silicic Acid Chromatography on Ab Responses

Human BM derived lipids recovered following silicic acid chromatography were tested for their ability to suppress primary Ab responses of tonsillar cells. Unfractionated lipids and those of collected fractions, F1 to F3, were added to PFC cultures at the indicated dilutions at culture initiation. The day 7 anti-SRBC specifc Ab response of cells cultured in the absence of lipids is indicated (□).

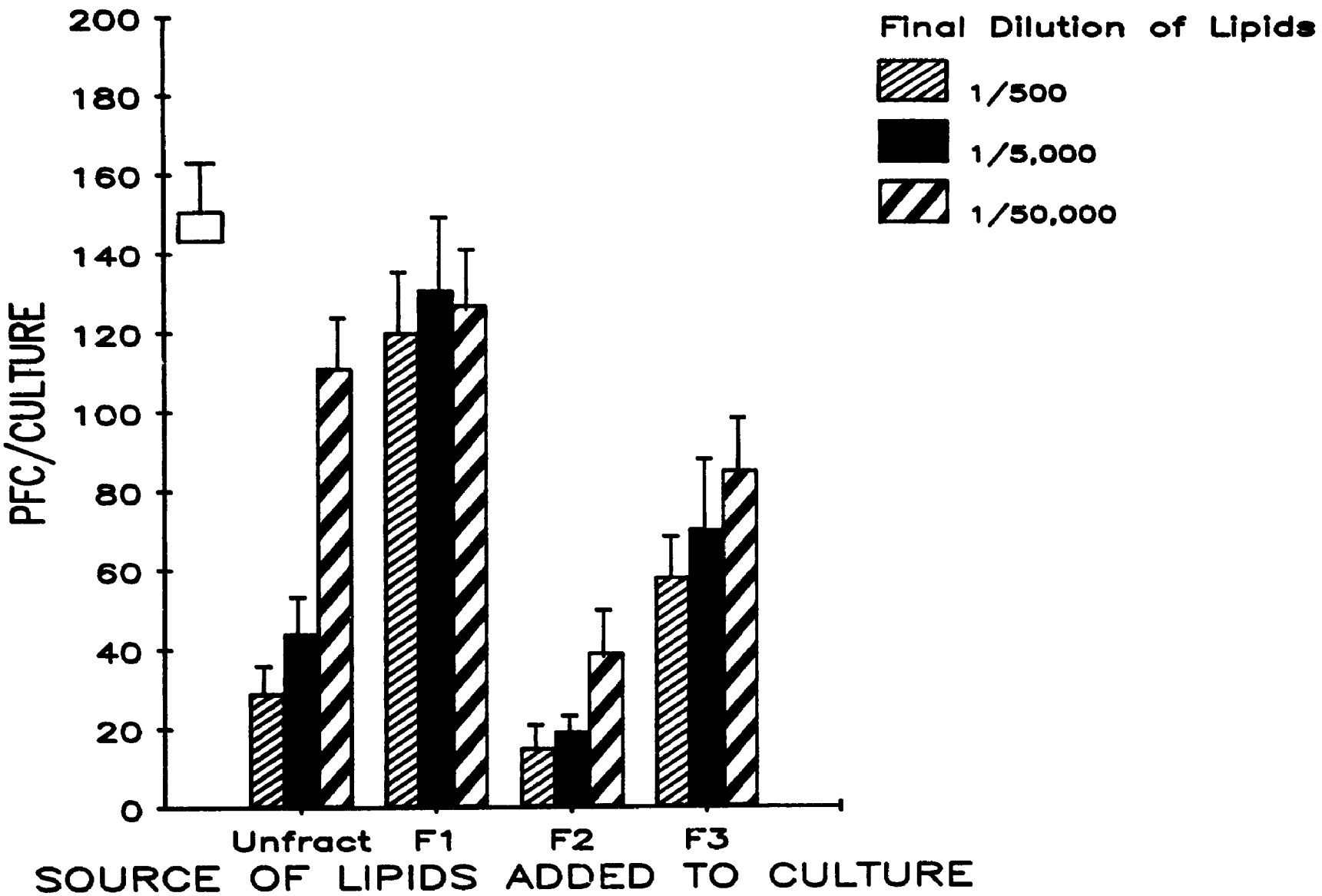
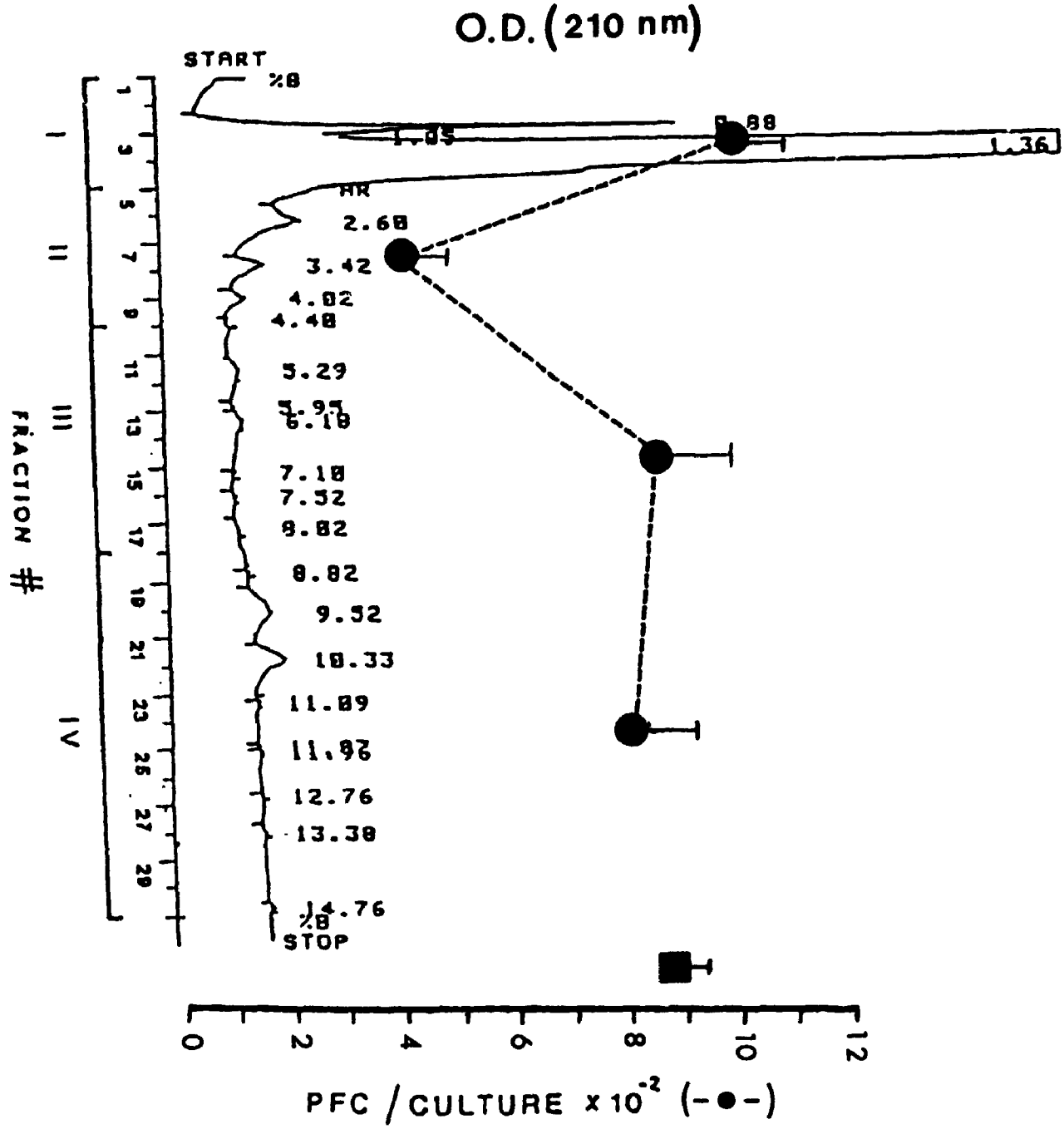

Final Dilution of Lipids
1/500
1/5,000
1/50,000
PFC/CULTURE
200
180
160
140
120
100
80
60
40
20
0
Unfract
F1
F2
F3
SOURCE OF LIPIDS ADDED TO CULTURE

FIGURE 3.6

Reverse Phase-HPLC of BM Derived Lipids

Total lipids extracted from human BM culture supernatants were fractionated by RP-HPLC using a gradient solvent system. The gradient ran over a 15 minute period, starting with a mixture of 90% methanol and 10% isopropanol, and ending with a solvent mixture of 20% methanol and 80% isopropanol. The 30 lipid fractions initially collected were pooled into four larger ones (FI, FII, FIII and FIV) and then diluted in complete culture medium before being added to PFC cultures. The PFC responses of tonsillar cells in the presence of Ag but no lipids ( ■ ) and those of cultures receiving lipids from each of the RP-HPLC generated fractions, FI to FIV, ( ● ) is indicated by superimposing it onto the HPLC chromatograph. The PFC values reported are the maximally suppressed responses observed from testing the lipid fractions at three dilutions: 1:500, 1:1,000 and 1:5,000.


O.D. (210 nm)
START
%B
1.05
.88
1.36
HR
2.68
3.42
4.02
4.48
5.29
5.95
6.18
7.18
7.52
8.82
8.82
9.52
10.33
11.09
11.96
12.76
13.38
14.76
%B
STOP
FRACTION #
I
II
III
IV
0
2
4
6
8
10
12
PFC / CULTURE x 10⁻² (-●-)

This latter finding also serves as a control to demonstrate that not all lipids were suppressive of the Ab response.

### 3.3.9 Fractionation of $^{3}$H-Acetate Labeled BDSF

In an effort to biosynthetically label BM derived lipids with a radioactive precursor, $^{3}$H-acetate was added to BM cell cultures being used to generate BDSF. Having determined the relative elution properties of BDSF from RP-HPLC, it was of interest to see whether lipids that had incorporated exogenous $^{3}$H-acetate resided in the same suppressive HPLC fraction II. Radioactively labelled BDSF was fractionated by RP-HPLC, as in previous runs, and the thirty fractions generated were collected and individually monitored for radioactivity. The results of such experiments are presented in Figure 3.7. These data demonstrate that 45% of the total radioactivity is associated with fraction 7, whose lipids are a major component of the highly suppressive HPLC fraction II described in section 3.3.8.

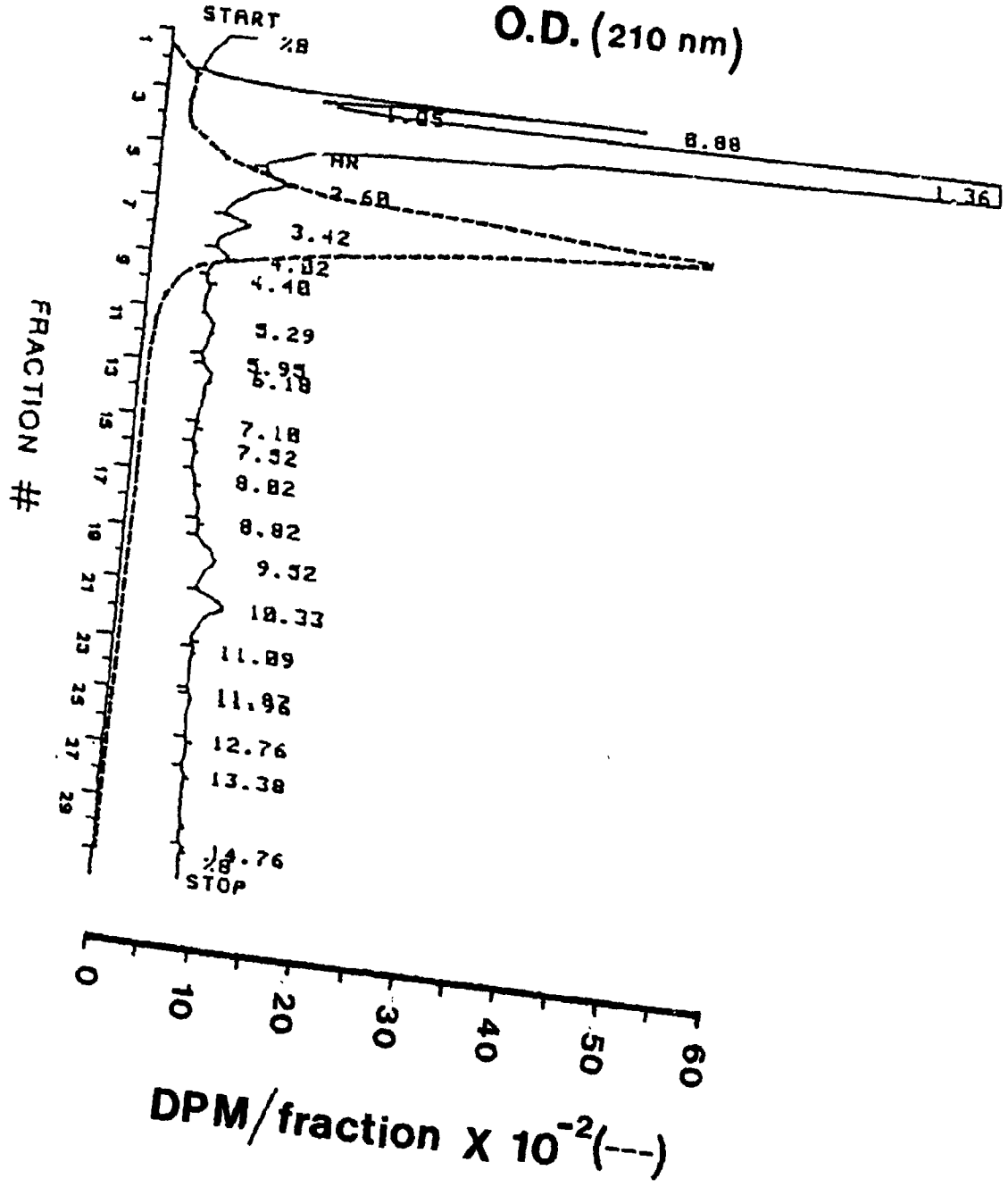
### 3.3.10 Enrichment for BDSF by Thin Layer Chromatography (TLC)

In an effort to associate suppressor activity with a particular lipid, TLC was used as a means of separating the various BM derived lipids. Preparative TLC plates were spotted with BM derived lipids and then allowed to migrate using a relatively polar solvent mixture consisting of chloroform, isopropanol, ethanol and 1 M acetic acid (20:20:60:10). The plates were first lightly stained by exposure to iodine, and then cut into 5 distinct strips that represented the five different lipids visualized. The silica in each glass strip was subjected to a lipid extraction. The lipids from 5 different areas of the TLC plate

FIGURE 3.7

Reverse Phase-HPLC of $^{3}$H-Acetate Labeled BM Derived Lipids

$^{3}$H-acetate labeled lipids, extracted from human BM culture supernatants, were fractionated by RP-HPLC using the same solvent system described for Figure 3.6. Thirty fractions, of 1 ml volume each, were generated by collecting the column eluent at 0.5 minute intervals. The radioactivity associated with each of these fractions was assessed by mixing the eluted lipids with scintillation fluid and counting them on a beta counter. The radioactivity profile of all the fractions (---) has been superimposed onto the chromatograph.


O.D. (210 nm)
START
%B
1.05
8.88
HR
2.68
1.36
3.42
4.32
4.48
5.29
5.95
6.18
7.18
7.52
8.82
8.82
9.52
10.33
11.89
11.96
12.76
13.38
14.76
%B
STOP
FRACTION #
0
10
20
30
40
50
60
DPM/fraction X $10^{-2}$(---)

were tested individually for biological activity as well as being spotted on an analytical TLC plate for visualization. Figure 3.8 is a chromatograph demonstrating the migration pattern of the 5 lipids extracted from the silica strips as well as that of unfractionated lipids. Figure 3.9 is a summary of testing the 5 TLC separated lipid fractions in a PFC assay. The results suggest that only lipids in fraction 3 are significantly suppressive for Ab responses.

### 3.3.11 Effect of Depletion of HNK-1 BM Cells on BDSF Synthesis

In chapter 2, data was presented demonstrating that human BM NS activity was sensitive to anti-HNK-1 plus C′ treatment (Fig. 2.3). To definitively establish that BDSF was a product of the above NS cells, it was necessary to show that HNK-1 depleted BM cells would be unable to synthesize BDSF. Supernatants from untreated or anti-HNK-1 treated BM cell cultures were extracted for lipids and their activity assessed in an Ab culture system. Figure 3.10 demonstrates that BDSF derived from either untreated or C′ only treated BM cell cultures are both highly suppressive of the Ab response. On the other hand, anti-HNK-1 depleted BM is completely devoid of PFC suppressor activity at all doses tested.

As a further confirmation that anti-HNK-1 treatment was abrogating BDSF production, supernatants from the latter cultures were examined by analytical TLC. The chromatograph of such lipids after visualization with iodine vapours, Figure 3.11, demonstrates that the top band of this normally seen pair of migrating bands is missing. In addition, the bottom lipid band is present at a reduced level (lane A). This pair of lipid bands migrates to the same area of the TLC as the suppressive lipids recovered from fraction 3 of the TLC separated lipids (section 3.3.10).

FIGURE 3.8

Fractionation of BM Derived Lipids by TLC

Human lipids derived from BM culture supernatants were spotted onto a preparative TLC plate and developed in a solvent system containing chloroform, isopropanol, ethanol and acetic acid (20:20:60:10). Using a separate lane, where total lipids had been spotted and subsequently stained with iodine, as a position indicator of the individual lipids, the plate was divided into 5 regions, F1 through F5, and the silica from each area collected. The lipids bound to the silica scrapings were individually extracted and then 5 $\mu$l aliquots from each fraction were run on an analytical TLC plate to determine the relative purity of each lipid fraction. The presented photograph of the latter chromatograph demonstrates the migration pattern of the following lipids: lane 1, F1 recovered lipids; lane 2, F2 recovered lipids; lane 3, F3 recovered lipids; lane 4, F4 recovered lipids; lane 5, F5 recovered lipids; lane 6, unfractionated BM lipids.

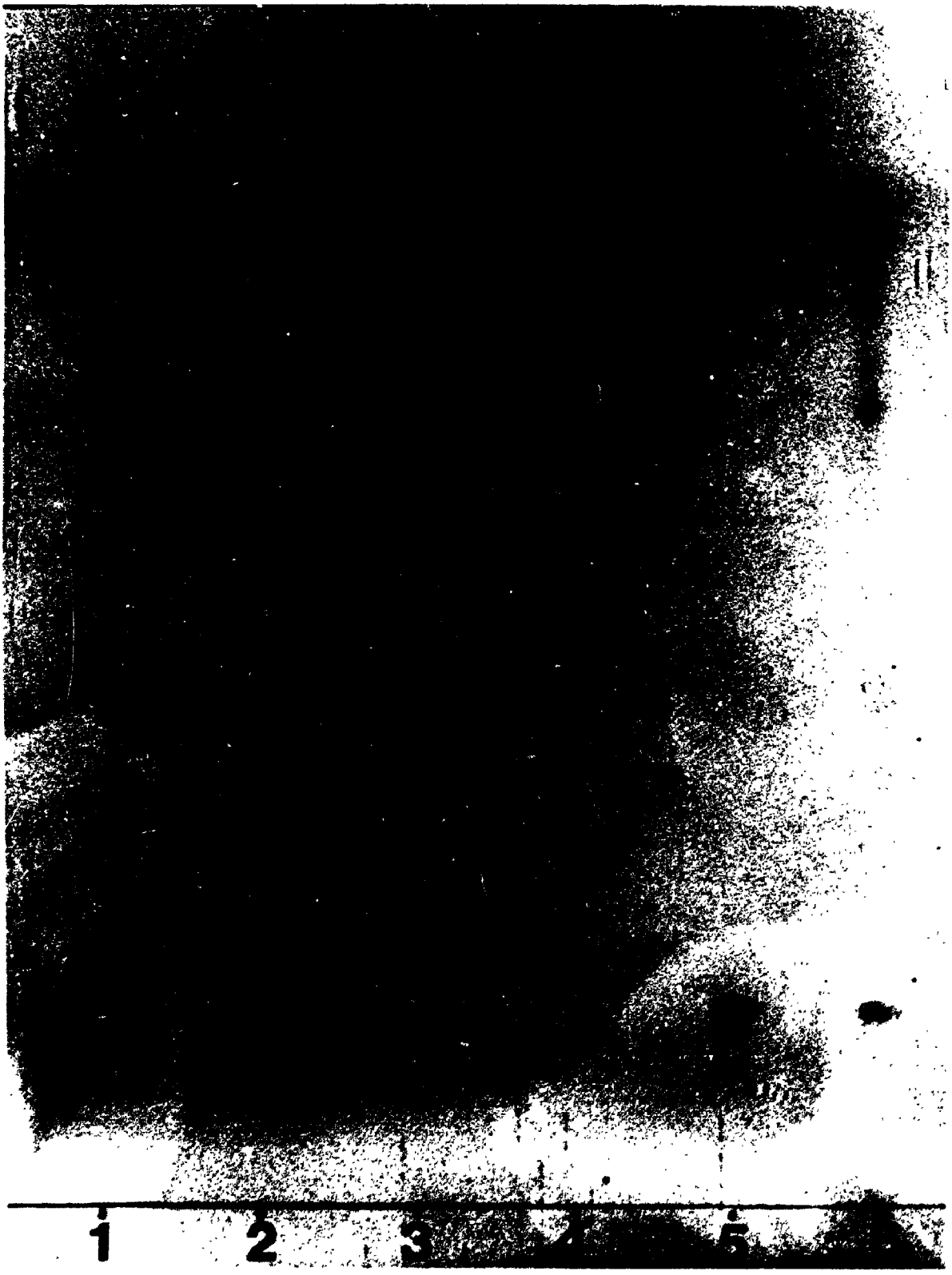

1
2
3
4
5

FIGURE 3.9

PFC Regulatory Activity of TLC Separated Lipids

BM derived lipids that had been fractionated by TLC and then recovered into 5 separate fractions (F1 to F5), were tested for biological activity in a PFC assay. Each lipid fraction was tested at three different doses and the final dilution of the lipids in culture is indicated. The ability of each lipid fraction to suppress the control PFC response of PBL cultured in the presence of SRBC (▲) is reported.

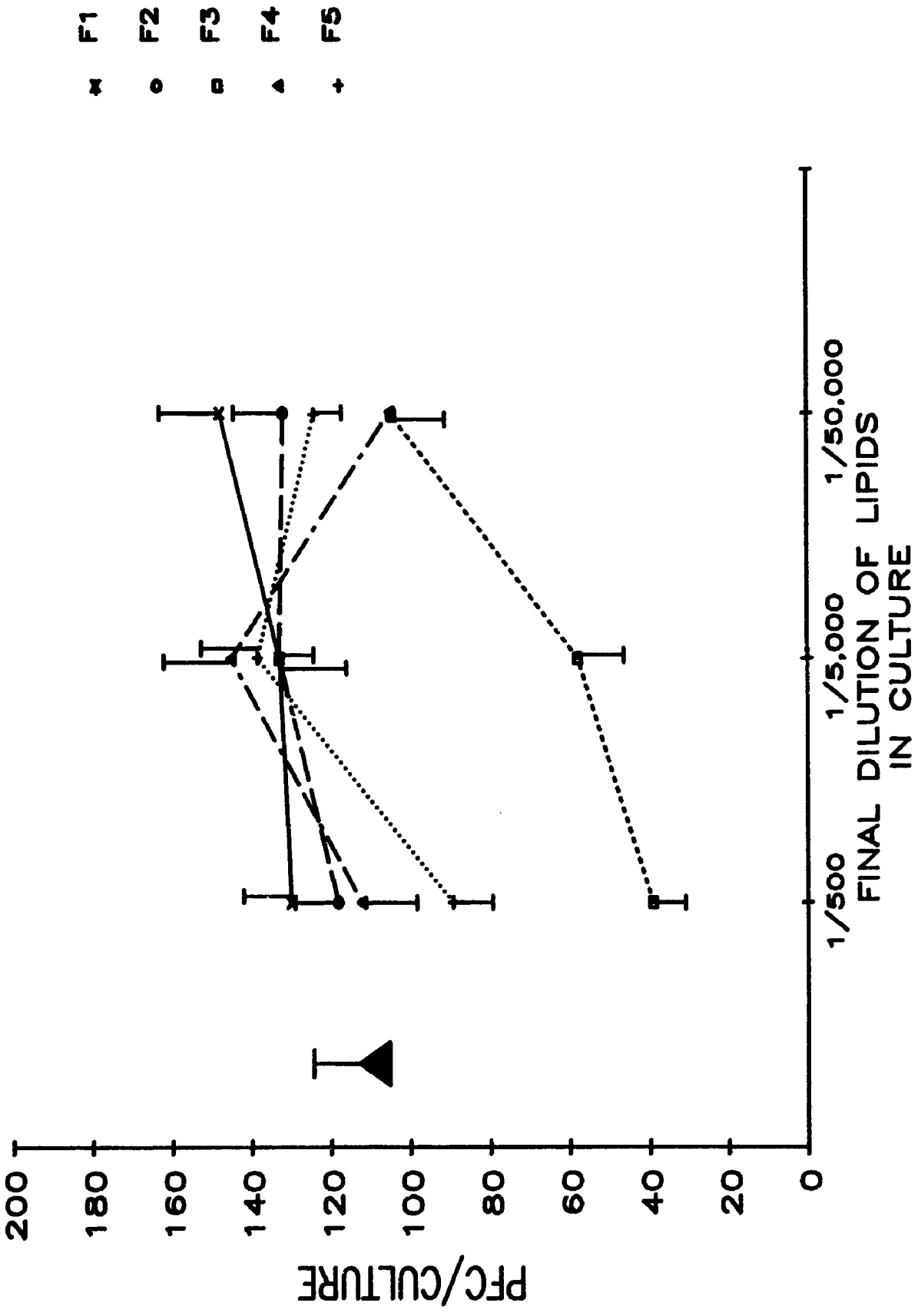

F1
F2
F3
F4
F5
PFC/CULTURE
200
180
160
140
120
100
80
60
40
20
0
1/500
1/5,000
1/50,000
FINAL DILUTION OF LIPIDS
IN CULTURE

FIGURE 3.10

Effect of NS Cells Depletion of BM on BDSF Production

The ability of NS depleted-BM cells to synthesize BDSF was tested by treating BM cells with the monoclonal anti-HNK-1 plus complement (C'). BDSF was then generated as usual by culturing either untreated BM cells, C' only treated BM cells, or anti-HNK-1 plus C' treated BM cells for 24 hrs and then extracting the lipids from the culture supernatants. Each of these extracts was tested at the three indicated dilutions for their ability to suppress a 7 days PFC response. The control responses of tonsillar cells cultured in the absence (○) and presence of Ag (●) are indicated.

