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HEMATOPOIETIC REGULATION BY CATECHOLAMINES

THESE

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I dedicate this thesis to my mother Dolores, and to my father Sergio

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HEMATOPOIETIC REGULATION BY CATECHOLAMINES

presented by MAURO TOGNI

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ABBREVIATION

2-ME	2-mercaptoetanol
6-OHDA	6-hydroxydopamine
ADH	adherent cells
α-MEM	α-minimal essential medium
ARs	adrenergic receptors
AUC	area under the curve
BM	bone marrow
BMT	bone marrow transplantation
CBP	Carboplatin
CMI	cell-mediated immunity
CNS	central nervous system
Con-A	Concavalin A
CSF	colony stimulating factor
DA	dopamine
DESI	desipramine
DHPG	3,4-dihydroxyphenylglycol
DNA	desoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
E	epinephrine
FITC	fluorescein isothiocyanate
FMLP	N-formyl/methionyl/leucyl/phenylalanine
GEMM	Granulocytes Erythrocytes Macrophages Monocytes
GH	growth hormone
GM-CFU	Granulocytes/macrophages colony formation unit

HIV	human immunodeficiency virus
HS	horse serum
HVA	Homovanillic acid
i.p.	intraperitonealy
IFN-α	interferon alpha
IFN-β	interferon beta
IFN-γ	interferon gamma
IL	interleukin
INDO	indomethacine
L	leukocytes
LCM	lung conditioned medium
LPS	lipopolysaccaride
MACS	Magnetic Activated Cell Separation
MHC	major histocompatibility complex
MLR	Mixed-lymphocyte reaction
mRNA	messenger ribonucleic acid
n-ADH	non-adherent cells
NE	norepinephrine
NK	natural killer cells
P	Platelets
PBL	peripheral blood leukocytes
PBM	Peripheral Blood Mononuclear cells
PBS	Phosphate buffered saline
PCR	polymerase-chain reaction
PE	Phycoerythrine

PHA	Phyto Hemagglutinin
PHE	phentolamine-HCl
PKC	Protein Kinase C
PKC-I	Protein Kinase C inhibitor
PRA	Prazosin
PRO	propanolol
PT	pertussis toxin
RNA	ribonucleic acid
RT	Room Temperature
RT-PCR	reverse transcriptase polymerase-chain reaction
SCG	supracervical ganglia
SEA	soluble egg antigens
SP	substance P
SRBC	sheep red blood cells
TBI	total body irradiation
TCGF	T-cell growth factor
TD	thymus-dependent
TGF-β	transforming growth factor-β
Th1	T-helper cell type 1
TNF-α	tumor necrosis factor alpha
TNF-β	tumor necrosis factor beta
VIP	vasoactive intestinal peptide
VMA	vanillylmandelic acid
YO	yohimbine

I. INTRODUCTION

1. *NEUROIMMUNOMODULATION*

The term neuroimmunomodulation has been coined to define the interaction between the neural and neuroendocrine system and the immune system. For a long time the immune system was considered to be independent and self-regulatory even if the existence of a link between the nervous and the immune systems was already postulated early this century. In 1903 Sajous[1] suggested that the thymus gland functions as an endocrine organ, and that various thymic peptides may modulate lymphocyte function directly and/or influence the immune system via hormonal and neural pathways. Consistently, in 1910 Ott and Scott[2] reported that thymic extracts induced milk ejection in the goat, an action that is actually known to be mediated by the neurohypophysal peptide oxitocin. This was the first evidence that a non-nervous organ could have "nervous" function. Much later, in 1985,Roszman et al.[3,4] demonstrated that lesions in the central nervous system (CNS) could influence the immune system. In particular Roszman showed that lesions of the hypothalamus resulted in a decreased number of nucleated spleen cells and thymocytes. These two evidences strongly suggested the existence of a bidirectional, reciprocal communication between the immune systems and the central nervous system. Here, I will briefly review the current evidence in this rapidly growing field of research.