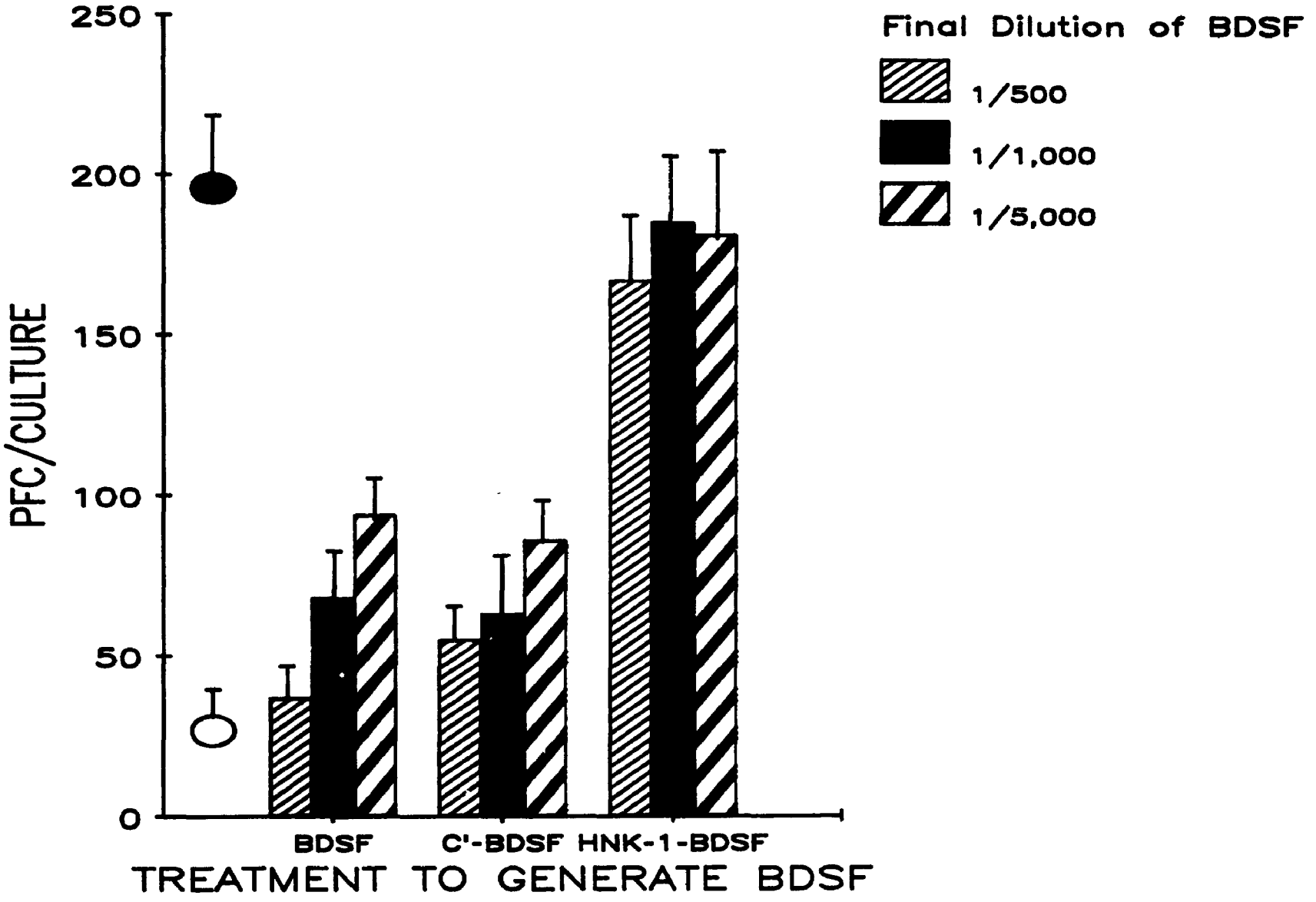

250
200
150
100
50
0
PFC/CULTURE
Final Dilution of BDSF
1/500
1/1,000
1/5,000
BDSF
C'-BDSF
HNK-1-BDSF
TREATMENT TO GENERATE BDSF

FIGURE 3.11

TLC of BDSF Recovered from NS Depleted BM Cells

Lipids generated from cultures of anti-HNK-1 plus C′ treated BM cells (lane A), C′ only treated BM cells (lane B), or untreated BM cells (lane C) were analylized by TLC. The photograph also demonstrates the TLC banding pattern of total lipids found in BM culture supernatants following exposure to iodine vapours. The arrows in lanes B and C indicate one specie of lipids that is present in these lanes but is absent in lipids recovered from NS depleted BM culture (lane A).


A
B
C

### 3.3.12 Labeled Lipids Have BDSF Activity

In a previous section (3.3.9) it was demonstrated that a population of lipids that were highly suppressive, contained a group of lipids that could incorporate $^{3}$H-acetate. To conclusively associate BDSF activity with such labeled lipids, TLC was used to isolate those lipids that had incorporated $^{3}$H-acetate. The iodine stained TLC plate and the autoradiograph of the same plate is presented in Figure 3.12. It is evident from the autoradiograph that only two of the numerous migrating bands incorporate the radioactive precursor.

To test whether these two labeled lipids were responsible for the observed suppressive effects, they were extracted from the TLC plate (Figure 3.13, lane A) and their biological activity assessed. As a control to demonstrate that any observed suppressive effects in lane A were not due to freely migrating $^{3}$H-acetate, the radioisotope was spotted in lane B of the TLC and ran under the same solvent conditions. From the autoradiograph it was evident that the free $^{3}$H-acetate did migrate to an area close to that of the labeled lipids. When labeled lipids were extracted from the TLC plate, silica was also taken from lane B at an equal distance from the origin. The recovered lipids were then tested for biological activity and the results are presented in Figure 3.13. The data demonstrate that only BM derived lipids from lane A are suppressive for Ab responses, while lipid extracts of lane B demonstrate no biological activity at both doses tested.

To assess which of the two labeled lipids was directly responsible for down regulating PFC responses, larger quantities of labeled lipids were loaded on preparative TLC plates and separated using a modified solvent system. The same labeled lipids that had been shown to be

FIGURE 3.12

Visualization of Labeled Lipids

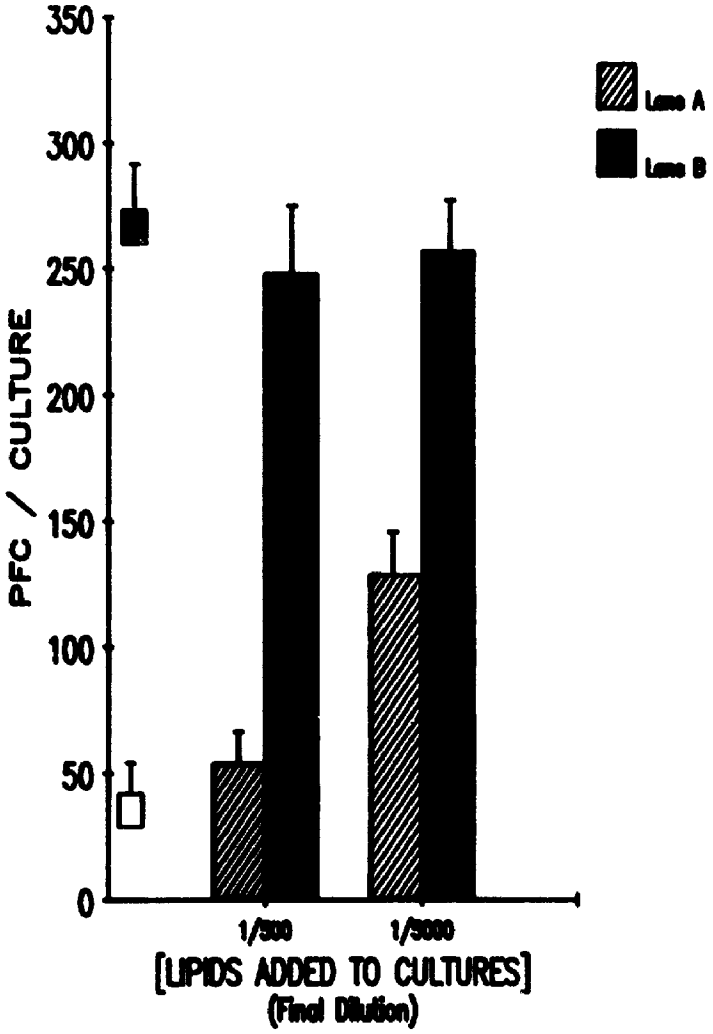
Labeled lipids were fractionated by TLC as described in sections 3.2.15 and 3.2.16 of Materials and Methods. To visualize those lipids that had incorporated the radioactive precursor $^{3}H$-acetate during the *in vitro* culture, an autoradiograph of the TLC plate was developed. Lane A represents the thin layer chromatograph of total BN derived lipids that could be visualized by exposure to iodine. Lane B is a photograph of a 6 days exposed autoradiograph of the same TLC plate.


A
B

FIGURE 3.13

Recovery of BDSF Activity from Labeled Lipids

Labeled lipids were first fractionated by TLC as described in sections 3.2.15 and 3.2.16 of Materials and Methods. An autoradiograph of the plate was then used to locate the two labeled lipids. Silica was collected around the area where the lipids of interest had migrated (lane A) and also from an area of equal migrating distance of a sham TLC fractionation of free $^{3}H$-acetate (lane B). Lipids extracted from the silica were tested at two different doses for their ability to suppress the tonsillar PFC response. The control Ab responses with (■) and without Ag (□) are also indicated.


350
300
250
200
150
100
50
0
PFC / CULTURE
Lane A
Lane B
1/500
1/5000
[LIPIDS ADDED TO CULTURES]
(Final Dilution)


A
B

suppressive (Fig. 3.13) were individually extracted from the TLC plate. Although both lipids are suppressive for PFC responses, a titration of their activities, shown in Figure 3.14, suggests that the band migrating towards the top of the plate in lane A of Figure 3.13 (FR1), is approximately 40 times more active than the bottom band (FR2). This is assuming that both bands are equally susceptible to labelling with $^{3}H$-acetate. Of interest is the fact that the most suppressive band, (FR1), is the same band that seems absent in the monoclonal depletion studies that removed NS activity from BM cell preparation (section 3.3.11).

### 3.3.13 Reactivity of TLC Fractionated Lipids with Biochemical Reagents

Having determined which of the TLC separated lipid bands were primarily responsible for BDSF activity, spray reagents were used to define some of the other possible biochemical characteristics of these lipids. A summary of the reagents used and the reactivity pattern for the two suppressive lipids is shown in Table 3.4. From the accumulated results the evidence supports the idea that BDSF is an unsaturated lipid that seems to lack sugar residues or choline residues. It had no reactivity with the reagents that detect the A, B, E, and F series of prostaglandins. However both lipid bands are strongly reactive with the molybdenum blue reagent which is specific for phospholipids. The fact that both labeled lipids, which were demonstrated to be suppressive to varying degrees (section 3.3.12), had an identical reactivity pattern with all reagents may indicate that these two lipids are related in structure. This may also explain their close migration pattern.

FIGURE 3.14

Assessment of the Individual Suppressor Actvity of the Two Labeled Lipids

The two species of lipids that incorporated $^{3}H$-acetate were individually titrated for suppressor activity. Initially the lipids were separated on TLC using a modified solvent system. With the aid of an autoradiograph, the individual lipids were recovered from TLC by performing a chloroform-methanol extraction on the collected silica. The lipid that is shown to migrate towards the top of the plate in Figure 3.12 (lane B), was designated as fraction 1 (FR1) and the bottom lipid, fraction 2 (FR2). Both labeled lipids were tested at five different dilutions for their ability to suppress the generation of a primary PFC response. The quantity of each lipid recovered was estimated by determining the radiation associated with each sample. The amount of each lipid added to cultures was extrapolated from the radioactivity measurements. The control PFC response by PBL in this experiment was 188 $\pm$ 12.

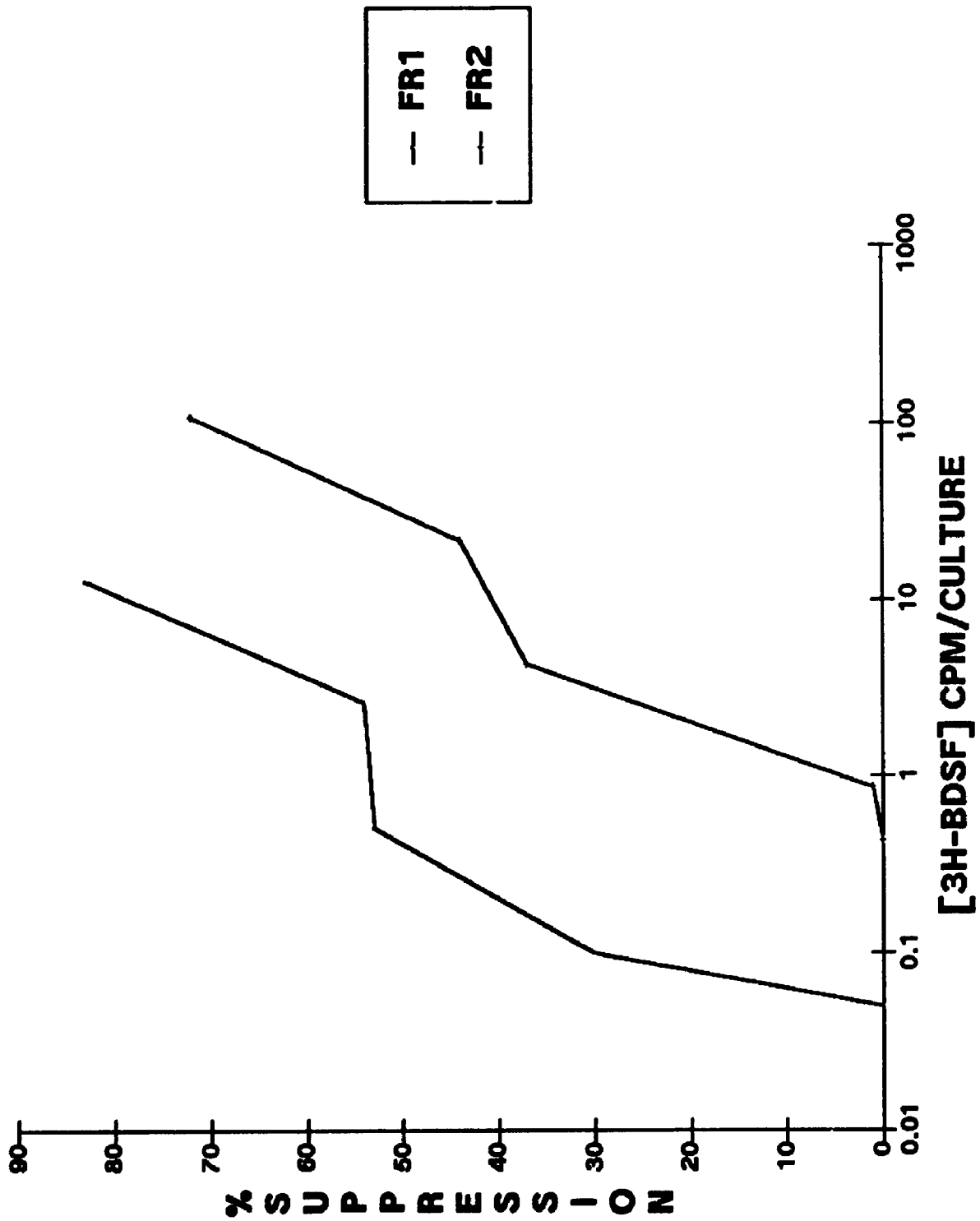

FR1
FR2
% SUPPRESSION
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
100
1000
[3H-BDSF] CPM/CULTURE

TABLE 3.4

Biochemical Characteristics of the Two Suppressive Lipids

| Reagent Used | Detectable Chemical Groups | Reactivity of Lipids | |
|---|---|---|---|
| | | FR1 | FR2 |
| Cyclodextrin | Saturated lipids | - ve | - ve |
| | Unsaturated lipids | + ve | + ve |
| Iodine Vapours | Unsaturated lipids | + ve | + ve |
| Orcinol | Sugars | - ve | - ve |
| Triphenyl Tetrazolium Chloride | Reducing sugars | - ve | - ve |
| Molybdenum blue | Phospholipids | + ve | + ve |
| Cupric Acetate | Prostaglandin A | - ve | - ve |
| | Prostaglandin B | - ve | - ve |
| | Prostaglandin E | - ve | - ve |
| | Prostaglandin F | - ve | - ve |

Total lipids from BM culture supernatants were fractionated by TLC prior to exposing them to the various biochemical reagents to detect chemical groups associated with the migrating lipids. The chemical reactions reported are for both of the lipid bands that were shown to be suppressive for Ab responses in section 3.3.12. FR1 and FR2 refer to the top and bottom migrating position of the two respective lipids in lane B of Figure 3.14. Each reaction was scored by consulting the information pamphlets for each reagent. This experiment was repeated twice.

### 3.3.14 Target of BDSF Action

#### 3.3.14.1 BDSF Can Be Transferred to Cultures by Non E-Rosetting Responding Cells

In an attempt to define the cell population initially involved in binding BDSF, E-rosetting was used to fractionate the responding cells. Following the fractionation, $E_R$+ or $E_R$- cells were incubated for 18 hours with BDSF and then added back to the cultures along with their complementary cell population. Table 3.5 shows that only the $E_R$- cells can transfer the BDSF suppressive activity. The suppression achieved by this method is in fact of greater magnitude than that observed by adding equal doses of BDSF directly to cultures.

#### 3.3.14.2 Labeled BDSF Binds to Non E-Rosetting Responding Cells

Since the initial evidence suggested the target of BDSF to reside in the $E_R$- fraction of responding cells, an attempt was made to confirm this by other techniques. $^3$H-acetate labeled BDSF was incubated with E-rosetted tonsillar cells, and then the radioactivity associated with either the cell pellet or the supernatant was quantitated. In Table 3.6 evidence is presented which supports previous findings that BDSF initially binds to $E_R$- cells. The radioactivity associated with the $E_R$- cell fraction is nearly 2 times that found with unfractionated cells and it is approximately 3 times greater than that found with the $E_R$+ cell fraction. The elevated radioactivity associated with the $E_R$- cell pellet coincides with the decreased radioactivity found in the supernatant of the same fraction. On the other hand, both supernatants from unfractionated and $E_R$+ cells had significantly higher levels of $^3$H-BDSF. The above pattern

TABLE 3.5

Ability of $E_R$- Responding Cells
To Transfer BDSF Suppressor Activity

| Culture Conditions | PFC/Culture (+/- S.D.) | % Suppression |
|---|---|---|
| Cells + Ag | 202 ± 31 | --- |
| Cells + Ag + BDSF(1/1000) | 92 ± 6 | 54[a] |
| $E_R$+ + Ag | 19 ± 9 | --- |
| $E_R$- + Ag | 70 ± 8 | --- |
| $E_R$+ + $E_R$- + Ag | 229 ± 41 | -13[b] |
| $E_R$+(BDSF) + $E_R$- + Ag | 244 ± 32 | - 7[c] |
| $E_R$+ + $E_R$-(BDSF) + Ag | 62 ± 9 | 73[d] |

---

Unfractionated or E-rosetted tonsillar cells were cultured in vitro for 7 days with SRBC to generate a primary Ab response. Where indicated, E-rosetted cells were incubated for 18 hrs at 37°C with a 1/1000 dilution of BDSF, washed with Hanks'BSS and then added to tissue culture wells. The anti-SRBC PFC response of four separate experiments is reported.

[a] Statistical difference from unfractionated cultures without BDSF, $p < 0.0005$.

[b] Statistical difference from unfractionated cultures without BDSF, $p > 0.1$.

[c] Statistical difference from E-rosetted cultures in the absence of BDSF, $p > 0.2$.

[d] Statistical difference from E-rosetted cultures in the absence of BDSF, $p < 0.0005$.

TABLE 3.6

BDSF Preferentially Binds
To Non E-Rosetting Cells

| Cell Populations Used in the Incubations with $^{3}$H-BDSF | % OF TOTAL RADIOACTIVITY IN: Cells | Supernatants |
|---|---|---|
| UNFRACTIONATED PBL | 22 | 78 |
| E-ROSETTING PBL | 13 | 87 |
| NON E-ROSETTING PBL | 41 | 59 |

$7.5 \times 10^{6}$ unfractionated or E-rosetted PBL were incubated with $^{3}$H-acetate labeled BDSF, for 2 hrs at 37°C. The dose of BDSF added per culture was equivalent to a 1/250 dilution, which corresponded to 2000 DPM. The cells were pelleted and the radioactivity associated with either the cells or the supernatants was determined on a beta counter. This experiment was repeated 3 times.

of reactivity suggest that BDSF preferentially binds to $E_R$- peripheral cells.

3.3.14.3 Ability of Macrophages to Transfer BDSF Activity

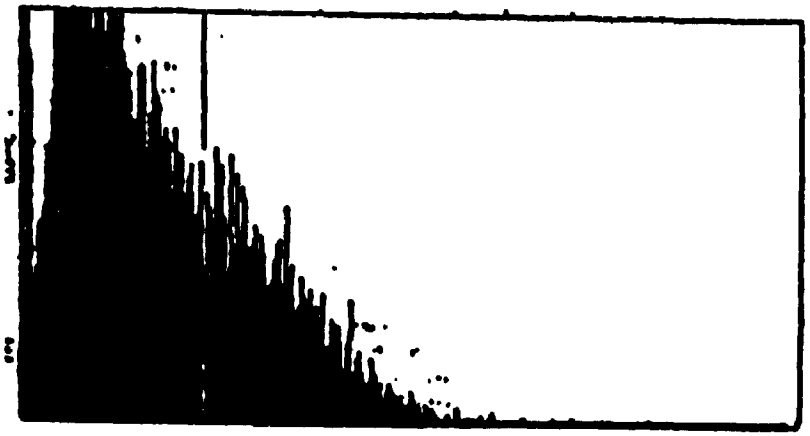
In view of the heterogeneous nature of $E_R$- cells, which included cells of both B lineage and myeloid lineage, experiments were designed to more specifically address the issue of which cell type may be the direct target of BDSF. Through the use of monoclonal Ab treatments, $E_R$- cells were depleted of either B cells or macrophages. Figures 3.15 and 3.16 demonstrate that Ab depletions yielded cell preparations that were highly enriched for either B cells or macrophages, as assessed by FACS analysis. The above populations of cells were used in a Con A proliferation assay, to test the target specificity of BDSF and the results are summarized in Table 3.7. First of all, the data indicate that macrophages are essential to generate an optimal Con A response. More importantly, the combination of cell mixtures required to observe a suppressed response is one including $E_R$- from which B cells had been depleted (or macrophage enriched) and that were exposed to BDSF prior to addition to culture. These cells could transfer suppressor activity when mixed with the complementary cells required for the generation of a Con A response. Incubation of BDSF with a population of cells enriched for B cells (and depleted of macrophages) was ineffective in transferring suppressor activity into the same test system. Noteworthy is that the degree of suppression achieved by either directly adding BDSF to the cultures or by incubating macrophages with BDSF and then adding them to cultures, is of the same magnitude.