1.1 Neural and hormonal factors that affect hematopoiesis

Sympathetic system and catecholamines

In 1983 Besedovsky et al.[5], showed that CNS functions were affected during the immune response. This group measured a decrease of norepinephrine (NE) level in the hypothalamus 4 days after immunization with a T-dependent antigen, suggesting that activation of the immune system directly modulates the CNS activity. Another demonstration of a link between the nervous and the immune system may be found in the studies of Felten et al.[6] which showed the presence of a direct innervation of lymphoid tissues. Such innervation is dense as many fibers enter the lymphoid tissues, and bi-directional, because of the presence of various innervation types (noradrenergic, cholinergic, peptidergic) and of different origin (for example the mouse tibia receive fibers from two sources, sympathetic fibers from the femoral artery/vein and other fibers from the sciatic nerve[7,8]) suggesting the presence of both afferent and efferent nerve supply. Not only nerve fibers enter the lymphoid organs but it has been reported that lymphocytes and other blood cells present specific receptors for catecholamines.[9,10] This suggests that the innervation entering the bone marrow (BM) acts not

only on blood vessels but also on hematopoietic cells. Moreover it has been reported that also lymphoid cells produce catecholamines (their complete metabolic pathway was detected) and that catecholamines may affect the functions of such cells.[11,12] Taken together, these findings reveal the existence of a complex and finely tuned network between the immune and the nervous systems.

Neuropeptides

Among the peptidergic mechanisms which may affect the immune machinery, substance P (SP) seems to play an important role. SP is an 11 amino acid neuropeptide first identified in 1931 by its capacity to induce contraction of guinea pig ileum.[13] During the following decades, its role has been studied extensively. SP was described to be released from nerve ending and to elicit diverse biological activities (Table. 1). SP has been reported to influence lymphocyte and monocyte metabolism. First of all, SP was reported to stimulate chemotaxis of human and guinea pig monocytes *in vitro* with an EC_{50} value of approximately 0.1 pM.[14] The chemotactic effect of SP could be blocked by D-amino acid analogs of SP but not by antagonists of the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP), suggesting SP specificity. SP elicits not only mononuclear leukocyte chemotaxis but also generation of newly synthesized inflammatory mediators from macrophages activated by bacteria (i.e. nitric oxide).[15]

Action	Target tissue
Smooth muscle contraction	Intestines, pulmonary airways
Vasodilatation	Systemic arterioles
Vasoconstriction	Cerebral arteries
Increased microvascular permeability	Skin
Increased secretion	Salivary glands, tracheal epithelium, nasal epithelium

Table 1: Direct effect of SP on non-neural tissues

Several other macrophage responses are also evoked by SP, including the down-regulation of membrane-associated enzymes and the release of inflammatory products derived from the lipoxygenase and cyclooxygenase pathways.[15,16] Furthermore, SP can also enhance immune functions by regulating the production of cytokines from macrophages. Studies with human blood monocytes have shown that

nanomolar (nM) concentration of SP can stimulate the production of interleukin-1 (IL-1), tumor necrosis factor (TNF-α), and interleukin-6 (IL-6).[17,18]

As SP, other molecules that are normally related to nerve activation like vasoactive intestinal peptide (VIP, a 28 amino acids peptide) have been reported to modulate BMfunction. VIP was first isolated from intestinal extracts and identified as an hormone on the basis of its vasodilatatory effects.[19] Then it was recognized as a neurotransmitter and has been implicated in a great variety of biological processes as reported in table 2.[20,21]

Target/function	Effect of VIP
Monocytes	VIP modulate monocyte migration[22]
Mast cells	VIP stimulates histamine release in human skin mast cells[23]
lymphocytes *in vitro* *Mitogen responses*	VIP inhibits the response of PBM to mercuric chloride[24] VIP inhibits the response of murine lymphocytes to Con-A and soluble egg antigens (SEA)[25]
Mixed-lymphocyte reaction	VIP inhibits murine one-way MLR[26]
Immunoglobulin prodction	VIP inhibits response of PBM to pokeweed mitogen[27] VIP inhibits Ig production in Con-A stimulated murine lymphocyte cultures[28]
lymphokine production	VIP inhibits production of IL-2 in Con-A stimulated murine lymphocyte cultures[29]
LGL activity	VIP inhibits NK activity in PBM[30]
lymphocytes *in vivo*	VIP infusion inhibits the output of lymphocytes from sheep lymph nodes[31] Decreased expression of VIP receptor on murine T cells decreases the rate of migration of the cells into Peyer's patches and mesenteric nodes[32]