FIGURE 3.15

FACS Analysis of B1 Depleted Tonsillar Cells

Tonsillar cells used in BDSF target studies were depleted of B cells by treating 2 X $10^7$ $E_R$- cells, in a 1 ml volume, with 20 $\mu$l of B1 monoclonal for 1 hr at $4^oC$ and then adding rabbit-low-tox complement (1:11 final dilution) for 1 hr at $37^oC$. The efficiency of the depletion was assessed by performing a FACS analysis for B1 staining untreated or Ab + C' treated cells. For cell sorter analysis, 1.5 X $10^6$ cells were incubated with 5 $\mu$l of either FITC conjugated B1 monoclonal or FITC conjugated mouse IgG Ab, for 30 minutes at $4^oC$. The cells were washed and loaded onto an EPICS V cell sorter for fluorescence analysis. The presented profiles are as follows: 1, $E_R$- cells stained the mouse IgG (29%); 2, $E_R$- cells stained with B1 (86%) ; and 3, $E_R$- cells that had been B1 depleted, stained with the B1 monoclonal (28%). The profiles represent the relative cell number (Y axis) vs the relative fluorescence intensity (X axis).

1



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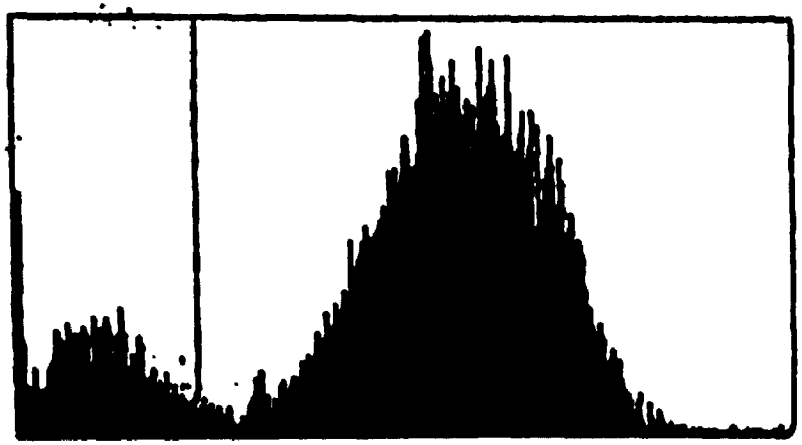

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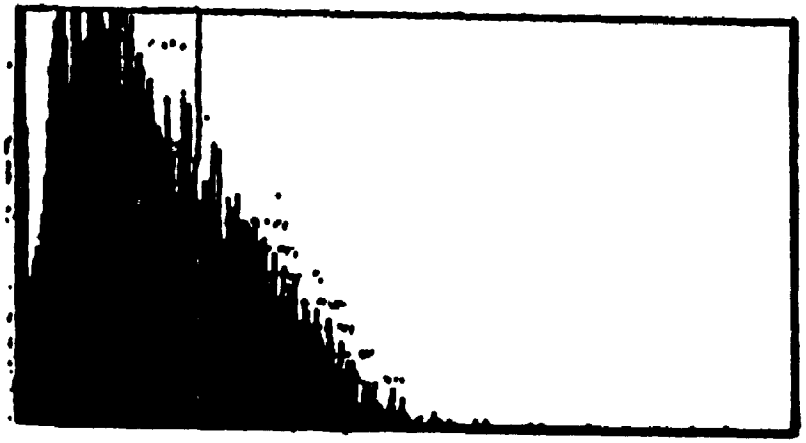
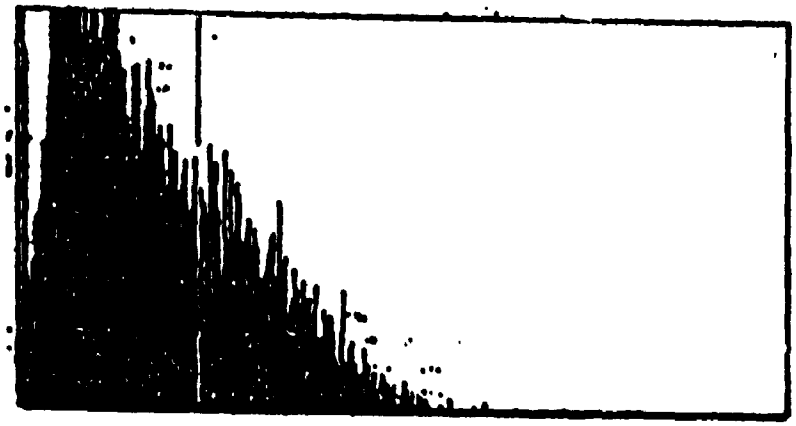


FIGURE 3.16

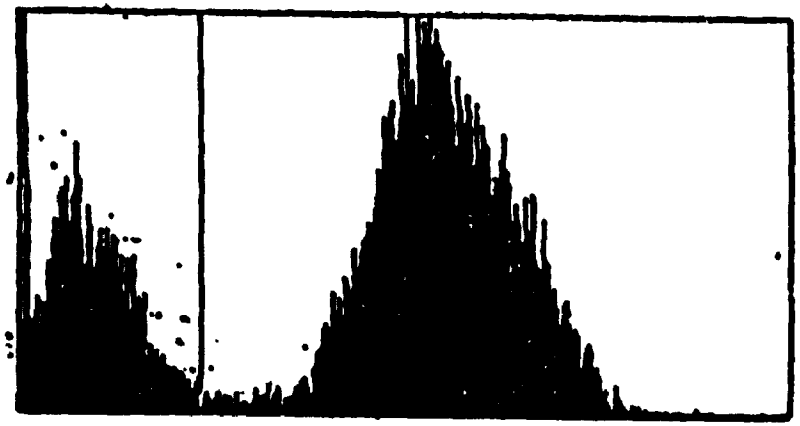
FACS Analysis of OKM1 Depleted Tonsillar Cells

Tonsillar cells used in BDSF target studies were depleted of macrophages by first treating 2 X $10^7$ $E_R$- cells, in a 1 ml volume, with 10 $\mu$l of OKM1 monoclonal for 1 hr at $4^oC$ and then adding rabbit-low-tox complement (1:11 final dilution) for 1 hr at $37^oC$. The efficiency of the depletion was assessed by performing a FACS analysis for OKM1 staining on either untreated or Ab + C′ treated cells. For cell sorter analysis, 1.5 X $10^6$ cells were incubated with 5 $\mu$l of either FITC conjugated OKM1 monoclonal or FITC conjugated mouse IgG Ab, for 30 minutes at $4^oC$. The cells were washed and loaded onto an EPICS V cell sorter for fluorescence analysis. The presented profiles are as follows: 1, $E_R$- cells stained the mouse $^r$gG monoclonal (29%); 2, $E_R$- cells stained with OKM1 (73%); and 3, $E_R$- cells that had been OKM1 depleted, stained with the OKM1 monoclonal (30%). The profiles represent the relative cell number (Y axis) vs the relative fluorescence intensity (X axis).

1



2



3

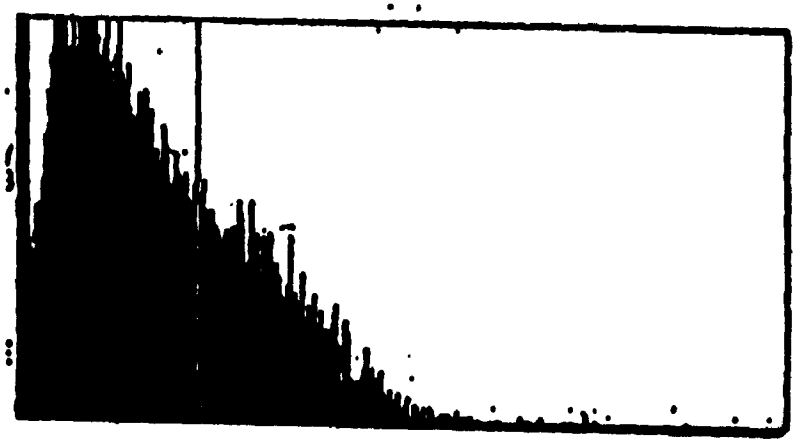


TABLE 3.7

Ability of Macrophages To Transfer

BDSF Suppressor Activity

| Culture Conditions | CPM/Culture $\bar{X} \pm$ S.D. | Percent Suppression |
|---|---|---|
| $E^+ + E^-$ | 5,587 ± 334 | ---- |
| $E^+ + E^- +$ Con A (5 μg/ml) | 85,388 ± 7,488 | ---- |
| $E^+ + E^-$(B depl) + Con A | 96,563 ± 7,290 | - 13 |
| $E^+ + E^-$(MØ depl) + Con A | 58,898 ± 5,337 | + 31[a] |
| $E^+ + E^-$(B depl) + $E^-$(MØ depl) + Con A | 90,177 ± 11,634 | + 7[b] |
| $E^+ + E^-$(B depl) + $E^-$(MØ depl) + Con A + BDSF | 30,162 ± 3,387 | + 67[c] |
| $E^+ + E^-(\text{B depl})_{BDSF} + E^-$(MØ depl) + Con A | 45,335 ± 1,715 | + 50[c] |
| $E^+ + E^-$(B depl) + $E^-(\text{MØ depl})_{BDSF}$ + Con A | 94,950 ± 4,777 | - 5[d] |

Tonsillar cells were fractionated by E-rosetting and then used in a Con A proliferative assay. The number of cells plated/well were as follows: E+ and E-, 2.5 X $10^5$; E-(B depl), 1 X $10^5$; and E-(MØ depl), 1.5 X $10^5$. BDSF was used in culture or in incubations with cells at a final dilution of 1:750. Experiments were repeated three times.

[a] Statistical difference from E-rosetted cultures with Con A, $p < 0.001$.

[b] Statistical difference from E-rosetted cultures with Con A, $p > 0.2$.

[c] Statistical difference from E+ cultures that also received E-(B depl) + E-(MØ depl) cells + Con A, $p < 0.0005$.

[d] Statistical difference from E+ cultures that also received E-(B depl) + E-(MØ depl) cells + Con A, $p > 0.2$.

### 3.3.15 BDSF Suppresses Interleukin-1 Synthesis

The results in section 3.3.14 suggested that the suppressive effects of BDSF may have been mediated through its action on a macrophage population. Experiments were designed to test whether macrophage related functions were being affected by BDSF. One of the key mediators to begin the cascade of events that eventually leads to an Ab response and T cell activation is interleukin-1 (IL-1). This cytokine is a direct product of stimulated macrophages. To test whether BDSF was interfering with IL-1 synthesis, the human monocytic cell line U-937 was used as a test system. This cell line can be induced to release IL-1 by incubating it with the phorbol ester, phorbol-12-myristate-13-acetate (PMA). The supernatants from cultures of U-937 cells with PMA in the presence or absence of BDSF were tested for IL-1 activity by adding them to the LAF co-stimulator assay routinely used for IL-1 detection. Results presented in Table 3.8 indicate that a 1/500 dilution of BDSF suppressed the synthesis of IL-1 to 71% relative to control levels. This is a similar dose of BDSF that repeatedly resulted in suppressed PFC and Con A responses.

### 3.3.16 Reconstitution of a BDSF Suppressed Ab Response by IL-1

If one of the mechanisms by which BDSF mediates its suppressive action is to reduce IL-1 levels, then it was reasoned that it should be possible to reconstitute a BDSF suppressed response by the addition of exogenous IL-1 to cultures. These suggested experiments were carried out using recombinant human IL-1 beta as a source of exogenous lymphokine. In Figure 3.17 results are presented that clearly demonstrate the ability of exogenous IL-1 to restore a BDSF suppressed Ab response to control values. The addition of an equal dose of rIL-1 to cultures, in the

absence of BDSF, results in a slightly enhanced Ab response, as would be expected.

TABLE 3.8

BDSF Suppresses the PMA Induced IL-1 Synthesis

| Culture Conditions to Generate IL-1 | + PMA (10 ng/ml) | IL-1 Activity (CPM/Culture ± SD) | Percent Suppression |
|---|---|---|---|
| U-937 supernatant | - | 32,153 ± 4532 | --- |
| U-937 + BDSF(1/500) | - | 35,211 ± 3336 | - 9[a] |
| U-937 + BDSF(1/5000) | - | 28,779 ± 4041 | 10[a] |
| U-937 | + | 65,720 ± 3001[b] | --- |
| U-937 + BDSF(1/500) | + | 41,797 ± 2797 | 71[c] |
| U-937 + BDSF(1/5000) | + | 65,007 ± 5496 | 2[d] |

U-937 cells were cultured at a density of 1 X $10^5$/well in 96-well plates for 24 hrs in the presence or absence of PMA. The ability of the cell-free culture supernatants to stimulate thymocyte proliferation in the presence of suboptimal doses (2.5 μg/ml) of PHA was used as an indicator of IL-1 levels in the U-937 cultures. The control responses in the IL-1 assays were as follows: PHA, 1807 ± 20; rIL-1 (50 U/ml), 5312 ± 875; PHA + rIL-1 (50 U/ml), 36,542 ± 4459. Experiments were repeated four times.

[a] Statistical difference compared to the U-937 constitutive IL-1 synthesis, $p > 0.1$.

[b] Statistical difference compared to the constitutive IL-1 synthesis, $p < 0.0005$.

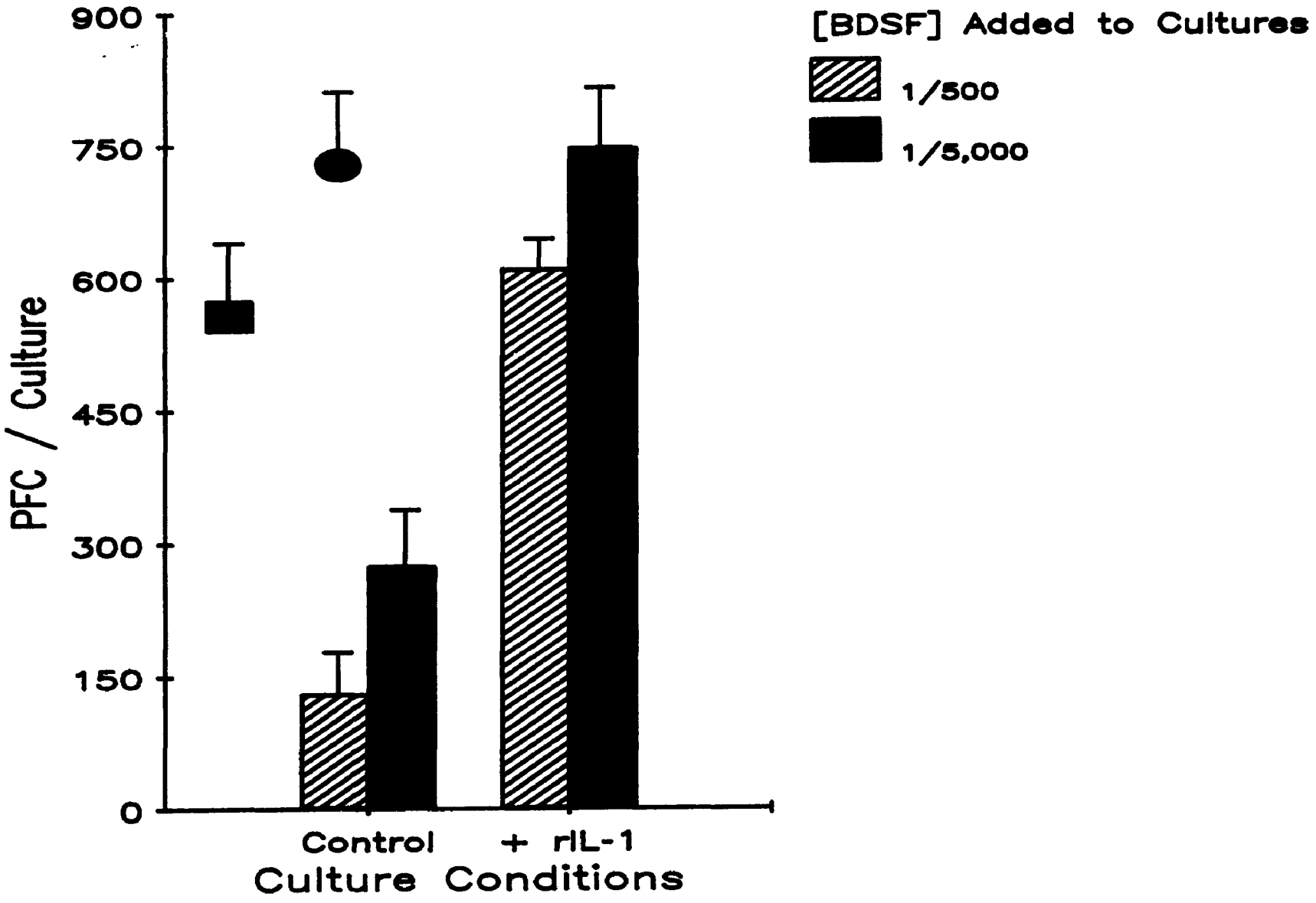
[c] Statistical difference compared to the PMA induced IL-1 synthesis, $p < 0.0005$.

[d] Statistical difference compared to the PMA induced IL-1 synthesis, $p > 0.3$.

FIGURE 3.17

Reconstitution of a BDSF Suppressed Response by IL-1

PFC responses generated with tonsillar cells in the presence of Ag (■) were suppressed by the addition of the two indicated doses of BDSF. The latter cultures were then supplemented with a predetermined dose of human recombinant IL-1-beta (20 U/ml) at culture initiation. The anti-SRBC specific Ab responses were monitored on day 7. The PFC response of cultures receiving only IL-1, in the absence BDSF, is indicated (●).


900
750
600
450
300
150
0
PFC / Culture
Control
+ rIL-1
Culture Conditions
[BDSF] Added to Cultures
1/500
1/5,000

## 3.4 Discussion

Although a consensus exists in the literature regarding the ability of BM-derived NS cells to suppress various immune responses, the mechanism by which this occurs remains uncertain. Data presented in this chapter suggest that NS cells release a highly suppressive molecule in BM culture supernatants that has a similar spectrum of action and kinetics as NS cells. The time restricted action of Bone marrow Derived Suppressor Factor (BDSF), to within 24 hours of culture initiation, is in full agreement with previous observations from this laboratory demonstrating similar kinetic behaviour of BM suppressor cells (Bains et al., 1982; Duwe and Singhal, 1979a). Noteworthy are the findings that culturing of elutriated BM fractions, enriched for Ab suppressive activity, allows the recovery of biological activity directly from such culture supernatants. The absence of this activity in cultures of other elutriated BM fractions, in conjunction with the observed increased potency of suppressor activity in supernatants of NS enriched cultures, collectively support the notion that NS cells appear to mediate their suppressive effects through the release of BDSF. A more formal proof of this hypothesis was arrived at through experiments demonstrating that NS-depleted BM was clearly unable to synthesize BDSF activity. Furthermore, biochemical analysis of these supernatants showed the absence of the highly suppressive lipid band, associated with BDSF activity, on TLC plates. These latter findings are of relevance when one considers that only the suppressive lipids (BDSF) incorporated a radioactive lipid precursor molecule ($^{3}$H-acetate). This implies that BDSF is newly generated in culture and likely belongs to a group of lipids that are rapidly being renewed. Furthermore this argues that BDSF is not the byproduct of aging BM cells that would release their

internal stores of lipids into the supernatant during the *in vitro* culture period.

In spite of the lipid nature of BDSF, the factor seems capable of acting in aqueous liquid cultures. Although the biochemical structure of BDSF is presently not known, all experimental evidence suggests that the mediator is a relatively polar lipid molecule. The phosphate groups detected on BDSF invariably contribute to the polar nature of the factor and to its solubility in polar solvents. However, one cannot dismiss the possibility that BDSF may make use of a protein carrier molecule, while in liquid culture, to facilitate its diffusion. The carrier determinant could be easily provided *in vitro* by the abundant proteins associated with the required serum supplement in culture media. This lipoprotein complex would have increased solubility in aqueous media. Such a mechanism has been reported for a lipid that inhibits lymphocyte proliferation and makes use of a protein carrier to eventuate its effects (Cornelius and Normann, 1988).

Although BDSF was routinely recovered from supernatants of BM cultures, it did not preclude the factor from also expressing its inhibitory activity while still on the surface of the BM cells synthesizing it. The ability of the factor to act in this fashion would also minimize any difficulties in solubility encountered by BDSF. Of interest and relevance to this study is the report by Stallcup *et al.* (1986) who demonstrated a lipid-like mediator derived from macrophages that could inhibit various immune functions. Noteworthy was the finding that this molecule was biologically active while still on the surface of the cells producing it, thereby invoking a cell-cell contact mechanism for suppression. If BDSF could operate in a similar fashion, this would endow

BDSF with a mechanism to restrict its potent suppressor activity to only nearby cells without the induction of undesirable systemic effects.

Perhaps it is not surprising that the macrophage was found to be the target of BDSF action, since it is one of the few cells commonly required for both Ab responses and Con A proliferation. In addition, the kinetics of BDSF action, restricting it to the first 24 hours of culture, predicted that the target of BDSF would have to be a cell required early in immune responses. These findings are fully consistent with reports demonstrating that IL-1, a key product of macrophages, is an essential lymphokine for the development of human Ig producing cells (Lipsky et al., 1983; Gerrard and Fauci, 1982) and in the generation of Con A responses (Palacios, 1982). Furthermore, the requirement for IL-1 seems to be restricted to the first 24 hours of culture (Lipsky et al., 1983). Finally, the documented evidence that macrophages are regulators of body lipid metabolism and that they continuously interact with lipids within their environment (Yui and Yamazaki, 1986) predisposes these cells to interact with lipid factors. This point is reinforced by the observations of Abbott and Myers (1986) who described a naturally occurring macrophage suppressor factor (MSF) in spleens of mice that was also lipid in nature. Of relevance to this study is that the latter lipid factor could inhibit macrophage functions. Whether BDSF interacts with a specific receptor on the surface of macrophages at this stage is not clear, however the selective binding of BDSF to these cells would argue for a recognition structure present on this lineage of cells.

The binding of BDSF to macrophages is likely to alter cellular functions. The lipid nature of BDSF would facilitate the interaction of the factor with membrane lipids, with the likely result of disrupting

normal membrane signalling events or interference with membrane associated enzymatic activities. The ability of lipids to alter normal macrophage and lymphocyte functions, like cell adhesion, lymphocyte migration, the ability to bind lymphokines as well as cellular proliferation, is well documented (Novo et al., 1987; Yohe and Ryan, 1986; Lingwood and Hakomori, 1977). In addition, protein kinase C activity, which is a key activation pathway for both B and T cells, has been shown to be regulated by membrane phospholipids (Rando, 1988).

Of interest is the fact that BDSF displays no HLA or Ag specificity in its action, but requires the presence of Ag to trigger the system and direct its inhibitory action. The selectivity of BDSF action may reside in its ability to deliver a strong suppressive signal to a system that has been recently activated, while not affecting resting cells.

Regarding the unique nature of BDSF, it is crucial to point out that although numerous suppressive molecules have been described over the years, very few factors have been shown to be BM-derived or that are constitutively produced. Of the human suppressor factors described, (Fleisher et al., 1981; Wilkins et al., 1983; Wolf et al., 1978; Schumacher et al., 1974; Shou et al., 1980), all can be distinguished from BDSF by various criteria, including molecular weight, source of cells producing it, requirement of cellular activation for factor synthesis, biochemical properties and kinetics of action. Comparison under all of these parameters renders BDSF a novel immunosuppressive factor.

The ability to recover BDSF activity from BM cells cultured in the presence of indomethacin, as well as the lack of reactivity of the suppressive lipid with the cupric acetate reagent, both argue against a role of prostaglandins in BDSF mediated suppression. This is in agreement

with reports from Choi et al. (1988) demonstrating the inability to fully abrogate murine NS activity through the use of the prostaglandin synthetase inhibitor indomethacin. Inasmuch as leukotrienes stimulate IL-1 synthesis (Rola-Pleszczynski and Lemaire, 1985) while BDSF suppresses the latter lymphokine, it is inconceivable that leukotrienes may play a significant role in BDSF mediated suppression. The ability of prostaglandins and leukotrienes to enhance and suppress numerous immune functions has been well established (reviewed by Meade and Mertin, 1978; and Rola-Pleszczynski, 1985), however the presented experimental evidence would suggest that they likely do not play a major role in BM-derived suppressor activity. The regulatory effects of BDSF on IL-1 do not dispute the accepted findings that prostaglandins of the E2 series can inhibit IL-1 synthesis (Kunkel et al., 1986) but merely provide an alternate mechanism for the regulation of IL-1 within the BM compartment. B cells initiate their maturation process under the influence of various cytokines produced by the BM stromal environment, which includes fibroblasts, macrophages and adipocytes (reviewed by Kincade et al., 1989). Therefore any factor capable of regulating stromal cell activity or their products, like IL-1, has the potential to affect B cell genesis and other hematopoietic functions.

One of the questions that arises is whether the BM has the necessary machinery for the generation of a primary Ab response, in the event that it encounters Ag. BM being the source of all circulating B cells contains cells at different stages of maturation and some are likely to be Ag responsive. Mature T cells, although limited in numbers, seem to traffic through the BM from peripheral organs. Lastly, there would be a requirement for Ag presenting cells. A variety of functional macrophages

exist within the BM. BM macrophages have been shown to be capable of efficient accessory cell function for T cell proliferative responses following stimulation with GM-CSF (Fischer et al., 1988). Human stromal macrophages or marrow histiocytes are also specialized mature macrophages with the ability to affect erythroid maturation (Lee et al., 1988). Additionally, IL-1 has been shown to be synthesized by BM stromal cells (Sieff et al., 1988; Fibbe et al., 1988) and the activation of macrophages can be regulated by stromal cells-derived lymphokines, like interferon-gamma, tumour necrosis factor and GM-CSF (Haq and Maca, 1986; Sisson and Dinarello, 1988). This entire body of evidence strongly argues that accessory cell functions can develop within the BM compartment. In addition, if one considers the direct effect of IL-1 on B cells, such as the ability to induce kappa chain expression and maturation in pre-B cells (Giri et al., 1984), the scenario seems amenable to the development of a primary Ab response. If the above assumption is correct that BM is capable of responding to Ag challenge, how do we reconcile the fact that this organ exists in an apparent state of anergy for IgM synthesis. The findings of this study would propose that the regulation of BM IL-1 levels may be an important mechanism in controlling the expansion of Ag triggered B cells and in maintaining BM IgM unresponsiveness.

This study does not exclude the possibility that BDSF may also regulate other lymphokines, other than IL-1, especially those of BM stromal cell origin, like the CSFs, IL-6, IL-7 and transforming growth factor-beta (TGF-beta) (Kincade et al., 1989); however it does provide the foundation for further investigations on the complex regulatory events that continuously occur within the BM to maintain normal hematopoiesis.

How BDSF may physically accomplish the apparent reduction in IL-1 activity found in culture supernatants is not totally clear; however speculation would favour an effect at the level of mRNA synthesis, since evidence indicates that IL-1 production is regulated at the transcriptional level (Oppenheim et al., 1986). An alternative mode of action is that BDSF may interfere with the external signalling events required to initiate IL-1 synthesis from macrophages. A number of lymphokines have been shown to stimulate IL-1 synthesis, such as M-CSF, IFN-gamma and TGF-beta (Oppenheim et al., 1986; Larrick, 1989). Disruption of any of these signals could potentially result in reduced IL-1 levels. For example, inhibitors of either protein kinase C or calcium/calmodulin-dependent kinase are capable of blocking IL-1 synthesis (Kovacs et al., 1987).

Most of the inhibitors of IL-1 recently reviewed by Larrick (1989) are poorly defined, none are of BM origin, and the majority have been demonstrated to exclusively inhibit the T cell proliferative effects of IL-1. The present study suggests that BDSF is not interfering with the ability of thymocytes to respond to IL-1, since BDSF does not prevent the reactivity of thymocytes to the IL-1 constitutively produced by the U-937 cell line. This latter point distinguishes BDSF from the numerous proteins that have IL-1 inhibitory activity. A final point to consider is that BDSF may potentially inactivate IL-1 by binding to it directly in the supernatants. Although this explanation is merely speculative and unlikely, further investigations are required in order to fully dismiss it.

In summary, one of the mechanisms by which BDSF suppresses Ab responses and Con A induced proliferation seems to be by limiting IL-1 levels. In the periphery IL-1 makes use of a self-induced inhibitor, in the form of prostaglandin E2, for regulation. Whether the same regulatory network operates within the BM is not clear, however it is conceivable that IL-1 levels within the BM compartment also require a control mechanism. Since IL-1 is a key cytokine in the progression of events that allow expansion of immune cells, it is plausible that the BDSF mediated regulation of IL-1 may represent one of the necessary control mechanisms required to maintain BM homeostasis.

# Chapter 4

# THE IMMUNOENHANCING ACTIVITY OF HUMAN BONE MARROW

### 4.1.1 Introduction

Although the majority of reports on BM regulatory functions have emphasized the presence of suppressor elements within the BM (reviewed in chapter 1), a limited number of studies have alluded to an immunoenhancing activity (Saffran et al., 1986; Duwe and Singhal, 1978; Bains et al., 1986). This raises the question whether a second antagonistic regulatory activity is present within the BM compartment. Investigations on the naturally occurring BM suppressor cells, led to the isolation of a factor from this tissue that could augment Ab synthesis of peripheral lymphocytes and DNA proliferation of BM cells and thymocytes. This factor appears to be the product of a subset of resident BM cells and shows characteristics dissimilar to many of the presently well defined cytokines. A number of lymphokines have been described to affect B-cell development and hematopoiesis (Arai et al., 1986; Gordon and Guy, 1987). Many of these mediators are synthesized by mitogen or Ag activated cells in peripheral lymphoid organs.

The focus of this chapter is to better characterize the immunoenhancing activity of human BM cells and the factor, Bone marrow Derived Enhancing Factor (BDEF), derived from the same cells. To assess the unique nature of BDEF in relation to a number of described stimulatory factors, a brief overview of the better characterized lymphokines will be presented. The rapidly advancing field of lymphokines has been extensively addressed in the literature and the reader is directed to the indicated reviews in the following text for a more comprehensive discussion of each cytokine.

### 4.1.2 Interleukin 1 (IL-1)

The primary source of IL-1 is the macrophage, however cells of epidermal, epithelial, lymphoid and vascular origin can synthesize IL-1 (Dinarello, 1988). IL-1 is probably the most pleiotropic of all the lymphokines, since it has the ability to affect not only immunological functions, but also haematological, neurological, metabolic and endocrinological activities. The numerous biological effects of IL-1 are summarized in reviews by Dinarello (1988) and Oppenheim et al. (1986). IL-1 is a proteinaceous molecule of MW 17,500 daltons (Da) with the ability to enhance the proliferation of T and B cells responding to their respective growth factors. Other activities also associated with IL-1 include: augments PFC responses; acts as an endogenous pyrogen; increases the binding of NK cells to their tumour targets; and stimulates hepatocytes to produce acute phase proteins (Oppenheim and Gery, 1982).

Two biochemically distinct forms of IL-1 have been described, an IL-1 beta (Auron et al., 1984) and an IL-1 alpha (Lomedico et al., 1984). The molecules are encoded for by separate genes and they are both biologically active, having the same spectrum of activities. The regulation of IL-1 synthesis is thought to be at the transcriptional level, since the amount of mRNA for each of the two species of IL-1 is directly proportional to the amount of IL-1 alpha and beta produced by activated monocytes (Oppenheim et al., 1986).

The membrane associated form of IL-1 initially reported by Kurt-Jones et al., (1985) is also biologically active. IL-1 has been shown to enhance the transcription of genes encoding IL-2, IL-3, IL-2 receptor, Ly-1, IL-6 and c-myc in a murine T-cell lymphoma (Mizel, 1987). The concentrations of IL-1 required for biological activity ranges between 1

nM and 1 pM (Dinarello, 1988). Products of the lipoxygenase pathway, like the leukotrienes, stimulate IL-1 synthesis, while derivatives of the cyclooxygenase pathway, like prostaglandins E2, are induced by IL-1 (Dinarello, 1988). IL-1 is thought to be a central component within the interleukin network mainly because of its wide spectrum of activation and its potent inducing action on other interleukins (Wong and Clark, 1988).

### 4.1.3 Interleukin 2 (IL-2)

IL-2 is a T-cell derived lymphokine that promotes the proliferation of activated T cells, enhances thymocyte mitogenesis, provides T-cell help for the generation of PFC responses in nude mice and induces CTL activity from thymocytes and nude spleens (Watson et al., 1979: Gillis et al., 1979; Gillis et al., 1980). The MW of human and mouse IL-2 are 15,000 and 20,000 to 35,000 Da respectively (Gillis, 1983). Its action is not solely restricted to T cells, since B cells express IL-2 receptors and proliferate in its presence (Zubler et al., 1984). NK cells are also responsive to the action of IL-2 by exhibiting elevated cytolytic action (Henney et al., 1981).

### 4.1.4 Interleukin 3 (IL-3) and Colony Stimulating Factors (CSFs)

CSFs can be divided into four types: IL-3 or multi-CSF, granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF) and granulocyte-macrophage-CSF (GM-CSF). They are all known to control the proliferation of granulocytes and macrophages (Arai et al., 1986). The molecular weights of the four CSFs are as follows: IL-3 has a MW range of 19,000 to 28,000 Da, GM-CSF has a MW of 23,000 Da, G-CSF is 25,000 Da, and M-CSF ranges from 45,000 to 70,000 Da plus it is the only CSF composed of 2 subunits (Nicola, 1987).

Only two of the four CSFs have a relatively lineage specific action. G-CSF and M-CSF give rise preferentially to granulocytic and macrophage type colonies respectively. In contrast, IL-3 and GM-CSF can support the growth of colonies containing various cell types, including neutrophils, granulocytes, macrophages and eosinophils (Clark and Kamen, 1987). T cells can produce IL-3 and GM-CSF.

Ihle et al. (1983) initially demonstrated the multiple lineage effect of IL-3 by successfully growing different hematopoietic colonies from mouse BM. The action of IL-3 was later defined as the ability to support both the growth and differentiation of BM progenitor cells that are committed to monocytic, granulocytic, erythroid, and megakaryocytic cell lineages (Rennick et al., 1985). The distinguishing feature between mouse and human IL-3 is that the latter will not support the growth or differentiation of mast cells (Saito et al., 1988). No evidence to date indicates that BM cells are capable of synthesizing IL-3 (Kodama et al., 1986).

### 4.1.5 Interleukins 4 and 5 (IL-4 and IL-5)

The biochemical properties and biological activities of mouse and human IL-4 and IL-5 have been reviewed in detail by Yokota et al. (1988). In general, $CD4^+$ T cells activated either by Con A, PHA, or PMA plus a calcium ionophore can secrete IL-4 as well as IL-2 and IFN-gamma. Human IL-4 has a MW of 15,000 daltons (Yokota et al., 1988). Of interest are the findings that IL-4 can induce class II MHC antigens on murine thymus macrophages (Ransom et al., 1987) and BM macrophages (Zlotnik et al., 1986). Both of these effects result in an enhanced Ag presenting ability of the macrophages. IL-4 is also capable of costimulating macrophage and

mast cell colony growth of bone marrow precursors that have been prestimulated with IL-3 (Rennick et al., 1987). Pre-incubation of resting B cells with IL-4 enhances their response to anti-IgM treatment (Yokota et al., 1988). IL-4 has also been shown to be involved in the regulation of Ig isotype expression since it induces IgE and IgG1 production but suppresses IgG3, IgG2a, IgG2b and IgM synthesis (Snapper and Paul, 1987). In addition, IL-4 promotes the differentiation and proliferation of OKT3+ cells from BM (Saito et al., 1988).

Both human and mouse IL-5 have a MW of 14,000 daltons with some minor variations due to glycosylation differences (Yokota et al., 1988). IL-5 preferentially enhances IgA synthesis but not IgG or IgM (Yokota et al., 1987). IL-5 was originally regarded as a late acting factor for B cell differentiation. Its key biological properties are: to replace T cells in the generation of an Ab response, to induce the maturation of resting B cells into IgM-secreting cells and to induce expression of IL-2 receptors on B cells (Takatsu et al., 1988). Of particular interest is the ability of IL-5 to directly act on BM cells to promote the growth of eosinophilic colonies (Yamaguchi et al., 1988).

### 4.1.6 Interleukin 6 (IL-6)

IL-6, also known as B-cell stimulatory factor-2 and IFN-beta, has a multiplicity of biological activities that are not species restricted (Wong and Clark, 1988). IL-6 can be expressed by fibroblasts, endothelial cells and mononuclear phagocytes and has a MW of 34,000 Da in humans (Wong and Clark, 1988). IL-6 was found to stimulate IL-2 synthesis from T cells (Garman; et al., 1987) as well as to act as a co-differentiating factor for the development of cytolytic T lymphocytes (CTL) from immature

thymocytes (Takai et al., 1988). IL-6 has been demonstrated to induce B cell differentiation and IgG secretion (Hirano et al., 1986), to act as a growth factor for hybridomas (Van Damme et al., 1987) and EBV transformed B cells (Tosato et al., 1988), and to have antiviral activity (Zilberstein et al., 1986). IL-6 also has effects on hematopoietic stem cells. Ikebuchi et al., (1987) discovered that IL-6 increases the rate by which IL-3 dependent BM blast cells form colonies. The pleiotropic effects of IL-6 make it a key member of the lymphokine network with potent hematopoietic regulatory activities.

### 4.1.7 Interleukin 7 (IL-7)

IL-7 supports the proliferation of pre-B cells but does not affect the proliferation of mature lymphocytes. Murine IL-7 has a MW of 25,000 daltons (Namen et al., 1988b) and can induce the proliferation of both B220 negative and positive B cells derived from long term BM cultures (Namen et al., 1988a). The primary source of IL-7 is BM stromal cells, with no detectable biological activities in spleen cells or thymocytes; in spite of the observation that mRNA transcripts encoding the IL-7 gene have been detected in all of these tissues (Namen et al., 1988a). The fact that stromal cells form a three-dimensional framework in the BM intersinusoidal spaces (Dorshkind, 1987) strengthens the observations that BM stromal cells play an integral role in regulating BM hematopoiesis (Dexter et al., 1985).

### 4.1.8 Interferons (IFNs)

Interferons are broadly divided into three groups: IFN-alpha of leukocyte origin, IFN-beta from fibroblasts, and IFN-gamma or immune IFN,

which is produced by lymphocytes when specifically activated by antigens or mitogens (The Interferon Nomenclature Committee, 1983). Although only IFN-gamma has been conclusively shown to have immunoenhancing effects on mature B cells resulting in the augmentation of *in vivo* PFC responses (Nakamura *et al.*, 1984), all three species of IFNs have synergistic suppressive effects on hematopoietic stem cells. They have all been reported to suppress colony forming unit-granulocyte-macrophage (CFU-GM), burst forming unit-erythroid (BFU-E) and colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) colony growth from human BM precursors (Broxmeyer *et al.*, 1985). Interferon-gamma has a MW of 25,000 Da (Gordon and Guy, 1987) and it appears to be the only molecule that can stimulate the oxidative metabolism of macrophages, which is associated with activated cells (Hogg, 1986). This IFN also been shown to regulates Ig isotype expression; it stimulates IgG2a synthesis while concurrently inhibiting IgG3, IgG1, IgG2b and IgE synthesis (Snapper and Paul, 1987). IFN-gamma synthesis is inducible by IL-1 (Puri *et al.*, 1987; Wilson *et al.*, 1988). In general the role of IFN-gamma has been suggested to be the preparation of mature resting B cells to respond to the proliferative signals of IL-2 (Romagnani *et al.*, 1986).

### 4.1.9 Tumour Necrosis Factor-alpha (TNF-alpha)

Tumour necrosis factor-alpha (TNF-alpha) is the product of LPS activated macrophages and lymphocytes. Its designation as cachectin is a result of the wasting symptoms that are elicited following elevation of TNF-alpha levels. This is a direct consequence of the ability of TNF-alpha to suppress lipoprotein lipase activity which results in an elevation of plasma triglycerides and a reduced ability to use fat

deposits for energy (Rouzer and Cerami, 1980). TNF-alpha, primarily known for its toxic effects on malignant cells, has been reported to activate osteoclasts and elicit IL-1 synthesis from endothelial cells (Dinarello et al., 1986; Nawroth et al., 1988). In fact, TNF-alpha and IL-1 share various biological properties and their actions synergize when both factors are present in culture (Dinarello, 1988).

Human TNF-alpha has a MW of 45,000 Da and is made up of 3 separate subunits of 17,000 Da each. TNF-alpha has multiple effects on the immune system that initially seem contradictory. For example, it can inhibit the growth of myeloid cells (Murphy et al., 1988) while at the same time it is fully capable of stimulating the production of hematopoietic growth factors like M-CSF, G-CSF and GM-CSF (Zucali et al., 1988). In fact the protection of TNF-alpha treated mice against the lethal effects of radiation is likely due to its ability to enhance the growth of colony forming units-culture (CFU-C) and colony forming units-spleen (CFU-S) from recovering animals (Slordal et al., 1989). Of interest is the differential effects TNF-alpha on T and B cells. It has been shown to enhance the proliferative response of T cells to IL-2 (Scheurich et al., 1987), however it does suppress B cell proliferation and differentiation (Kashiwa et al., 1987).

Closely linked to the gene for TNF-alpha on human chromosome 6, is another gene that encodes for the biologically active polypeptide lymphotoxin (LT). Although the two molecules (TNF-alpha and LT) share similarities at the amino acid level and use the same cell surface receptors, LT is distinguished from TNF-alpha by the fact that it is strictly a product of lymphocytes (Cuturi et al., 1987). The numerous

biological effects of TNF-alpha have been recently reviewed by Beutler and Cerami (1989).

### 4.1.10 Aims of Chapter 4

This chapter will deal with the experimental evidence to support the idea that within the BM compartment a population of cells with lymphocytic morphology have an inherent immunoenhancing ability which results in augmentation of Ab responses as well as direct mitogenic activity for both BM cells and thymocytes. The data will also demonstrate that this enhancing activity is mediated by a factor, BDEF, released into the supernatants of BM cultures. By virtue of its biological and physicochemical characteristics BDEF appears to be unlike most other lymphokines. The thrust of this chapter will be the characterization of this BM derived immunoenhancing activity with particular emphasis on the purification of BDEF.

4.2.1 Preparation of Human Bone Marrow Derived Enhancing Factor (BDEF)

Bone marrow cells recovered from density gradient separations were cultured in Nunc tissue culture dishes (Gibco) at a concentration of 1 X $10^7$/ml for 24 hours in serum free RPMI. The supernatant from such cultures was collected and the cells replated for a further 24 hours in fresh RPMI-1640 medium. Pooled supernatants from these cultures were used as a source of Bone marrow Derived Enhancing Factor (BDEF).

4.2.2 Preparation of BDEF

BDEF was enriched by concentrating the cell free BM derived supernatants on an Amicon ultrafiltration unit using a YM10 filter with a nominal cutoff of 10,000 daltons (Da) (Amicon Corp., Oakville, Canada). The retentate, which represented 1/10th of the original supernatant volume, was dialyzed against 100 volumes of PBS to remove remaining low molecular weight molecules. This preparation contained molecules of molecular weight (MW) >10,000 Da.

4.2.3 Purification of BDEF by HPLC

For High Pressure Liquid Chromatography (HPLC) BDEF preparations were first lyophilized, resuspended in double distilled water, placed in a 1,000 Da MW cutoff dialysis membrane (Spectrapor, Los Angeles, CA) and dialyzed against PBS for 48 hours, at $4^oC$. The protein concentration of these preparations was assessed by the micro-Lowry technique as described by Lowry et al., (1951). Samples of 100 µl volume, containing 285 µg of protein, were run on a Waters M-45 HPLC (Waters Associates, Milford, MA)

using a Waters I-300 column at a flow rate of 1 ml/min. Degassed PBS was used as running buffer. Fractions were collected according to the protein profile of the eluted material. After several runs, similar fractions were pooled, lyophilized, resuspended in a minimal volume of water and then dialyzed against 1000 volumes of PBS for 48 hours at $4^{o}C$. Fractions were filtered and stored at $-20^{o}C$ until further use.

### 4.2.4 Enrichment of BDEF by DEAE Chromatography

Diethylaminoethyl (DEAE) cellulose (0.92 meq/g) (Sigma) was first swollen by resuspension in 0.5 N HCl. The settled cellulose was resuspended in double distilled water and allowed to settle. Fresh water was added until the pH reached 5.0. The settled cellulose was resuspended in 0.5 N NaOH and then washed with distilled water until the final pH was 7.5. The swollen cellulose was equilibrated with a low ionic strength eluting solution (3.5 mM NaCl, pH 7.8) and then poured into the barrel of a plastic syringe such that the packed volume was approximately 4.0 ml. The DEAE column was thoroughly rinsed with 3.5 mM NaCl prior to loading the BDEF sample. A 1 ml sample of BDEF, with a protein content of 19 $\mu$g, was allowed into the column. Using solutions of increasing ionic strength, bound proteins were eluted. The linear gradient of the eluting solutions ranged between 3.5 and 1000 mM NaCl. Fractions of 2.5 ml each were collected, lyophilized, dialyzed against 0.01 M PBS, filter sterilized and then used in biological test assays at equal protein concentrations. The entire procedure was carried out at room temperature.

### 4.2.5 Con-A Sepharose Chromatography

Fractionation of BDEF by Con-A sepharose chromatography was carried out by incubating 3.5 ml of Con-A sepharose 4B slurry (Sigma), which had a protein binding capacity of 50 mg, with 9 mg of human BM derived proteins. This was done in a 15 ml conical tube mounted on a rotating wheel to facilitate the mixing of the sepharose with the proteins. The incubation lasted 10 hrs at $4^{o}C$. Unbound proteins (Con A -ve) were recovered by collecting the supernatant after the sepharose was allowed to settle. This procedure was repeated several times until 40 ml of buffer (0.02 M Tris-HCl plus 0.5 M NaCl, pH 7.4) was collected. Proteins that were bound to the Con A sepharose (Con A +ve) were eluted by incubating the matrix with 0.5 M alpha-methyl-mannoside, overnight at $4^{o}C$. The following day, the supernatant was collected and concentrated by lyophilization and then dialysis against 2 liters of 0.01 M PBS. Of the recovered proteins, 75% were in the non-Con A binding fraction and 25% were in the Con A binding fraction. A sham elution was carried out using PBS, to serve as a control for any effects due to Con A that may have leached off the column.

### 4.2.6 Purification of BDEF by Affinity Chromatography

Three grams CNBr-activated Sepharose 4B (Pharmacia) was reacted with 50 mg of DEAE purified IgG fraction of rabbit antibodies (Ab). The coupling reaction was allowed to occur overnight at $4^{o}C$ and the efficiency was calculated to be 97%. The slurry was poured into a glass column and washed 3 times with alternating cycles of 0.1 M $CH_3COONa$-$CH_3COOH$ buffer, pH 4.0 and 0.1 M Tris buffer, pH 8.0 both containing 0.5 M NaCl. The column was equilibrated with running buffer, 0.01 M PBS + 0.2% $NaN_3$, and

then loaded with 830 µg of proteins containing BDEF activity. Unbound proteins were washed off the column using 20 volumes of running buffer and then with 25 ml of 0.5 M NaCl solution. Bound proteins were eluted with 100 ml of 0.1 M glycine-HCl buffer, pH 2.5. Eluted protein fractions were immediately equilibrated with 1 M Tris-base such that their final pH was 7.5. Eluted proteins were concentrated by Amicon ultrafiltration on a YM-2 1,000 MW cutoff membrane, filter sterilized and stored at -20°C.

4.2.7 Preparation of Antibodies Against BM Proteins:

Supernatants from BM cultures were lyophilized, and 500 µg of proteins were loaded onto preparative PAGE gels. Bands of interest, which had been visualized by lightly staining with Coomassie blue, were cut from the gel and electroeluted according to the method of Hunkapiller et al., (1983). The concentrated proteins were mixed 1:1 with complete Freund's adjuvant (CFA) (Gibco) and then injected subcutaneously into individual rabbits. The animals were boosted 2 times at 2 week intervals and then bled one week after the final injection. The serum obtained was enriched for IgG Ab by ammonium sulphate precipitation and DEAE chromatography.