Table 2: Modulation of lymphoid cell functions by Vasoactive Intestinal Peptide: VIP: Vasoactive Intestinal Peptide, PBM: Peripheral Blood Mononuclear cells, NK: Natural Killer cells, MLR: Mixed lymphocytes Reaction

Melatonin

Already in 1973 Deguchi et al. reported that the pineal neurohormone melatonin is able to synchronizes the organism with the photoperiod.[33,34] In the following years, it has been reported that melatonin plays also an immunomodulatory role.[35] In particular it modulates the antibody response. This role is now well recognized.

It has been reported that T-helper cells bear G-protein coupled melatonin receptors and, perhaps, melatonin nuclear receptors.[36] In addition to the modulation of antibody production, activation of melatonin receptors enhances the release of T-helper cell type 1 (Th1) cytokines, such as IFN-γ and IL-2, as well as of novel opioid cytokines which crossreact immunologically with both interleukin-4 (IL-4) and dynorphin B.[37-39] Melatonin has also been reported to enhance the production of IL-6 from human monocytes. These mediators may counteract secondary immunodeficiencies, protect mice against lethal viral and bacterial diseases, synergize with IL-2 in cancer patients and influence hematopoiesis.[40] Hematopoiesis is apparently influenced by the action of the melatonin-induced opioids on kappa-opioid receptors present on stromal BMcells. Most interestingly, IFN-γ and CSF may modulate the production of melatonin in the pineal gland. A hypothetical pineal-immune-hematopoietic network is, therefore, taking shape. In conclusion, melatonin seems to be an important immunomodulatory hormone which deserves to be further studied, in order to identify its relevance in immune-based diseases, its therapeutic indications, and its adverse effects.

Other neural and hormonal factors

Several other neural or hormonal substances has been reported to affect hematopoiesis. Sexual hormones increases the immune reactivity in female; glucocorticoids and growth hormone are fundamental in thymus and T cell development. Enkephalins and Endorphins can either stimulate or suppress immune function depending on the doses.

After this short overview of the different effects of neural and hormonal substances on the immune-hematopoietic system one can clearly appreciate the complexity of the network between these two systems. Moreover it can be inferred that only a fine regulation of the interaction between these systems may result in a correct functionality of both systems. Therefore, lack of a basic neural, hormonal or immune factors might cause or be associated to disease states. In addition, a wrong balance between neural, hormonal and immune factors may be involved in a series of immuno-based or hematopoietic diseases. The relevance of a complete understanding of this complex network seems therefore evident. This might allows therapeutic approaches based on the balancing or replacement of two or more endogenous substances and not (as it appears today) by “inserting” exogenous and foreign substances, with the risk of adverse secondary effects.

1.2 *Cytokines*

The old adage “don’t judge a book by its cover” is certainly true when applied to cytokines. Although cytokines were originally defined as host defense proteins, they clearly have many other functions. Historically, humoral factors which regulate lymphocytes and were contained in the supernatant of mitogen- or allogeneic cell-stimulated lymphocytes were called lymphokines. On the other side factors of the same nature but regulating monocytes and macrophages were called monokines. The specific name of such substances was composed by the name of the target cell followed by the biological activity of the factor. For example a factor that stimulates T-cell proliferation would be named T-cell growth factor (TCGF). The discovery of tumour-cell lines on one side, and of lymphokine-dependent-cell lines on the other side, provided a method for obtaining cytokines in great amount, and in the same time a good method for testing their activities. With these methods it became clear that differently named lymphokines were just representing different activities of the same factor. Later a standardized nomenclature was developed, and the factors were called “Interleukines”, for their activity of signaling between leukocytes. Then cytokines was used in general to define both lymphokines and monokines. The first interleukin, IL-1, was shown to be responsible of the activity of at least eight of the previously reported factors. More recently, recombinant DNA techniques, allowed the purification and identification of the cytokines which are listed in table 3. In this table a series of cytokines, called colony stimulating factors (CSF), is skipped. CSF are a family of acidic glycoproteins, named after their ability to stimulate growth and differentiation of distinct hematopoietic cell lineages. Four distinct CSF have been identified: multilineage CSF (multi CSF), that correspond to IL-3; granulocyte-macrophage CSF (GM-CSF); macrophage CSF (M-CSF); granulocyte CSF (G-CSF). As suggested by the name, these substances stimulate the growth of specific lineages, and, with interleukins, participate in the balancing of the hematopoietic system. As neural and hormonal factors, it seems clear that, cytokines have effects not only on the immune system, but also on other systems (tab 3). For example, IL-1 acts on the hypothalamus, IL-11 on hepatocytes, or IL-15 on the intestinal epithelium. Moreover, it seems clear that it is not the production or secretion of one of these molecules that regulates a given function, but that it is the balance between two or more of these molecules that matters.