4.2.8 Processing of Antisera

Blood collected from rabbits was allowed to clot by incubating it first at 37°C for 20 min and then at 4°C overnight. The next day, the samples were centrifuged at 2,500 rpm for 20 min and the blood clot discarded. From the remaining sera Igs were precipitated by first taking a 50% ammonium sulphate fraction and then a 40% ammonium sulphate precipitable fraction. Recovered Igs were dialyzed extensively against PBS before being loaded onto a DEAE column for IgG enrichment.

### 4.2.9 DEAE Chromatography

DEAE cellulose (Sigma, St. Louis, MO) was swollen as described above. The cellulose was resuspended in 0.02 M PBS containing 0.02% $NaN_3$ (pH 7.5), and then poured into a glass tube to form a bed volume of 6 ml. The column had a calculated retention capacity of 900 mg of total protein. Once the column had equilibrated with the PBS, 500 mg of rabbit Ab was slowly loaded onto the column while at RT. The column was run at 0.35 ml/min and 30 fractions, of 2 ml each, were collected using PBS as the running buffer. IgG was recovered in the void volume fractions while IgM, which had bound to the column, had to be eluted with 0.5 M NaCl. Each fraction was scanned at 280 nm on a Beckman DU-8B spectrophotometer (Beckman) and fractions that fell within the first absorbance peak were pooled and used as a source of IgG enriched Ab.

### 4.2.10 ELISA

Proteins of interest were resuspended in PBS and plated into 96-well flat bottom Nunc plates (Gibco) and let stand for 24 hrs at $4^{o}C$. The plates were then washed 3 times with washing buffer that consisted of 0.01 M PBS supplemented with 0.2% gelatin (Fisher, Don Mills, Canada), 0.02% $NaN_3$, and 0.05% Tween-20 (Sigma). The plates then received 200 $\mu$l of blocking buffer (0.01 M PBS + 1% gelatin) and incubated for 2 hrs at RT. After the incubation the plates were washed once and then 100 $\mu$l of appropriate Ab diluted in washing buffer was added to each well. This reaction was allowed to go overnight at $4^{o}C$ and the following day the plates were washed 3 times prior to adding 100 $\mu$l of a 1:2000 diluted goat anti-rabbit IgG alkaline phosphatase conjugated Ab (Bio-Can, Mississauga, Canada). This was incubated for 3 hours at RT before washing the plates

3 times. Finally, each well received 100 µl of a 1 mg/ml solution of p-nitrophenyl phosphate disodium (Sigma) in substrate buffer that contained: 4.9 ml diethanolamine (Sigma), 0.5 ml of 2% $NaN_3$, 5 mg $MgCl_2 \cdot 6H_2O$ and 46 ml $H_2O$ (pH 9.8). The substrate was left in the plates for 0.5 hr at RT before stopping the reaction by the addition of 50 µl 3.0 M NaOH. The plates were scanned at 405 nm using a Multiskan microplate reader (Flow Laboratories, Mississauga, Canada) to quantitate the colour reaction.

4.2.11 Western Blotting

Unfractionated BM derived proteins were run on a PAGE gel according to the method of O'Farrell, (1975). Each lane was loaded with 40 µg of total protein and once electrophoresis was completed the proteins were transferred to nitrocellulose (Schleicher & Schuell Inc., Keene, N.H.) using the Bio-Rad Trans-Blot cells (Bio-Rad, Mississauga, Canada). The transfer was carried out overnight at $4^{o}C$ with a current of 0.2 mA and 45 volts in 3 litres of buffer containing 9.06 g Tris-base, 42.23 g glycine, 3.0 g sodium dodecyl sulfate (Bio-Rad), and 600 ml methanol. Once complete, each lane of nitrocellulose was cut and soaked for 2 hrs in PBS-Tween buffer which contained 8.7 g/l NaCl, 6.1 g/l $KH_2PO_4$, 1.0 g/l NaOH, and 0.05% Tween-20 (pH 6.8). Appropriate IgG enriched Abs were diluted in PBS-Tween buffer to a concentration of 5 µg/ml and 2 ml of this added to each nitrocellulose lane. This was incubated at RT for 2 hrs and washed 5 times (10 minutes each) with the above buffer. Each lane was pulsed with 0.25 uCi of $^{125}I$-protein A in a total volume of 2 ml. The nitrocellulose strips were left at RT for 2 hrs, then washed 6 times with PBS-Tween buffer prior to air drying. The strips were aligned on paper and then place onto a X-OMAT AR X-ray film (Kodak, Rochester, NY) for 13

hrs at -70°C. Exposed films were processed using an automated Data IV X-ray film developer (Picker, Cleveland, OH).

4.2.12 Murine BM and Thymocyte DNA Proliferation Assays

For DNA synthesis assays, 5 X $10^5$ BM cells or thymocytes were cultured in flat bottom 96-well Nunc plates (Gibco). All cultures were plated in quadruplicate. After 24 hrs of culture, the cells were pulsed with 1 uCi of $^3$H-thymidine (New England Nuclear) and then 24 hrs later were harvested onto fiber filter paper using a Titertek Cell Harvester (Flow Labs.). Incorporation of $^3$H-thymidine was determined by liquid scintillation counting.

4.2.13 Statistical Analysis

The statistical analysis used is detailed in section 2.2.9.

### 4.3.1 Ability of BM Cells to Enhance PFC Responses

The addition of limited numbers of human BM cells to PFC cultures resulted in an enhanced Ab response. As shown in Figure 4.1, a ratio of BM cells to responding cells ranging between 0.2 to 0.5 clearly led to an enhanced PFC response. On the other hand, higher doses of BM cells resulted in a suppressed Ab response.

### 4.3.2 Enrichment of BM Derived Enhancing Activity by Elutriation

Centrifugal elutriation mechanically separates cells according to size and density parameters. Human BM cells were fractionated by elutriation in an effort to enrich for a population of cells that exhibited immunoenhancing activity. The cell size distribution profile of unfractionated BM and of the four fractions recovered after elutriation is shown in Figures 2.2a and 2.2b. Of the four arbitrary fractions collected, only fraction 2 contained Ab enhancing activity (Table 4.1). Fraction 2 contained 25% of the total nucleated BM cells and was enriched for cells of lymphocyte morphology. In spite of the heterogeneous nature of elutriated fraction 2 BM cells, these cells were capable of generating a soluble immunoenhancing activity after 24 hrs of culture. These culture supernatants displayed Ab enhancing activity at much lower doses than those required when using supernatants from cultures of unfractionated BM cells (Table 4.2).

FIGURE 4.1

Regulation of PFC Response by BM Cells

Tonsillar cells (1 X $10^6$) were cultured with SRBC for 7 days to generate an in vitro primary Ab response (●). To these cultures, varying doses of human BM cells were added at culture initiation. The anti-SRBC specific PFC response of these cultures was assayed on day 7 (■). These results have been previously presented as part of Figure 2.1.

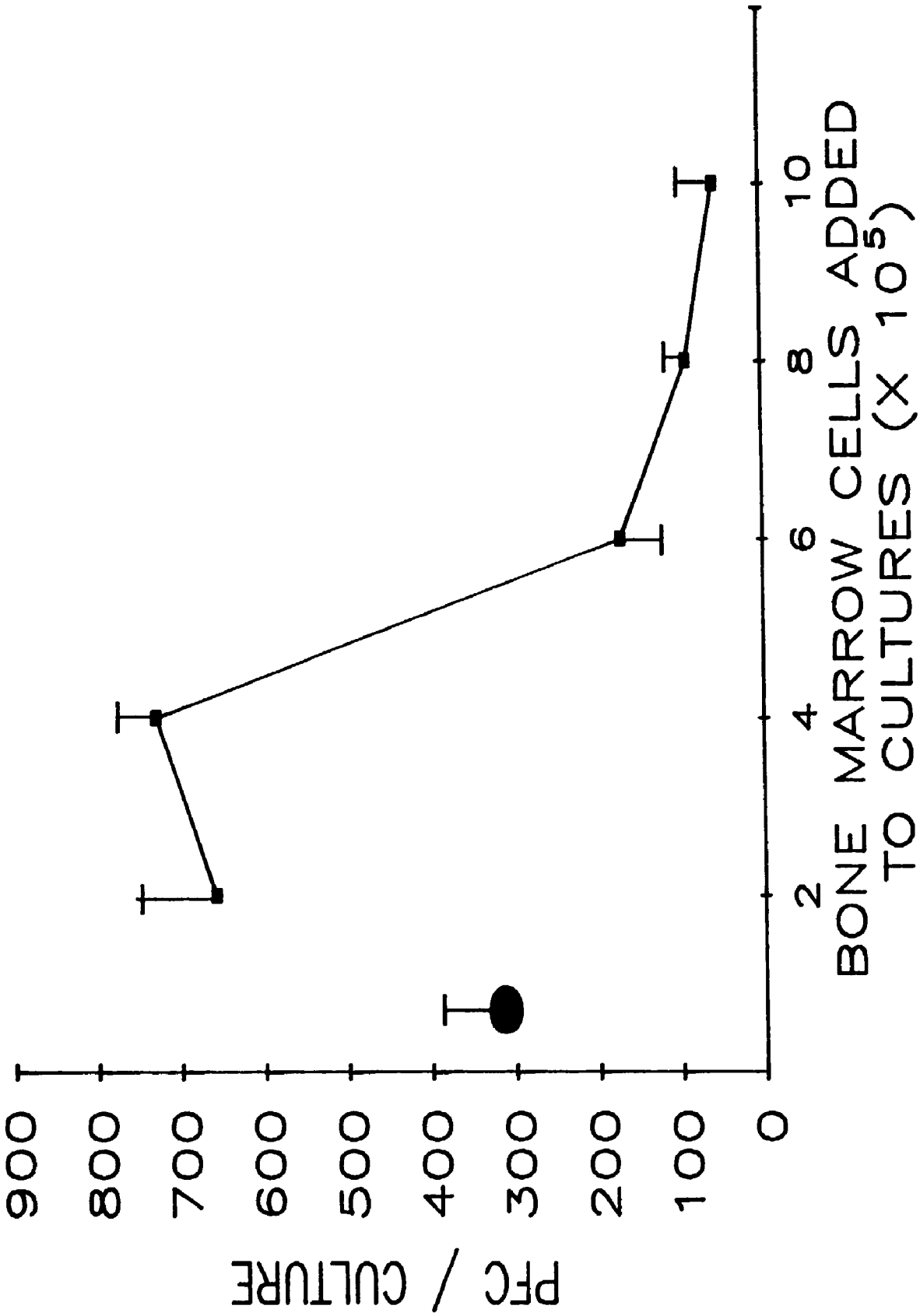

PFC / CULTURE
900
800
700
600
500
400
300
200
100
0
2
4
6
8
10
BONE MARROW CELLS ADDED
TO CULTURES (X $10^5$)

TABLE 4.1

Effect of Elutriated BM Cells on PFC Responses

| Type of Cells Added to PFC Cultures | PFC / Culture $\bar{X} \pm$ S.D. | % Shift from Control Response |
|---|---|---|
| UNFR BM | 365 ± 31 | - 55 |
| Elutr F1 | 800 ± 46 | - 2 |
| Elutr F2 | 1475 ± 176[a] | + 81 |
| Elutr F3 | 715 ± 52 | - 12 |
| Elutr F4 | 353 ± 37 | - 57 |

Unfractionated BM cells (6 X $10^5$) or elutriated BM cells (2 X $10^5$) were cultured with PBL and SRBC for 7 days to generate a primary Ab response. The control response in this experiment was 815 ± 56. These data have been presented before as part of Table 2.2.

[a] Statistical difference from the control PFC response, $p < 0.02$.

TABLE 4.2

Recovery of BDEF Activity in Supernatants of Elutriated BM Cell Fractions

| Cell Source of Supernatants | % of Total BM Cells Found in the Fraction | General Cell Morphology | Effect of Supernatants on PFC Response | |
|---|---|---|---|---|
| | | | Dose | % Shift |
| UNFR BM | 100 | --- | 0.1 | - 47 |
| Elutr F1 | 6 | Erythroid | 0.02 | + 6 |
| Elutr F2 | 25 | Lymphoid | 0.02 | + 65 |
| Elutr F3 | 27 | Monocytic | 0.02 | - 5 |
| Elutr F4 | 42 | Myeloid & Granulocytic | 0.02 | - 45 |

Supernatants were generated by culturing either unfractionated or elutriated BM cells at a density of 1 X $10^7$/ml in RPMI-1640 medium for 24 hours. The doses of supernatants are reported in volume equivalents (in ml) of original BM culture supernatants. This takes into account any concentration and dilution of the supernatants that occurred prior to being added directly to PFC cultures.

### 4.3.3 Enhancement of the Human and Murine PFC Response by BDEF

Supernatants recovered from cultures of unfractionated human BM cells contained an immunoenhancing activity that has been designated as BDEF (Bone marrow Derived Enhancing Factor). This activity is manifested as an enhancement of the *in vitro* Ab response of both human and murine cultures. BDEF from various individuals added to cultures of normal tonsillar cells in the presence of SRBC routinely resulted in a dose-dependent augmentation of the human PFC response (Figure 4.2a). In addition to enhancing human responses, human BDEF was also capable of enhancing the murine PFC response in a dose-dependent fashion (Figure 4.2b). Human and murine cells cultured in the presence of BDEF but in the absence of Ag failed to produce an Ab response higher than the background response of unstimulated cells.

### 4.3.4 Kinetics of BDEF Activity

The kinetics of BDEF activity were studied by varying the time at which the factor was added to Ab cultures. Table 4.3 shows that BDEF induces maximal enhancement of the PFC response when added within 24 hours of culture initiation. Addition of BDEF on subsequent days caused no significant deviation of the PFC response from control values.

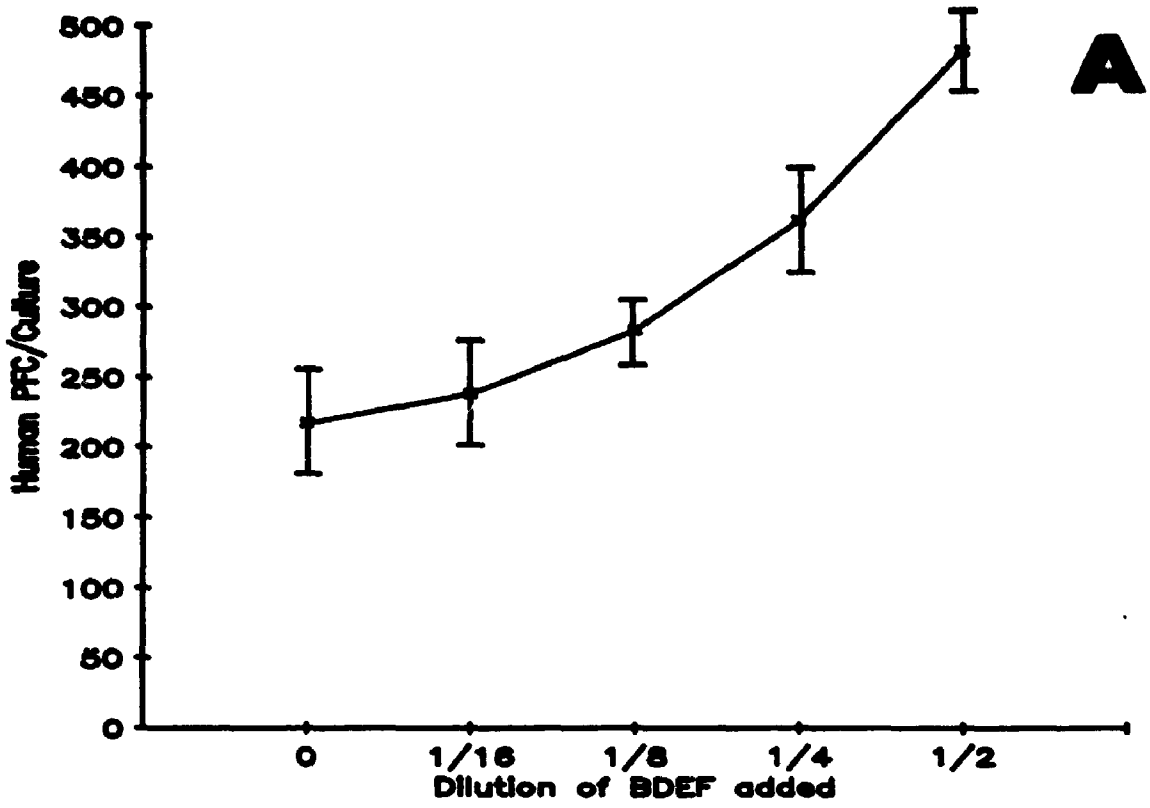
### 4.3.5 Target of BDEF

In view of the ability of BDEF to enhance Ab synthesis *in vitro*, an attempt was made to define the initial target of BDEF action. Table 4.4 summarizes results obtained through incubation studies. The responding human cells were initially fractionated by E-rosetting into $E_R$- and $E_R$+ and then incubated for 18 hrs in the presence of BDEF. The treated cell

FIGURE 4.2

Activity of BDEF on Human and Murine PFC Responses

BDEF produced from human BM cultures was tested at different doses for its ability to enhance the human (panel A) and murine (panel B) PFC responses. The factor was added at the initiation of cultures and the PFC assays enumerated on day 5 for the murine and on day 7 for the human cultures.


A
Human PFC/Culture
500
450
400
350
300
250
200
150
100
50
0
0
1/16
1/8
1/4
1/2
Dilution of BDEF added

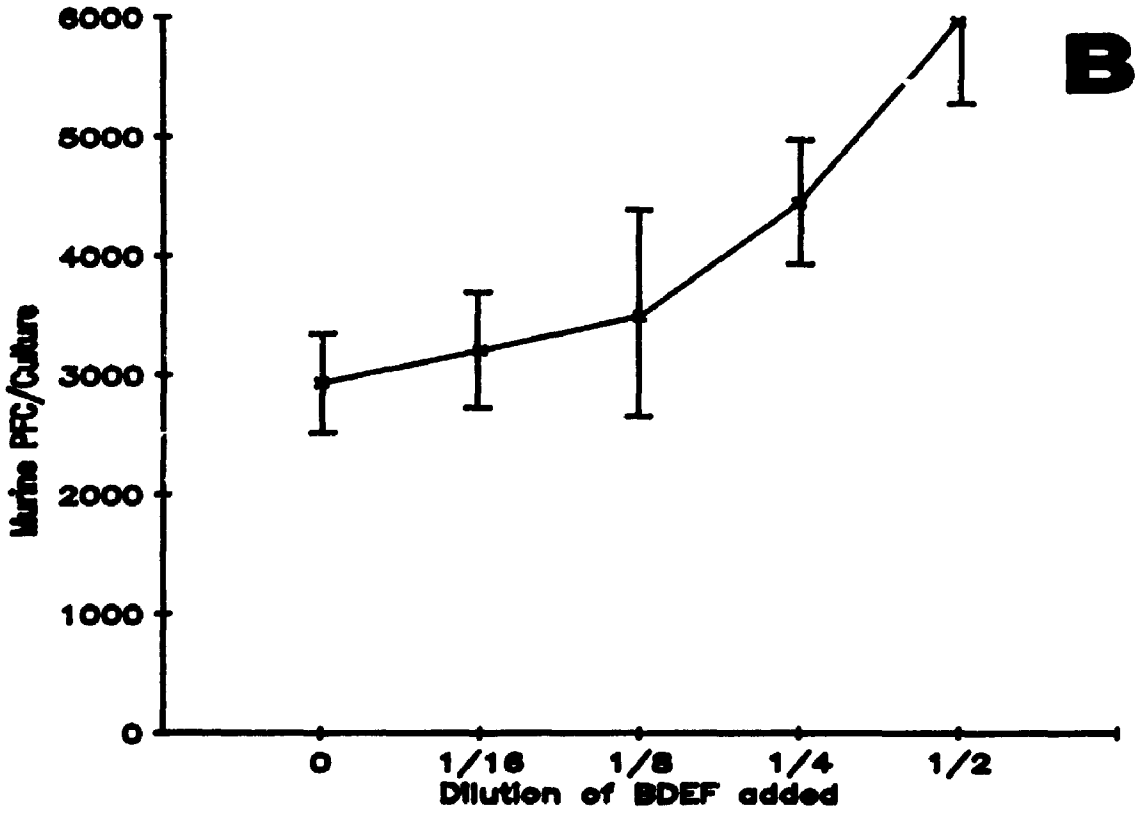

B
Murine PFC/Culture
6000
5000
4000
3000
2000
1000
0
0
1/16
1/8
1/4
1/2
Dilution of BDEF added

TABLE 4.3

Kinetics of BDEF Activity

| [a]Day of BDEF Addition | PFC/Culture ± S.D. | % Increase | P value |
|---|---|---|---|
| --- | 239 ± 33 | --- | --- |
| 0 | 631 ± 48 | 164 | <0.0005[b] |
| 1 | 543 ± 59 | 127 | <0.0005 |
| 2 | 251 ± 36 | 5 | < 0.3 |
| 3 | 222 ± 19 | -7 | < 0.2 |

[a] Tonsillar cells were cultured with SRBC for the generation of a PFC response. Where indicated, Amicon prepared BDEF was added to the cultures at a final dilution of 1/10.

[b] P value when compared to the control culture where no BDEF was added.

Experiments were repeated four times.

TABLE 4.4

Target of BDEF In PFC Responses

| Culture Conditions | PFC/Culture ± S.D. | % Increase | P value |
|---|---|---|---|
| PBL + Ag | 211 ± 17 | --- | |
| PBL + Ag + BDEF[a] | 407 ± 36 | 93 | <0.0005[b] |
| $E_R$+ + $E_R$- + Ag | 234 ± 28 | 11 | < 0.1[b] |
| $E_R$+(BDEF) + $E_R$- + Ag | 241 ± 28 | 14 | < 0.2[c] |
| $E_R$+ + $E_R$-(BDEF) + Ag | 458 ± 52 | 117 | <0.0005[c] |

[a] PBL were cultured in a 7 days PFC assay and BDEF was added directly to cultures at a final dilution of 1/10.

In the incubation assays, 5 X $10^5$ E-rosetted PBL were incubated with a 1/10 dilution of BDEF at 37°C for 18 hrs and then washed 2 times prior to being added back to cultures with an equal number of cells from the complementary E-rosetting fraction.

[b] P value compared to control cultures of unfractionated PBL.

[c] P value compared to control cultures of E-rosetted cells.

Experiments were repeated three times.

population was then added to its complementary population and cultured in the presence of Ag for Ab production. Results from Table 4.4 suggest that the immunoenhancing activity associated with BDEF is transferred along with the $E_R$- cells that had been incubated with the factor. $E_R$+ cells similarly incubated with BDEF are unable to transfer BDEF activity to the culture system.

### 4.3.6 Lack of IL-5 Activity of BDEF

In an effort to show that BDEF is unlike other known lymphokines, BDEF was tested to see whether it could reconstitute an Ab response for a T-dependent Ag (SRBC) in a T-cell depleted system. This latter activity is one of the key biological activities associated with IL-5. In Table 4.5 evidence is presented that BDEF is unable to replace T-cell function in a T-depleted Ab culture and therefore lacks IL-5 activity. TRF, or IL-5 activity, generated by culturing allogeneic cells in clearly able to reconstitute the same T cell depleted PFC response.

### 4.3.7 The Protein Nature of BDEF

To determine the chemical nature of BDEF, experiments were carried out that compared the Ab enhancing activity of crude BDEF against the activity recovered from ammonium sulphate precipitations. Table 4.6 demonstrates that a 1 μg/ml dose of crude BDEF results in only a 5% increase of the PFC response. On the other hand, a similar dose of BDEF derived from proteins precipitated with 45% ammonium sulphate results in a 39% increase of the PFC response. Finally, BDEF prepared from 90% ammonium sulphate precipitable fractions, yielded a maximal enhancing activity of 90% when tested at 1 μg/ml. The enrichment for BDEF activity

TABLE 4.5

Lack of T-Cell Replacing (IL-5) Activity of BDEF

| Culture Condition | PFC/Culture ± S.D. | P value |
|---|---|---|
| [a] $E_R$- + SRBC | 265 ± 44 | ------ |
| $E_R$+ + $E_R$- + SRBC | 515 ± 15 | <0.0005[b] |
| $E_R$- + SRBC + TRF[c] | 470 ± 13 | <0.0005[b] |
| $E_R$- + SRBC + BDEF[d] | 270 ± 12 | < 0.2[b] |

[a] E-rosetted cells were added to cultures at a concentration of 5 X $10^5$/well.

[b] P value compared to cultures of $E_R$- cells plus Ag.

[c] TRF was derived by culturing tonsillar cells as described by Muraguchi et al. (1981) in the presence of 0.1% PHA for 48 hrs, and then adding the processed supernatants at a concentration of 20% (v/v) at 48 hrs after culture initiation.

[d] BDEF prepared by Amicon ultrafiltration was added to cultures at a final dilution of 1/4.

Experiments were repeated three times.

TABLE 4.6

The Protein Nature of BDEF

| Culture Conditions | Source of BDEF | PFC/Culture $\bar{X} \pm$ S.D. | % Increase |
|---|---|---|---|
| PBL + Ag | ---- | 289 ± 44 | --- |
| PBL + Ag + BDEF (10 μg/ml) | Amicon | 543 ± 66 | 88 |
| PBL + Ag + BDEF (1 μg/ml) | Amicon | 303 ± 50 | 5 |
| PBL + Ag + BDEF (10 μg/ml) | 45% $(NH_4)_2SO_4$ | 590 ± 42 | 104 |
| PBL + Ag + BDEF (1 μg/ml) | 45% $(NH_4)_2SO_4$ | 401 ± 51 | 39 |
| PBL + Ag + BDEF (10 μg/ml) | 90% $(NH_4)_2SO_4$ | 717 ± 148 | 148 |
| PBL + Ag + BDEF (1 μg/ml) | 90% $(NH_4)_2SO_4$ | 550 ± 73 | 90 |

PBL were cultured to generate a primary Ab response against SRBC and where indicated BDEF, prepared by different techniques, was added directly to cultures at the indicated concentrations. BDEF was added at culture initiation. This study was repeated three times.

is more evident by analyzing the percent increase in PFC activity per microgram of protein. It is obvious that the 90% ammonium sulphate protein fraction has a higher immunoenhancing activity per unit mass than either crude BDEF or 45% ammonium sulphate derived BDEF (Table 4.6). The ability to recover BDEF activity in ammonium sulphate precipitates and the enrichment for this activity in such preparations all suggest that BDEF is of protein nature.

### 4.3.8 Con-A Sepharose Chromatography of BDEF

As a means of determining whether the BDEF molecule possessed carbohydrate groups, Con-A sepharose affinity chromatography was used. The Con-A lectin that is chemically bonded to the sepharose beads has affinity for carbohydrates with terminal alpha-D-mannosyl and alpha-D-glucosyl residues. A summary of results from testing the biological activities of Con-A sepharose fractionated BDEF on either the Ab response or the mitogenic activity for murine thymocytes is presented in Figures 4.3 and 4.4 respectively. Both testing criteria, indicate an increase in specific enhancing activity of proteins eluted from the Con-A sepharose column. In the PFC assay, a 1 μg/ml dose of Con-A binding proteins enhance the response by 16%. Conversely, an equal dose of either unfractionated BDEF or Con-A non-binding proteins were unable to significantly alter the PFC response from control values (Figure 4.3). A similar pattern of reactivity is observed when the same samples are tested for mitogenic potential. Data in Figure 4.4 clearly support the findings that BDEF activity resides in the Con-A binding protein fraction and suggest that BDEF may have carbohydrate moieties attached to it.

FIGURE 4.3

Fractionation of BDEF Activity for Ab Responses
by Con A-Sepharose Chromatography

Crude BDEF was fractionated on a Con A-Sepharose column as described in the Materials and Methods (section 4.2.5) and then tested in a PFC assay, using tonsillar cells, at the indicated doses. The control Ab responses of cells cultured with Ag only (□) or with Ag plus a preparation from a sham elution of the column (△) are also reported.

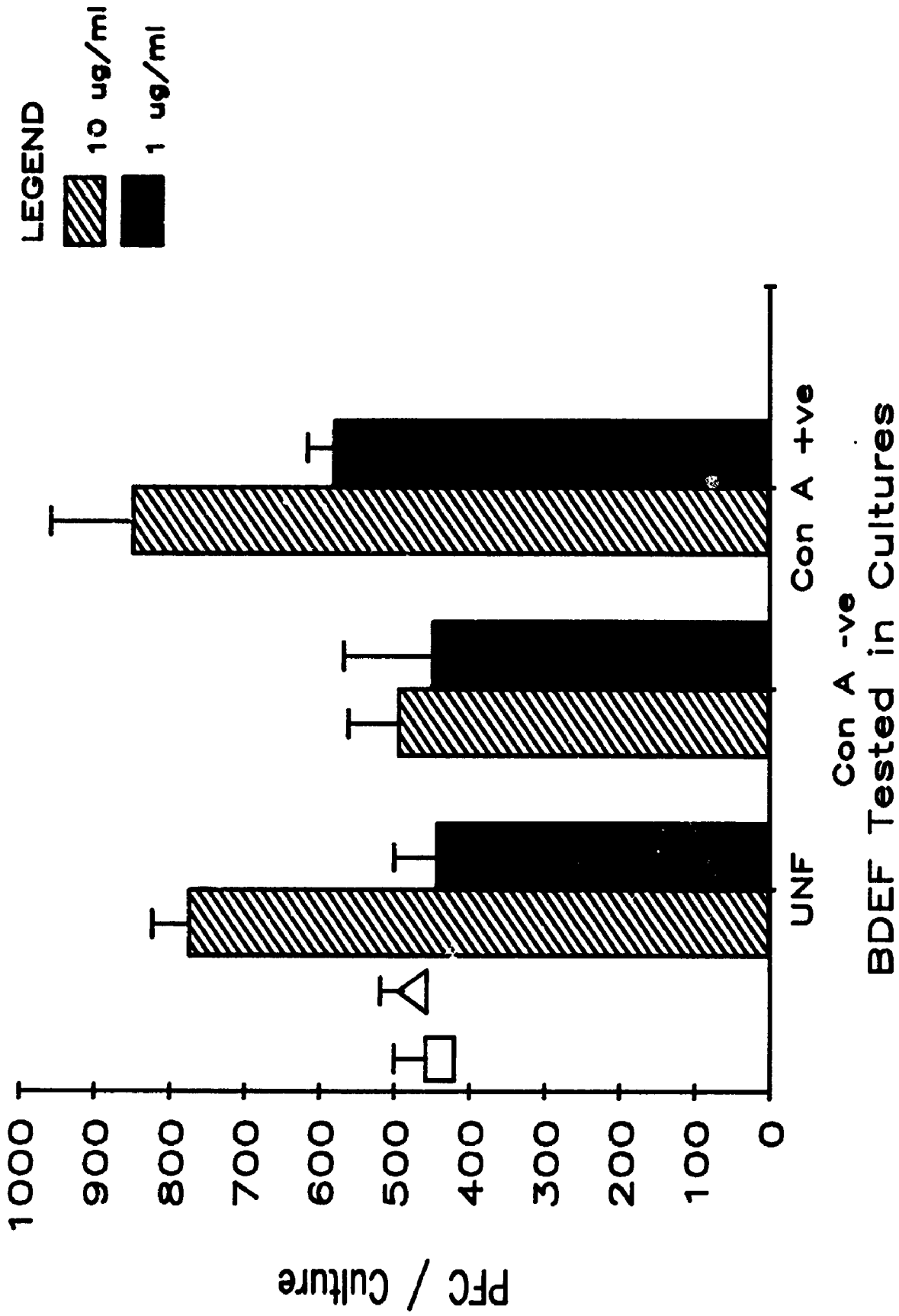
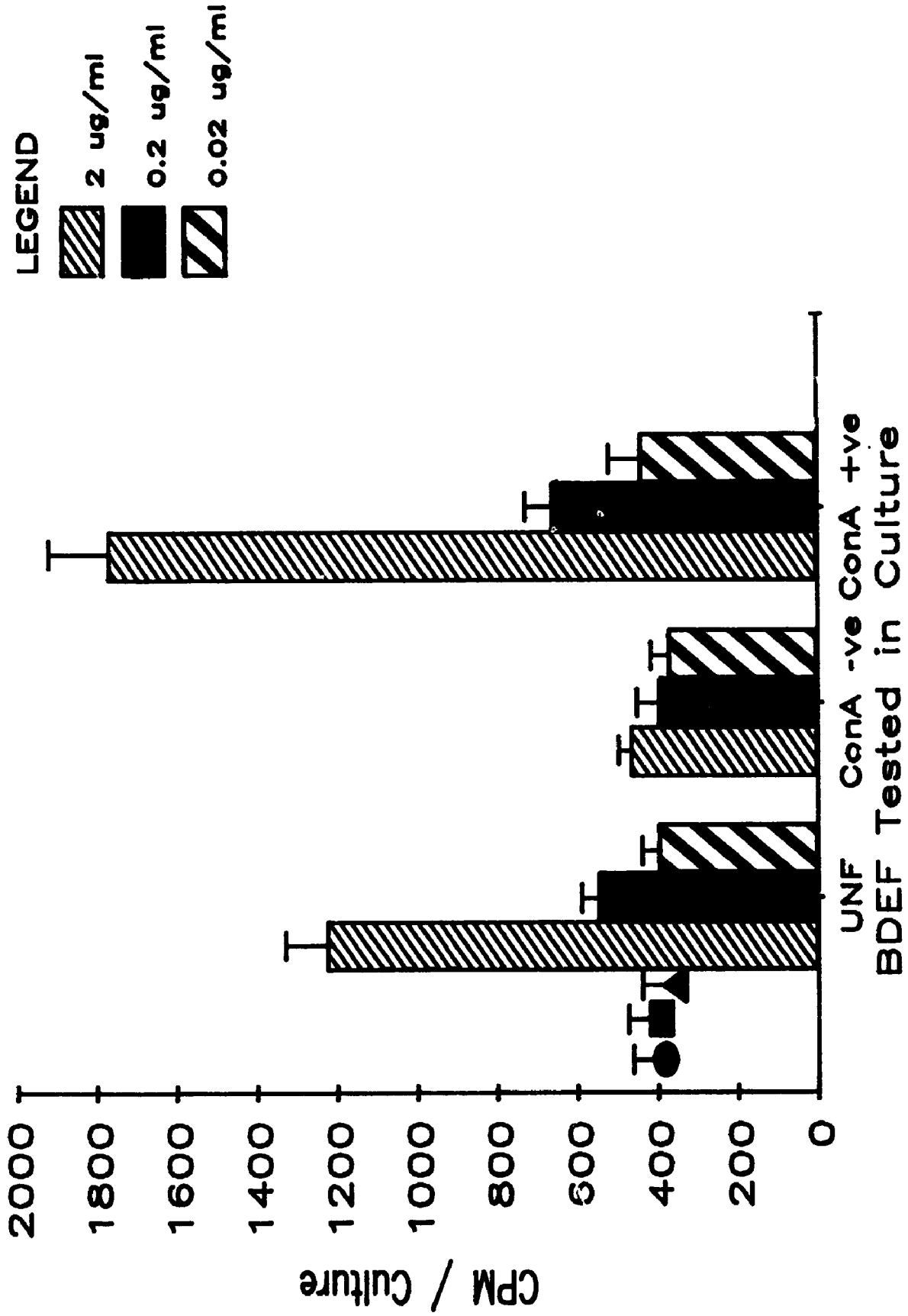

LEGEND
10 ug/ml
1 ug/ml
PFC / Culture
1000
900
800
700
600
500
400
300
200
100
0
UNF
Con A -ve
Con A +ve
BDEF Tested in Cultures

FIGURE 4.4

Fractionation of BDEF Mitogenic Activity
by Con A-Sepharose Chromatography

Balb/c thymocytes were plated in flat bottom 96-well plates at a density of 5 X $10^5$/well using RPMI-1640 medium supplemented with 10% FCS and 5 X $10^{-5}$M 2-mercaptoetharol. Unfractionated or Con A-Sepharose chromatography fractionated BDEF was added to each well of quadruplicate cultures at the indicated doses. Cultures were incubated for 24 hrs prior to pulsing them with 1 μCi of $^3$H-thymidine. Twenty-four hrs later the cultures were harvested and DNA synthesis assessed by liquid scintillation counting. The reported controls are as follows: background proliferation of thymocytes ( ● ), proliferation of thymocytes in the presence of 50 μl of a sham eluted preparation ( ■ ), and the proliferation of thymocytes in the presence of the competitor sugar used to elute bound proteins from the column (0.1 M alpha-methyl-mannoside) ( ▲ ).


LEGEND
2 ug/ml
0.2 ug/ml
0.02 ug/ml
CPM / Culture
2000
1800
1600
1400
1200
1000
800
600
400
200
0
UNF ConA -ve ConA +ve
BDEF Tested in Culture

### 4.3.9 DEAE Chromatography of BDEF

In a continuing attempt to understand more of the physico-chemical nature of BDEF, DEAE chromatography was utilized to determine the relative charge on the BDEF molecule. DEAE-sephadex separates molecules according to their ability to bind to the positively charged matrix. BDEF fractionated on such a column was tested in both the Ab and mitogenic assays at equal doses and the results are found in Figures 4.5a and 4.5b respectively. In both test systems, BDEF activity resided largely in fraction 1 (F1), with some activity remaining in fraction 2 (F2). It was calculated that the two fractions had been eluted from the DEAE column with concentrations of NaCl ranging between 3.5 mM to 91.5 mM for F1, and 91.5 mM to 192.5 mM for F2. Furthermore, the PFC enhancing activity and the mitogenic activity in F1 were of greater magnitude than the same activities generated by unfractionated BDEF. These data suggest that the BDEF molecule has a relatively small negative charge.

### 4.3.10 Sensitivity of BDEF to Low pH

To further distinguish BDEF from other lymphokines, active BDEF preparations were exposed for 24 hrs to pH 2.2 by dialysis. In Figure 4.6 evidence is presented that BDEF is resistant to such treatment. Both doses of treated BDEF could enhance the Ab response. Their activity is statistically comparable to that of sham dialyzed BDEF.

### 4.3.11 Fractionation of BM Derived Proteins by HPLC

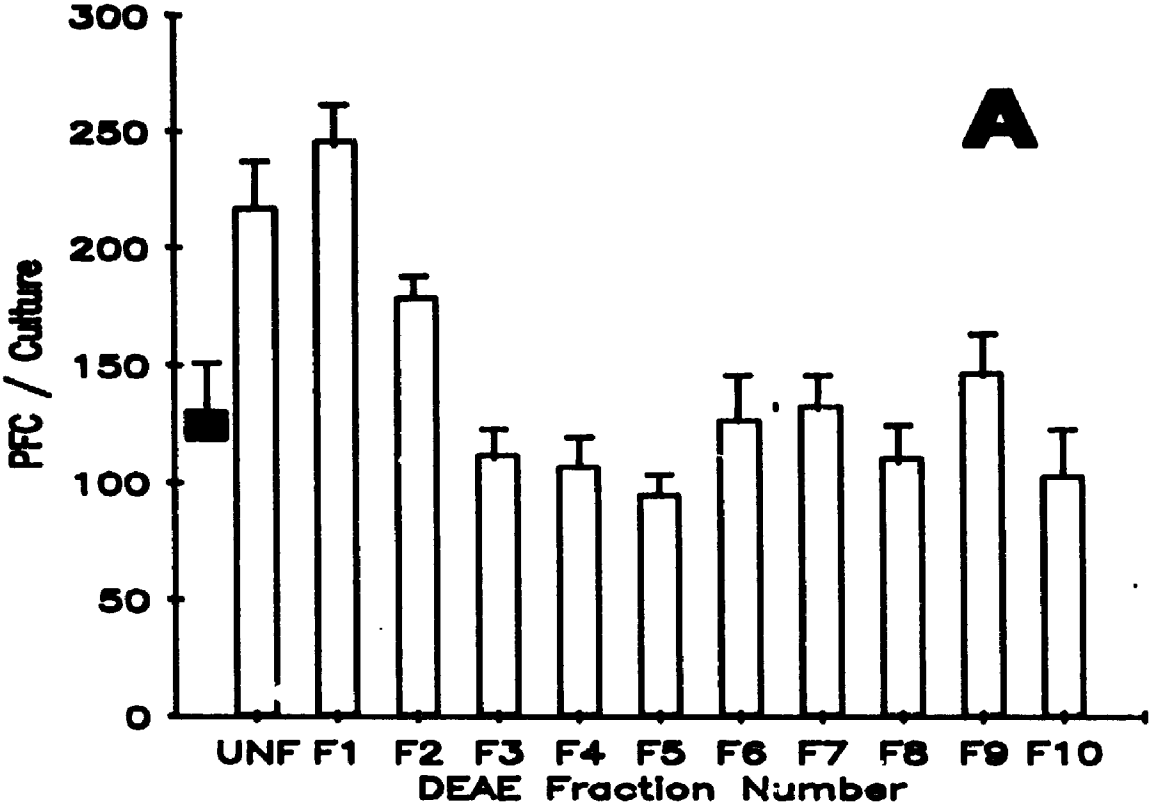
To further characterize BDEF, HPLC was used to fractionate proteins that had been isolated from human BM culture supernatants. The HPLC protein elution profile is shown in Figure 4.7a. The four major protein

FIGURE 4.5

Fractionation of BDEF Activity by DEAE Chromatography

A. BDEF fractionated by DEAE chromatography was tested for its ability to enhance the *in vitro* Ab response of PBL (■). Unfractionated or DEAE fractionated BDEF was added at culture initiation and was used at an equal dose of 1 μg/ml.

B. The ability of DEAE fractionated BDEF to stimulate the DNA synthesis of murine thymocytes was tested. Both unfractionated BDEF and proteins recovered from the DEAE column were tested in cultures at a concentration of 0.8 μg/ml. The control background proliferation of thymocytes is indicated (▲).


A
PFC / Culture
300
250
200
150
100
50
0
UNF F1 F2 F3 F4 F5 F6 F7 F8 F9 F10
DEAE Fraction Number

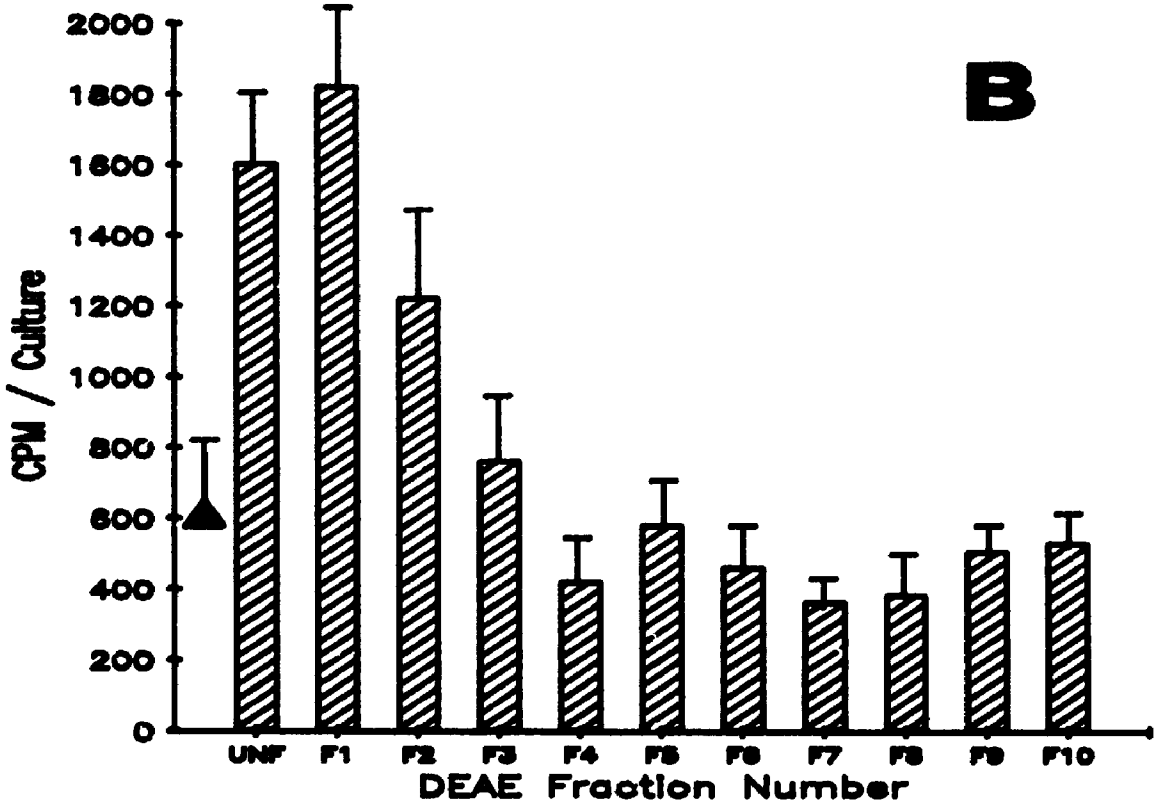

B
CPM / Culture
2000
1800
1600
1400
1200
1000
800
600
400
200
0
UNF F1 F2 F3 F4 F5 F6 F7 F8 F9 F10
DEAE Fraction Number

FIGURE 4.6

BDEF Is Not Sensitive to Low pH Treatment

One ml BDEF was placed in a dialysis membrane of MW cutoff 10,000 Da and exposed to either 0.01 M PBS (pH 7.4) or 0.05 M glycine-HCl buffer (pH 2.2) for 24 hrs at $4^{o}C$. The pH of each sample was then adjusted by further dialysis against 500 volumes of 0.01 M PBS (pH 7.2), prior to adding both samples, along with a control preparation of BDEF, to PFC cultures. The anti-SRBC specific Ab response of tonsillar cells, without the addition of BDEF, is also indicated (●).