In the last years, some evidence of the modulation of production of interleukines by neural factors has been reported. Huang et al. stimulated IL-6 production in lymphocytes by injecting rat with IL-1. They then demonstrated that NE in the dose range of 10^{-6}-10^{-4} M increased IL-6 levels in the supernatant of spleen lymphocytes obtained from rats treated with NE. In addition NE at doses 10^{-9}-10^{-7} M enhanced the effects of IL-1 on IL-6 release by spleen lymphocytes. This seems an important point to understand another level of the interaction between the nervous and immune system. In most cases, substances that act on one of the two

systems have a specific effect at one concentration and the opposite effect at other concentration (higher or lower). This is the case, for example, in the growth-factor-based treatment during chemoterapy. In fact the properties of such substances are responsible for the serious negative side effects.[41]

This again suggests that what is needed for a correct functioning of both the nervous and immune system is a fine regulation of their interaction. This interaction is further complicated by the complexity of the immune system which may be divided as follows:

- T and B lymphocytes
- Accessory non-lymphoid cells (epithelial cells, dendritic cells, macrophages and so forth)
- Hormones, released at remote site and entering the immune micromilieu via the blood
- Cholinergic, adrenergic, peptidergic and other neurons
- biologically active substances, such as Interleukins, neuropeptides

The fine interaction and interdependence of these components results also in the possibility for a component to counterbalance, at least in part, the possible lack of other components.

Cytokine	Secreted by	Major biological function	
		Target cells / tissue	Activity
Interleukin-1 (IL-1α, IL-1β)	Monocytes, macrophages, B cells, dendritic cells and other	T_H cells B cells NK cells Vascular endothelial cells Macrophages and neutrophiles Hepatocytes Hypothalamus	Co-stimulates activation Promotes maturation and clonal expansion Enhances activity Increases expression of ICAMs Chemotactic activity Induces synthesis of acute-phase proteins Induces fever
Interleukin-2 (IL-2)	T_H1 cells	Antigen-primed T_H and T_C cells Antigen-specific T-cell clones NK cells (some) and T_C cells	Induces proliferation Supports long term growth Enhances activity
Interleukin-3 (IL-3)	TH cells, NK cells, and mast cells	Hematopoietic cells Mast cells	Supports growth and differentiation Stimulates growth and histamine secretion
Interleukin-4 (IL-4)	T_H2 cells, mast cells, NK cells	Antigen-primed B cells Activated B cells Resting B cells Thymocytes and T cells Macrophages Mast cells	Co-stimulates activation Stimulates proliferation and differentiation; induces class switch to IgG1 and IgE Up-regulates class II MHC expression Induces proliferation Up-regulates class II MHC expression; increases phagocytic activity Stimulates growth
Interleukin-5 (IL-5)	T_H2 cells, mast cells	Activated B cells Eosinophils	Stimulates proliferation and differentiation; induces class switch to IgA Promotes growth and differentiation