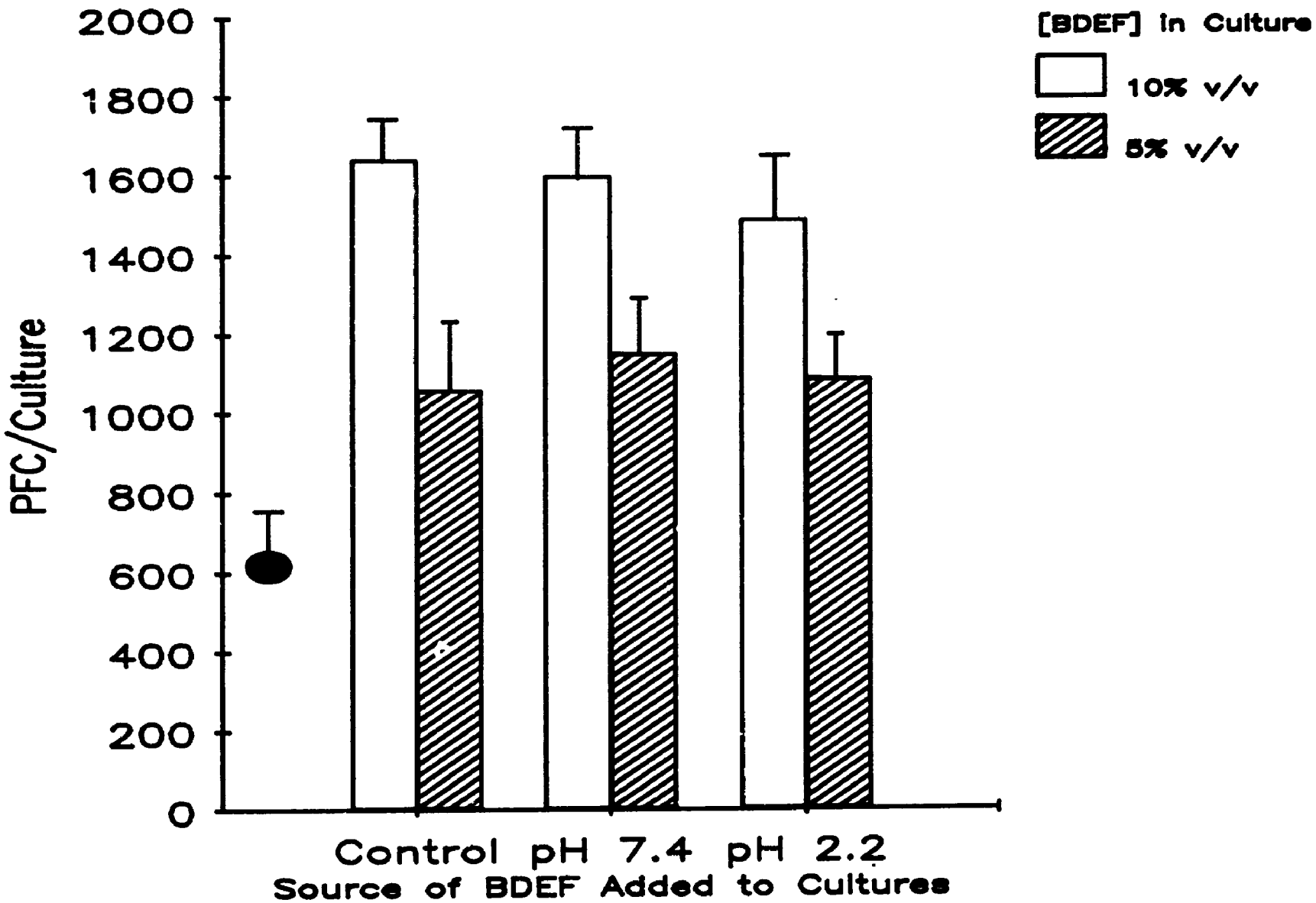
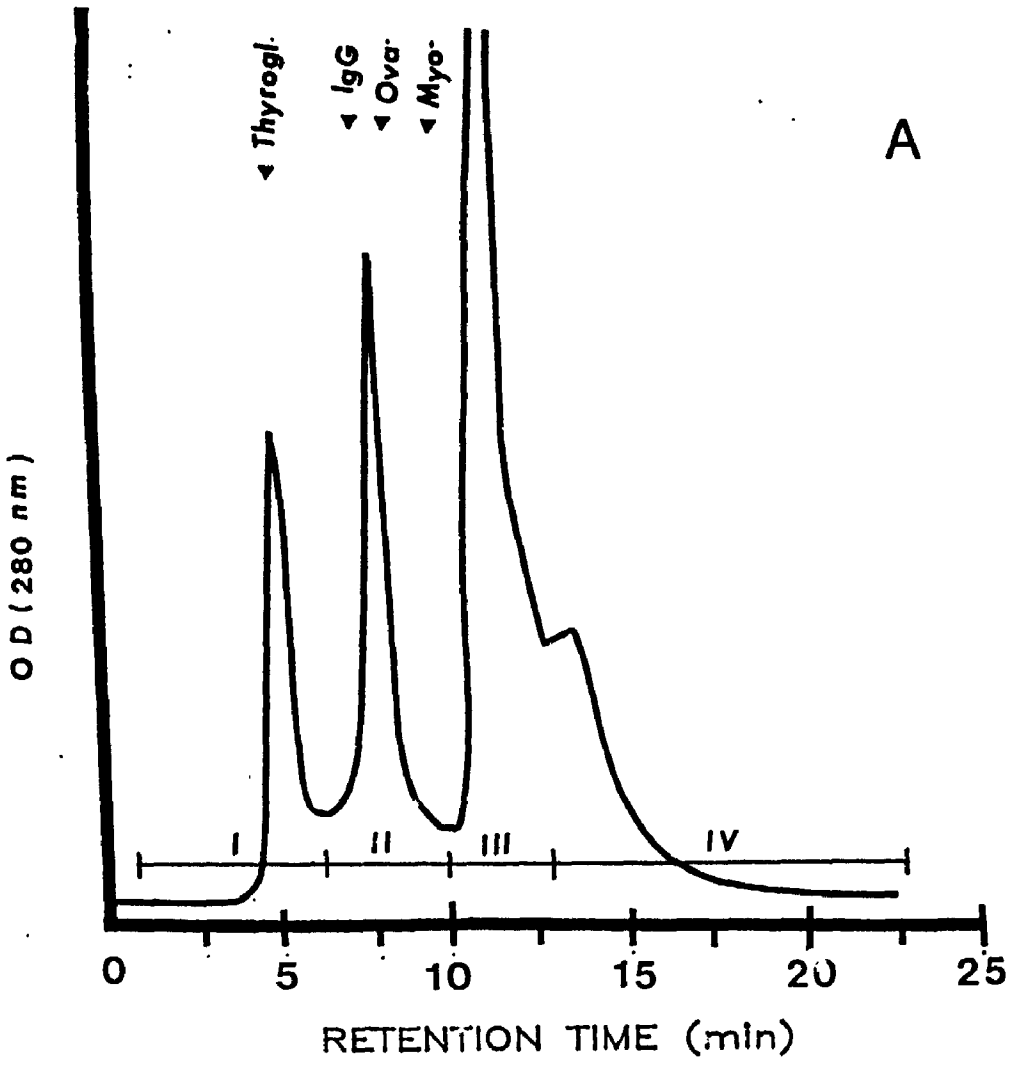

2000
1800
1600
1400
1200
1000
800
600
400
200
0
PFC/Culture
[BDEF] In Culture
10% v/v
5% v/v
Control
pH 7.4
pH 2.2
Source of BDEF Added to Cultures

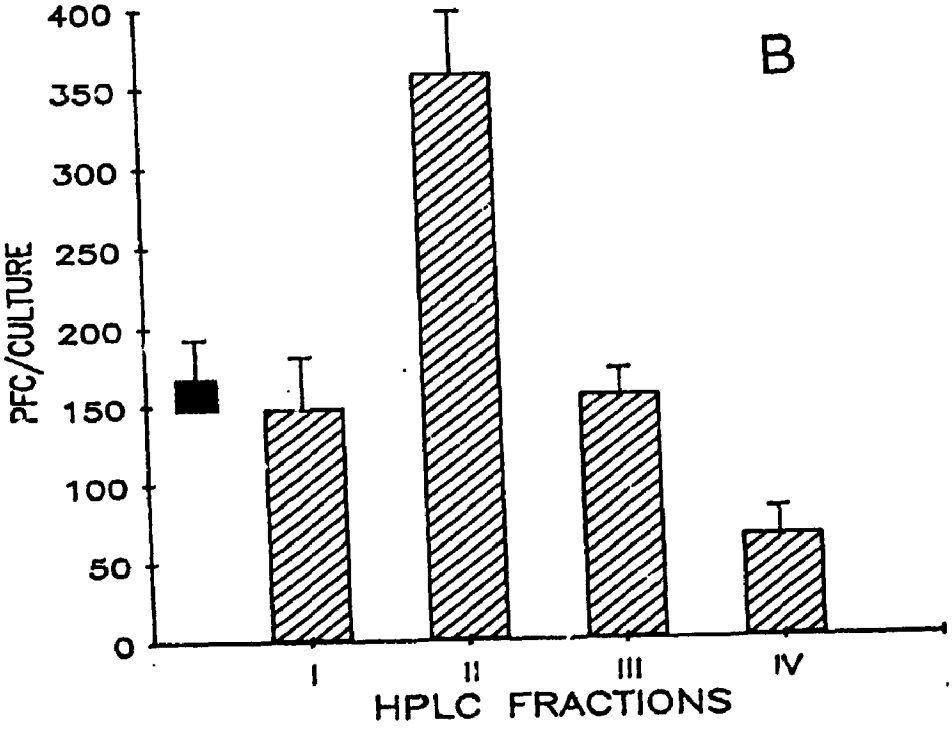
FIGURE 4.7

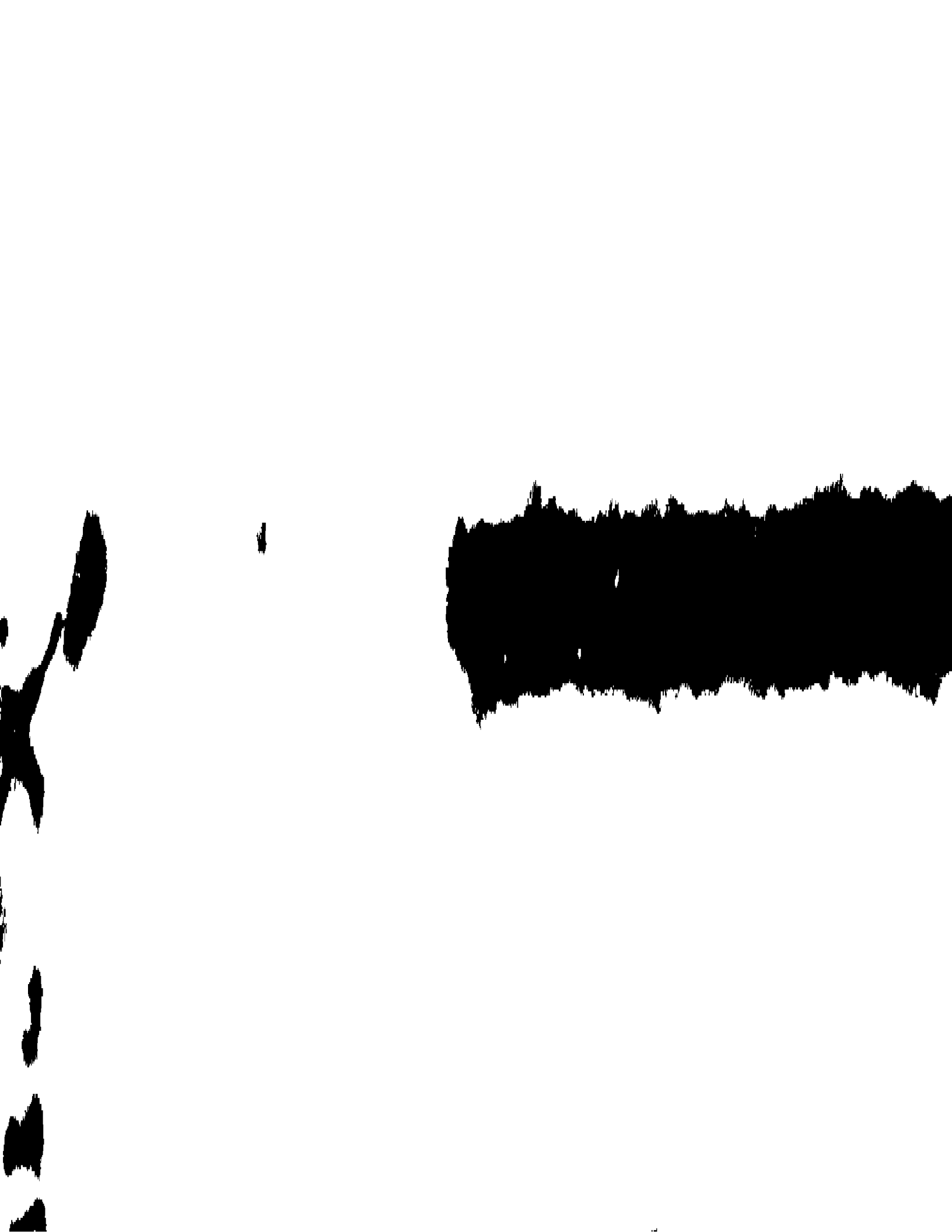
Fractionation of BDEF by HPLC

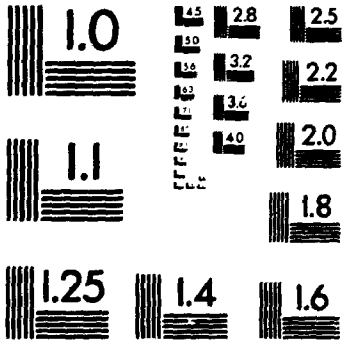
A. BM culture supernatants were concentrated by lyophilization and then dialyzed against 1000 volumes of PBS, prior to loading 100 µl samples (285 µg of protein) onto an HPLC. The elution profile was generated by scanning at 280 nm. The elution of the molecular weight markers is indicated on the profile. The molecular weight of the standards is as follows: thyroglobulin, 670,000; IgG, 158,000; ovalbumin, 44,000; myoglobin, 17,000. The four Roman numerals (I to IV) indicate the fraction number into which each of the indicated areas under the curves were pooled.

B. The four pooled fractions (I to IV) were individually tested for their ability to enhance the Ab response of tonsillar cells. Each fraction was tested at a dose of 10 µg/ml of culture. The control day 7 anti-SRBC specific PFC response of cells cultured in the absence of factors is indicated (■).


A
Thyrogl.
IgG
Ova-
Myo-
O D ( 280 nm )
I
II
III
IV
0
5
10
15
20
25
RETENTION TIME (min)


B
400
350
300
250
200
150
100
50
0
PFC/CULTURE
I
II
III
IV
HPLC FRACTIONS






peaks were collected individually and labelled as fractions I to IV. The fractions were concentrated and then tested at equal concentrations in a PFC assay. Figure 4.7b demonstrates that only fraction II significantly enhanced the PFC response by a magnitude of 1.5 times of the control values. Fractions I and III had no effect on the Ab response when tested at various dilutions. Fraction IV was found to be suppressive for the PFC response. This is consistent with suppression of Ab responses by a low-molecular weight factor also derived from BM cultures and described in chapter 3.

### 4.3.12 Generation of Abs Against two BM Proteins

HPLC fractionation of BM proteins revealed that the Ab enhancing activity was present in Fraction II. This fraction contained proteins of MW range of 60,000 Da. Inspection of whole BM proteins by PAGE analysis verified that two major protein bands were present in this area of the gel: one migrating to 60,000 Da (p60) and the other to 55,000 Da (p55) (Figure 4.8). The gels were run under reducing conditions. Not knowing which protein may be responsible for BDEF activity, polyclonal antibodies to both proteins, p60 and p55, were raised in rabbits. To determine if the Abs were specific for the two proteins of interest, whole BM proteins were run on PAGE gels, transferred to nitrocellulose, and then reacted with either Ab. Figure 4.9 demonstrates that each Ab recognized the protein to which they were immunized, with minimal crossreactivity to each other and no reactivity with other BM proteins. It is apparent in lanes A and B of western blot of BM proteins (Figure 4.9), that anti-p60 and anti-p55 only react against p60 and p55 respectively, while an IgG

FIGURE 4.8

SDS-PAGE of Proteins from BM Culture Supernatants

Proteins derived from BM culture supernatants were concentrated by lyophilization followed by dialysis against 1000 volumes of PBS. A 50 μg sample was loaded onto a 12% acrylamide SDS-PAGE gel. In a separate lane protein standards were run as MW markers. Following the run, the gel was stained for proteins by immersion in a solution containing 0.15% Coomassie Blue R-250 dissolved in 50% methanol and 7% acetic acid. The photograph demonstrates the stained gel with the BM proteins in lane A and the MW weight markers in lane B (with their respective molecular weights in kilodaltons).

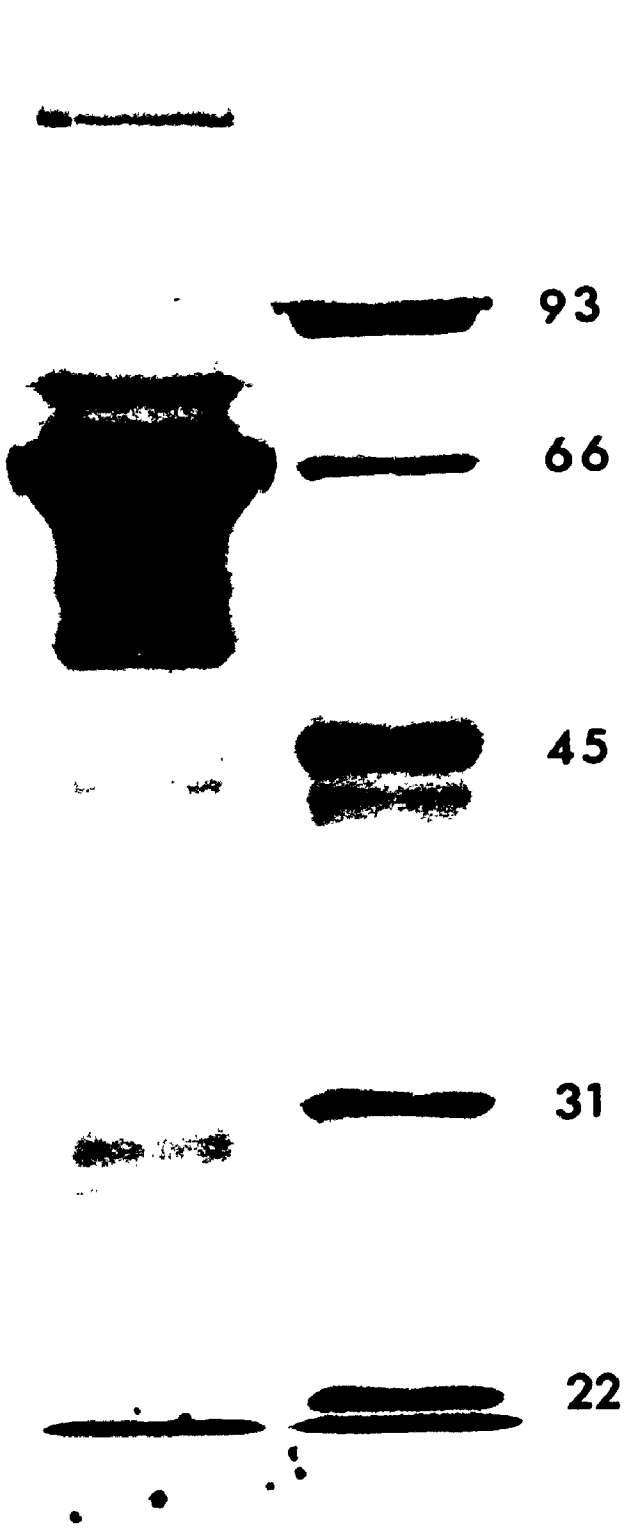

A
B
93
66
45
31
22

FIGURE 4.9

Specificity of Rabbit Abs for BM Derived Proteins

The two polyclonal Abs raised in rabbits were tested for reactivity against proteins derived from BM culture supernatants. Proteins of BM origin (40 $\mu$g) were first fractionated on a 12% acrylamide SDS-PAGE gel and then blotted onto nitrocellulose filter paper. The individual lanes were then incubated with 10 $\mu$g of each of the following Abs: lane A, anti-p60; lane B, anti-p55; and lane C, normal rabbit IgG. After washing, the filter strips were probed with $^{125}$I-protein-A for 2 hrs. The reactivity of each Ab was determined by exposing an X-ray film for 13 hours. The photograph of the developed X-ray film is presented.


C
22—

preparation from an unimmunized rabbit (NRS) has no reactivity against the same BM proteins (lane C, Fig. 4.9).

### 4.3.13 Ability of Abs to Recognize Native BM Proteins

The two antibodies described above were generated by immunization with denatured proteins, and it was evident that by western blotting they could recognize the denatured form of either p60 or p55. However, in order for these Abs to be useful in the purification of BDEF, it was necessary to assess whether they could also recognize the native forms of the two BM proteins. This was determined by ELISA. Microwell plates were coated with native BM proteins and then reacted with either anti-p60 or anti-p55. Table 4.7 demonstrates that anti-p60 and anti-p55 recognize the native BM derived proteins, while NRS displays no such reactivity.

### 4.3.14 Affinity Purification of p60 and p55

With the two Abs in hand, it became feasible to construct an affinity chromatography column with each Ab, in the hope of recovering highly purified native BM proteins. The two proteins, p60 and p55, were then tested for biological activity. The ability of each protein to enhance an *in vitro* Ab response is shown in Figure 4.10a. Furthermore, p60 and p55 were assayed for their mitogenic activity on BM cells (Fig. 4.10b) and thymocytes (Fig. 4.10c). It is clear that both the Ab enhancing activity and the mitogenic activities solely reside in the affinity purified p60 preparation. These data suggest that BDEF activity is associated with the protein p60. The other protein, p55, and a sham eluted preparation contained no immunoregulatory activity in any of the above tests. As a further confirmation that the observed immunoenhancing

TABLE 4.7

Reactivity of anti-p60 and anti-p55 Antibodies with Native Proteins from Human BM

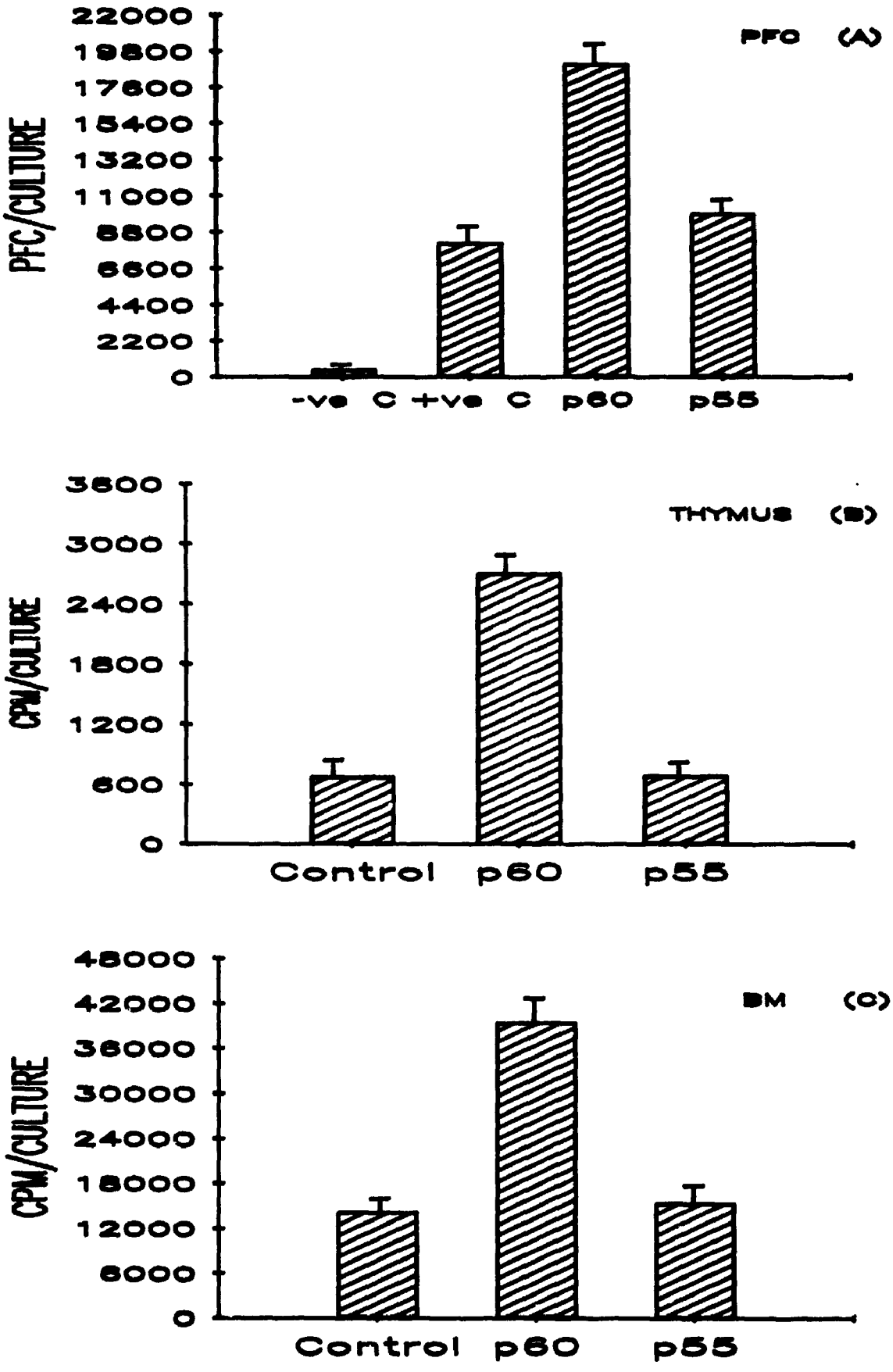
| [a]Concentration of Abs Used | O.D. (405 nm) | | |
|---|---|---|---|
| (μg/well) | anti-p60 | anti-p55 | NRS |
| 100 | > 2 | > 2 | 0 |
| 10 | 0.53 ± 0.02 | 0.32 ± 0.02 | 0 |

[a] Rabbit antibodies or normal rabbit serum (NRS) were added to wells of a 96-well plate that had been previously coated with 5 μg of total proteins derived from human BM culture supernatants. The reaction was developed using a goat anti-rabbit alkaline phosphatase conjugated Ab, as described in Materials and Methods (section 4.2.10). Experiments were repeated twice.

FIGURE 4.10

Biological Activity of Affinity Purified p60 Protein

Affinity purified protein p60 was tested for BDEF-like activity in a murine PFC assay (panel A), mitogenicity for murine thymocytes (panel B), and mitogenicity for murine BM cells (panel C). In all assays the purified protein was tested at a concentration of 1 μg/ml. In the PFC assay the response of murine splenocytes in the presence (+ve C) and absence (-ve C) of Ag is indicated. In the mitogenicity assays, the control responses indicate the background level of proliferation of either thymocytes or BM cells.


PFC (A)
PFC/CULTURE
22000
19800
17600
15400
13200
11000
8800
6600
4400
2200
0
-ve C
+ve C
p60
p55
THYMUS (B)
CPM/CULTURE
3600
3000
2400
1800
1200
600
0
Control
p60
p55
BM (C)
CPM/CULTURE
48000
42000
36000
30000
24000
18000
12000
6000
0
Control
p60
p55

activity was associated with p60, PAGE analysis was performed on the biologically active preparation. In Figure 4.11 evidence is presented that p60 was recovered in a relatively pure form with no visible evidence of other protein contaminants.

### 4.3.15 Inhibition of BDEF Activity by Anti-p60 Ab

Having provided evidence that BDEF was the protein p60, it was of interest to test whether anti-p60 could block the biological activity of BDEF. *In vitro* Ab cultures were set up in the presence of BDEF as well as anti-p60, anti-p55 or NRS. As seen in Table 4.8, varying doses of anti-p60 Ab abrogated the immunoenhancing activity of an optimal amount of BDEF in a dose dependent manner. On the other hand, anti-p55 or NRS exhibited no inhibitory activity for the BDEF enhanced response. The addition of anti-p60, anti-p55, or NRS to cultures in the absence of BDEF did not significantly affect the control PFC response.

FIGURE 4.11

Purity of Protein p60 Recovered from Affinity Columns

To assess the relative purity of protein p60 recovered from the affinity column, 20 µg of proteins per lane were loaded onto a 12% acrylamide SDS-PAGE gel. Following electrophoresis, the gel was stained using a Bio-Rad Silver Stain kit. A photograph of this gel is presented along with the protein standards and their respective molecular weight in kilodaltons in lane 1, total BM proteins in lane 2, and proteins eluted from the anti-p60 affinity column in lane 3.

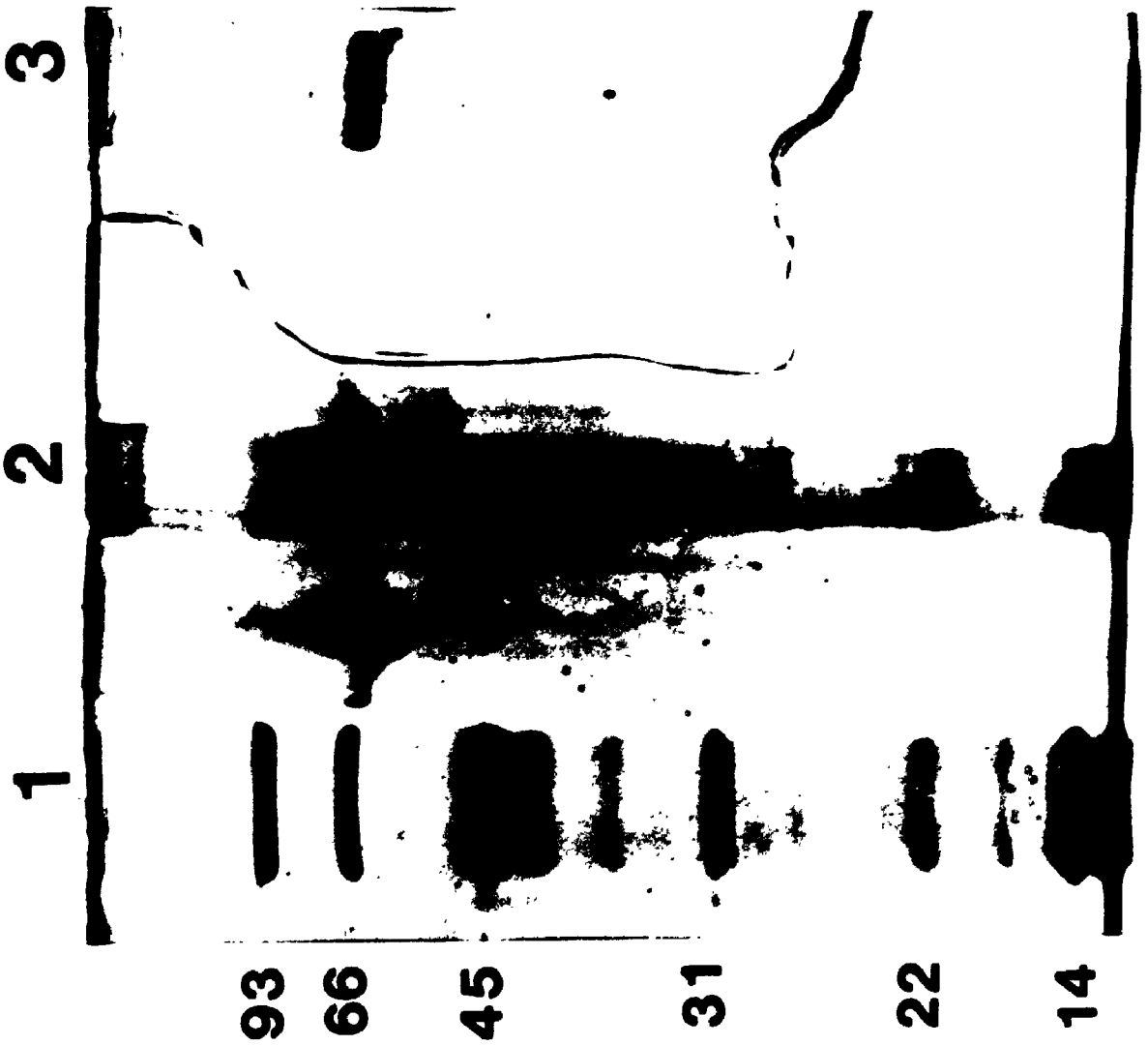

3
2
1
93
66
45
31
22
14

TABLE 4.8

Abrogation of BDEF Activity by Anti-p60 Ab

| Culture Conditions | PFC/Culture + S.D. | % Increase |
|---|---|---|
| Cells + Ag | 144 ± 17 | -- |
| Cells + Ag + BDEF | 271 ± 32 | 88 |
| Cells + Ag + BDEF + anti-p60 (50 μg) | 167 ± 22[a] | 16 |
| Cells + Ag + BDEF + anti-p60 (25 μg) | 204 ± 17 | 42 |
| Cells + Ag + BDEF + anti-p60 (12 μg) | 229 ± 27 | 59 |
| Cells + Ag + BDEF + anti-p55 (50 μg) | 239 ± 25[b] | 66 |
| Cells + Ag + BDEF + NRS (50 μg) | 254 ± 28[c] | 76 |
| Cells + Ag + anti-p60 (50 μg) | 137 ± 9[d] | -5 |
| Cells + Ag + anti-p55 (50 μg) | 131 ± 26[d] | -9 |
| Cells + Ag + NRS (50 μg) | 149 ± 18[d] | 3 |

PBL were used to generate a primary Ab response against SRBC. The source of BDEF was affinity purified protein p60 and it was used at a concentration of 1 μg/ml. DEAE purified antibodies were added to the cultures at the indicated doses at culture initiation.

[a] $P < 0.0005$ compared to control condition with BDEF.

[b,c] $P > 0.05$ compared to control condition with BDEF

[d] $P > 0.1$ compared to control cultures.

Experiments were repeated three times.

## 4.4 Discussion

The emphasis of this chapter is on the identification of a second immunoregulatory activity of normal human BM. This activity, which results in enhanced primary Ab responses, is attributable to a distinct population of BM cells of lymphoid morphology. The augmentation of Ab synthesis is mediated via a factor that is elaborated by either whole BM cells or fractions of BM cells enriched for enhancing activity. This is consistent with reports from this laboratory demonstrating the generation of a similar immunoenhancing activity from murine BM (Saffran et al., 1986; Duwe and Singhal, 1978). The mediator, named Bone marrow Derived Enhancing Factor (BDEF), appears to be a glycoprotein of molecular weight 60,000 Da with carbohydrate groups being of the alpha-D-pyranoside type. A unique feature of BDEF is its constitutive production from BM cells, as opposed to the many immunostimulatory molecules produced by mature or immortalized cells of peripheral tissues that normally require activation by either antigens, mitogens or phorbol esters (Arai et al., 1986; Oppenheim et al., 1979). One interpretation of this finding may be that molecules which contribute to the maintenance of normal BM functions, like BDEF, are required on a continuous basis and low levels are produced constitutively.

The ability of BDEF to augment both human and murine PFC responses implies that the factor is neither MHC nor species restricted in its spectrum of action. Furthermore, this ability to cross species barriers suggests that such molecules have been strongly conserved during the evolution of the immune system. This may be expected of lymphokines intimately associated with vital BM functions.

Another distinguishing feature of BDEF is its ability to be directly mitogenic for BM cells and thymocytes. This infers that the factor may not require target cells to have been previously activated in order to respond to BDEF signals. A question which arises from this postulate is how does one prevent BDEF from acting on all BM cells? The selectivity of BDEF action perhaps resides in either the limited range at which the factor can act within the BM compartment or to the factor's limited lifespan in vivo.

Although the above characteristics of BDEF seem unique, a direct comparison with some of the better characterized lymphokines is in order since this further highlights BDEF's novel nature. In spite of the fact that human IL-1 has been shown to be necessary and can augment Ig synthesis (Lipsky et al., 1983; Gerrard and Fauci, 1982), its MW of 17,000 Da distinguishes it from BDEF.

The much larger MW of BDEF, in conjunction with its inability to support the proliferation of the IL-2 dependent cell line CTLL-2 (Bains et al., 1986), clearly set it apart from IL-2. IL-3, on the other hand, has been shown to act as a multi-lineage colony stimulating factor (Rennick et al., 1985); however it has not been conclusively demonstrated that it is produced within the BM compartment. The fact that BDEF is directly mitogenic for thymocytes is also unlike IL-3 (Keller et al., 1985). IL-4, which has a MW of 15,000 Da, has been shown to augment the cellular proliferation of B cells, thymocytes and hemopoietic progenitor cells; however all of these effects occur in the presence of co-stimulatory signals (Yokota et al., 1988). In addition, the species restricted action of both IL-3 and IL-4 clearly distinguish them from BDEF.

The lack of sensitivity of BDEF to low pH (2.2) treatment would also argue that BDEF is not IFN-gamma. Furthermore, IFN-gamma is produced by mature T cells upon mitogenic stimulation (Morris, 1988), while BDEF originates from BM cells.

Although TRF/IL-5 does possess immunoenhancing activity, two features further distinguish it from BDEF. IL-5 on SDS-PAGE migrates to a MW of 25,000 to 30,000 Da (Takatsu et al., 1988). In addition, IL-5 is a late acting factor in the development of Ab responses, since it induces terminal differentiation of both resting and primed B cells (Takatsu et al., 1988). On the other hand, BDEF appears to be a monomeric protein of 60,000 Da, with no TRF/IL-5 activity, that acts during the initial 24 hours of culture.

IL-6 also has been shown to augment Ig secretion of S. aureus activated B cells (Sehgal et al., 1987), as well as to stimulate IL-2 production from T cells (Garman et al., 1987). In spite of this, its MW of 34,000 Da (Wong and Clark, 1988) clearly distinguish it from BDEF.

Another lymphokine known to have effects on both B and T cells is IL-7. Native IL-7 has a MW of 25,000 Da and has been shown to serve as a growth factor for early lymphoid cells of both B and T lineage (Henney, 1989). The proliferative effects of IL-7 on B cells seem restricted to precursor B cells ($B220^-$, cytoplasmic $IgM^-$) and to pre-B cells ($B220^+$, cytoplasmic $IgM^-$) (Henney, 1989). All available evidence indicates that IL-7 cannot induce differentiation of mature B cells. BDEF on the other hand, seems capable of promoting Ig synthesis from mature peripheral B cells. IL-7 has also been demonstrated to be directly mitogenic for $CD4^-$ $CD8^-$ T cells as well as activated mature T cells (Henney, 1989). Although some similarities may initially seem apparent between the biological

activities of BDEF and IL-7, upon closer scrutiny the combined effects of BDEF's mitogenic activity on thymocytes, where $CD4^-$ $CD8^-$ cells are a minority, and its ability to augment the Ig production of peripheral B cells argues for BDEF being a novel lymphokine unlike IL-7.

The discovery of BDEF further elucidates any difficulties in interpreting the initial demonstrations that BM cells could enhance Ab synthesis. The problems encountered with the latter observations were how to rule out any direct contribution of BM cells to the total Ab response that may overshadow any enhancing effects. This was initially argued on a quantitative basis, that is, the maximally enhanced Ab responses could not be fully accounted for by the individual responses of BM cells and tonsillar cells (see sections 2.3.6 and 2.4); indicative of an active enhancing effect. This evidence was indirect proof that enhancement of the Ab response had occurred. However, the fact that BDEF, a product of the same BM enhancing cell population, mediates a similar enhancement of the PFC response serves as a more direct proof for the ability of BM to augment Ab responses.

The finding that the enhancing activity of unfractionated BM is only evident at low ratios of BM to responding cells is indeed perplexing. To explain this phenomenon, one may speculate that the net immunoregulatory activity of BM is a result of the antagonistic signals between the resident enhancing activity (described in this chapter) and suppressive activity (described in chapters 2 and 3). However, in an abundance of unfractionated BM cells, the suppressive activity dominates, likely due to the increased potency of the suppressive over the enhancing activity. Furthermore, if the generation of the suppressive activity is assumed to require an interaction of BM NS cells with other BM cells to facilitate

BDSF production, this may explain the need for a relatively large number of BM cells in order to observe suppression. Alternatively, low doses of BM cells may provide an advantageous environment for the enhancing cell population and allow expression of its activity. The above hypothesis would predict that the enhancing activity is under less stringent regulatory controls, while the suppressor activity, due to its potent effects and potential for adverse reactions, should remain under more strict control.

Studies on the target of BDEF in the PFC response indicate that the cells residing in the E-rosette negative fraction of the responding population can bind BDEF. This would initially imply that B cells and macrophages may be the likely candidates. However, the observed mitogenic effect of BDEF on thymocytes must caution the above interpretation to include cells of the T lineage. T cells could potentially reside in the non-E-rosetting fraction of responding cells if they do not express the E-rosette receptor (CD2) or if the surface density of CD2 expressed on these cells was low, preventing them from rosetting efficiently.

In considering the target of BDEF, we must also take into account the findings of this laboratory demonstrating the action of BDEF on T-cell colony generation (Bains et al., 1986). This series of experiments demonstrated that the same factor could augment the rate of growth as well as the actual number of T-cell colonies that could be generated from human BM cells. These findings also support the hypothesis that BDEF affects cells of the T lineage. The role of BDEF within the BM, with respect to its action on T cells, may be reconciled by considering reports of limited amounts of T-cell development occurring outside of the thymus (Chervenak

et al., 1985; Dosch et al., 1985), and likely in the BM. BDEF may be one of the unknown factors being advocated to influence the expansion of these T-cell clones. This limited repertoire of T cells may provide the BM with a source of lymphokines and T helper function that would accelerate the generation of secondary Ab responses. This may be important since the BM has been shown to become a major source of Ig during secondary Ab responses (Benner et al., 1974a; Benner et al., 1974b; Benner et al., 1977).

The series of experiments in this thesis do not differentiate whether BDEF has one or multiple targets. It would not be surprising if the latter interpretation is correct, in view of the numerous lymphokines now being described to have multi lineage effects (Ihle et al., 1983; Garman et al., 1987; Dinarello, 1988).

The salient findings of this report are that a BM derived lymphokine is capable of augmenting Ig synthesis as well as of stimulating the proliferation of BM and thymus cells. The spectrum of action of BDEF is consistent with the pluripotent activities described for many lymphokines, especially those having effects on hematopoietic stem cells. However the mechanism by which BDEF eventuates its enhancement of Ab synthesis is not clear. One explanation that takes into account the broad spectrum of action of BDEF, postulates that the increased Ab response could simply be a result of the expansion of both T cells and B cells. This effect could in turn amplify any required interactions between all cells necessary to generate an optimal Ab response, such as Ag presentation, T-helper cell function and lymphokine production, leading to an elevated PFC response.

# Chapter 5

# CONCLUSION

## 5.1 The Physiologic Role of BDSF and BDEF

Direct investigations on BM functions have in general been hampered by this organ's complex nature and architecture. This in turn has biased investigations to the hematopoietic potential of BM, leaving the question of this organ's immunoregulatory functions unresolved. It was the aim of this thesis to directly address the latter issue.

The presented data suggest that two distinct immunoregulatory activities normally reside within the BM compartment. With regard to the natural suppressor activity of BM, one feature seems to stand out; NS activity appears to be restricted to tissues undergoing intense hematopoiesis. Organs like the BM and the neonatal spleen have an abundance of immature hematopoietic cells that are highly susceptible to tolerance induction. Although many arms of the immune system have been suspected of contributing to the maintenance of tolerance, the mechanisms responsible for the induction of tolerance remain poorly defined. That NS cells are capable of suppressing both B cell and T cell responses as well as facilitating allogeneic graft survival, make them ideal candidates for cells able to deliver tolerogenic signals to cells that have been recently activated by Ag.

In view of the evidence provided in this thesis for the ability of BDSF, a BM derived lymphokine, to regulate primary Ab responses and IL-1 synthesis, it is tempting to speculate that IL-1 is a key intermediate that may dictate whether BM is permissive for the development of an *in situ* primary Ab response. It is possible that the limited T-cell helper activity present within the BM may be of the TH2 type. Although such a phenotypic designation for T cells has so far not been described for the human system, as it has for the mouse, it does not preclude their

existence in humans. This assumption is a prerequisite in order to interpret those reports demonstrating that TH2 type cells are more likely to provide T-cell help for a primary Ab response than TH1 cells (Boom et al., 1987; Killar et al., 1988). When these observations are now considered in conjunction with those of Weaver et al. (1988) demonstrating the extreme dependency of TH2 type cells for membrane forms of IL-1 for their activation, an interesting picture arises. The preferred interpretation of this work is that BM may exclusively depend on TH2 cells for the generation of a primary Ab response. Therefore by restricting IL-1 synthesis one may ultimately determine whether T-cell help is provided for a primary Ab response.

Whether BDSF can perform its inhibitory function in vivo is not clear and remains a speculative issue. However, that IL-1 may be intimately associated with the mechanisms for tolerance induction is substantiated by the reports of Weigle et al. (1989) demonstrating that administration of IL-1 interferes with the in vivo induction of tolerance both at the B and T cell level. Therefore, the elucidation of BM regulatory mechanisms may prove useful in understanding how some forms of tolerance may be established in this organ.

The physiologic role of BM suppressor elements in vivo is not clear, however one may speculate that it is undesirable to allow B cells to fully differentiate within the BM compartment in response to Ag challenge. The occurrence of such an event would encompass adverse effects like the loss of a fraction of the protective B cell repertoire. This may occur as a result of exposure of immature B cells to Ag which in turn leads to the tolerance of these clones. Whether all Ags can gain access to the BM is presently not clear, but it is conceivable that the local concentration

of self Ags in the BM may be greater than that of foreign Ags. Under these conditions, B cells capable of responding to Ag and destined for transport to the periphery, could be expanded and thereby increase the frequency of autoreactive clones. However the suppressive action of BDSF may control the expansion of any B cells within the BM that have been activated by Ag stimulation, regardless of the nature of the Ag.

Regarding the immunoenhancing activity also mediated by BM cells, it is difficult at the present time to determine the physiologic relevance of this activity. Considering earlier reports from this laboratory (Bains et al., 1986; Saffran et al., 1986), BDEF, the enhancing mediator, may affect the proliferation and maturation of precursor T cells. This limited maturation of T cells outside of the thymus may represent a mechanism to generate receptors on precursor T cells for their eventual migration to the thymus, where T cell education is thought to occur.

Alternatively, the BM may have a need for a small number of functional T cells to facilitate the establishment of Ab production during secondary immune responses. BDEF may simply provide the stimulus necessary for the expansion of T cells with helper function to offset the disproportionate presence of CD8+ T cells within the BM.

The antagonistic action of the suppressive and enhancing activities of BM suggest that the two may act in concert to maintain homeostasis within the BM, at least at the level of primary Ab synthesis and possibly at the hematopoietic level by indirectly influencing the expansion or restriction of certain target cells.

## 5.2 Proposed Experimentation

This thesis has presented evidence for the identification and partial characterization of two novel regulatory activities of human BM. However, it raises a number of other questions and suggests some priorities that may further elucidate both the nature and the mechanism of action of both BDSF and BDEF.

In spite of the difficulties in positively enriching for BM cells responsible for the two activities, it is important that efforts be made to clone these cells. For the suppressor cells, by a series of enrichments, first by elutriation and then by cell sorting, using the established surface reactivities for the HNK-1 and SSEA-1 Ags, a relatively pure population of NS cells should be prepared for expansion in vitro, by culturing in media containing various colony stimulating factors. Alternatively, the same cells could be fused with an appropriate tumour partner, preferably of myeloid lineage, to form a hybridoma. The supernatants of these cultures could then be screened for BDSF activity. To this end, an Ab against BDSF could prove very useful for both screening BDSF secreting cell lines and to probe TLC plates for the lipid associated with BDSF activity. In view of the small molecular weight of BDSF, it would require the coupling BDSF onto a larger protein carrier. In efforts to understand more about the biochemical nature of BDSF, a series of labeled lipid precursors could be introduced into BM cultures to reveal the family of lipids related to BDSF. Since acetic acid is a precursor for many lipids, it would be more useful to use lipid precursors further along the biosynthetic pathway, like malonate or mavalonate, to further determine the group of lipids BDSF is associated with. In

addition mass spectroscopy could be used to determine its molecular weight.

To obtain more evidence that IL-1 synthesis is being inhibited by BDSF, it should be possible to demonstrate that the levels of mRNA specific for IL-1 correlate with the decreased levels of the lymphokine in the supernatants. This could be done by *in situ* hybridization initially on the U-937 cell line and then on cells from Ab cultures. To further demonstrate that BDSF is interfering with IL-1 signals, IL-1 analogs like forskolin, which have been shown to replace IL-1 cellular signals (Shiwakara *et al.*, 1988), could be used to restore a BDSF suppressed response.

Since Ab responses are dependent on numerous lymphokines that act in a cascade-like fashion, the effect of BDSF on other interleukins should be explored, in particular IL-6 and IL-2.

Regarding the enhancing factor, one of the priorities should be to derive an amino acid sequence of the protein. This would allow direct comparison with sequences of known proteins and therefore allow the placement of BDEF into a common family of proteins. In addition, the amino acid sequence would allow synthesis of a nucleotide probe that could be used to screen a BM gene library constructed for the eventual cloning of the gene encoding BDEF.

In view of the glycoprotein nature of BDEF, the role of carbohydrates in mediating the biological activity should be studied. This could be accomplished by treating BDEF with insoluble enzymes specific for different carbohydrate structures and then assessing whether the biological activity has been affected. This approach may also shed some light onto the nature of cell surface receptors for BDEF.

Finally, the effect of BDEF on different T cell precursor subsets should also be investigated. Since much is known about the series of events that occur in maturing T cells, with regard to the type of their surface receptors, it should be feasible to determine whether BDEF can affect rearrangement events for the gamma, delta, alpha or beta genes of the T-cell receptor.

In conclusion, this study has laid the foundation to the understanding of two novel regulatory functions of BM. Although these activities are restricted to this organ, their influence on cells destined to spend their life in the periphery make them important to warrant more in depth investigations of their function in immune regulation.

## APPENDIX 1

The formula used for calculating the "t" value to assess the level of significant difference between two sample means is as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s\sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

where: t = the value in the normal "t" distribution table associated with a probability level that two sample means are different.

$\bar{X}_1$ = sample mean of one set of observations

$\bar{X}_2$ = sample mean of a second set of observations

s = the weighted (pooled) standard of deviation of the sample differences between means (see below for formula)

$N_1$ = the number of observations in one experimental group

$N_2$ = the number of observations in the second experimental group

The weighted standard of deviation of the sample differences between means (s) is calculated by taking the square root of the samples variance ($s^2$):

$$s^2 = \frac{\Sigma (X-\bar{X}_1)^2 + \Sigma (X-\bar{X}_2)^2}{(N_1 - 1) + (N_2 - 1)}$$

where $s^2$ = weighted standard of variance of the sample differences of means

X = an individual sample observation

$\bar{X}_1$ = the sample mean of one set of observations

$\bar{X}_2$ = the sample mean of a second set of observations

$N_1$ = the number of observations in one experimental group

$N_2$ = the number of observations in the second experimental group

f = the degrees of freedom = the denominator of the above equation

## SAMPLE CALCULATION OF STATISTICAL SIGNIFICANCE

Raw data for Table 2.2:

Control response (PFC/Culture) of PBL: 801, 779, 959, 811, 727, 913.

$$\bar{X}_1 = 815 \pm 65$$

Response of cells receiving UNF BM cells: 367, 323, 403, 340, 396, 361.

$$\bar{X}_2 = 365 \pm 31$$

$$s^2 = \frac{(196+1296+1936+16+7744+9604) + (4+1764+1444+625+961+16)}{(6-1) + (6-1)}$$

$$= \frac{20792 + 4814}{10} = \frac{25666}{10} = 2566.6$$

$$\text{therefore } s = \sqrt{2566.2} = 160$$

$$\text{then} \quad t = \frac{815 - 365}{160 \quad 2+6}$$

$$= \frac{450}{160 \times 0.58} = 4.85$$

From the "t" distribution table, using 10 degrees of freedom and a one tailed test, the calculated t value (4.85) is greater than the value associated with a level of significance of $p < 0.005$, 3.169. Therefore we would reject the null hypothesis, that is that the two sample means are not different and accept the alternate hypothesis that they are statistically different at a confidence of $p < 0.005$.

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