Cytokine	Secreted by	Major biological function	
		Target cells / tissue	Activity
Interleukin-6 (IL-6)	Monocytes, macrophages, T_H2 cells, bone - marrow stromal cells	Proliferating B cells Plasma cells Myeloid stem cells Hepatocytes	Promotes terminal differentiation into plasma cells Stimulates antibody secretion Help in differentiation promotion Induces synthesis of acute-phase proteins
Interleukin-7 (IL-7)	Bone-marrow, thymic stromal cells	lymphoid stem cells Resting T cells	Induces differentiation into progenitor B and T cells Increases expression of IL-2 and its receptor
Interleukin-8 (IL-8)	Macrophages, endothelial cells	Neutrophils	Chemokine; chemotactic activity; induces adherence to vascular endothelium and extravasation into tissue
Interleukin-9 (IL-9)	T_H cells	Some T_H cells	Acts as mitogen, supporting proliferation in absence of antigen
Interleukin-10 (IL-10)	T_H2 cells	Macrophages Antigen-presenting cells	Suppresses cytokine production and thus indirectly reduces cytokine production by T_H1 cells Down-regulates class II MHC expression
Interleukin-11 (IL-11)	Bone-marrow stromal cells	Plasmocytomas Progenitor B cells Megakaryocytes Hepatocytes	Supports growth Promotes differentiation Promotes differentiation Induces synthesis of acute-phase proteins
Interleukin-12 (IL-12)	Macrophages, B cells	Activated T_C cells NK and LAK cells and activated T_H1 cells	Acts synergistically with IL-2 to induces differentiation into CTLs Stimulates proliferation
Interleukin-13 (IL-13)	T_H cells	Macrophages	Inhibits activation and release of inflammatory cytokines; important regulator of inflammatory response
Interleukin-15 (IL-15)	T cells	T cells, intestinal epithelium NK Activated B cells	Stimulates growth of intestinal epithelium, T cell proliferation Support proliferation Co-mitogen for proliferation and differentiation
Interleukin-16 (IL-16)	T cells (primarily $CD8^+$) Eosinophils	$CD4^+$ T cells Monocytes Eosinophils	Chemotaxis; induces expression of class II MHC; induces synthesis of cytokines; suppresses antigen-induced proliferation Chemotaxis; induces class II MHC Chemotaxis; induces cell adhesion
Interferon alpha (IFN-α)	Leukocytes	Uninfected cells	Inhibits viral replication
Interferon beta (IFN-β)	Fibroblast	Uninfected cells	Inhibits viral replication

		Major biological function	
Cytokine	Secreted by	Target cells / tissue	Activity
Interferon gamma (IFN-γ)	T_H1, Tc, NK cells	Uninfected cells, Macrophages Many cell types Proliferating B cells	Inhibits viral replication Enhances activity Increases expression of class I and class II MHC molecules Induces class switch to IgG2a; blocks IL-4-induced class switch to IgE and IgG1
Transforming growth factor β (TGF-β)	Platelets, macrophages, lymphocytes, mast cells	Monocytes and macrophages Activated macrophages Epithelial, endothelial, lymphoid, and hematopoietic cells Proliferating B cells	Chemotactically attracts Induces increased IL-1 production Inhibits proliferation, thus limiting inflammatory response and promoting wound healing Induces class switch to IgA
Tumor necrosis factor α (TNF-α)	Macrophages, mast cells	Tumor cells Inflammatory cells	Has cytotoxic effect Induces cytokine secretion and is responsible for extensive weight loss (cachexia) associated with chronic inflammation
Tumor necrosis factor β (TNF-β)	T_H1 and Tc cells	Tumor cells Macrophages and neutrophils	Has cytotoxic and other effects similar to TNF-α Enhances phagocytic activity

Table 3: Cytokines which are involved in the complex network regulating the development of cellular and humoral immune response, induction of the inflammatory response, hematopoiesis and cellular proliferation and differentiation. Cytokines act not only on hematopoietic cells, but also on hepatocytes, vascular endothelial cells, and last but not least on cells of the nervous system.

1.3 *HEMATOPOIESIS*

1.3.1 lymphoid organs

In 1868, Ernst Neuman recognized that cells of the immune system and blood cells require continuous replenishment during postnatal life. Neuman also recognized that this process occurred within the primary lymphoid organs (BM and thymus).

a) Bones are composed of cortex and medulla. The cortex is a strong layer of compact bone; the medulla is a honeycomb of spongy bone, the interstices of which are known as the medullary cavity and contain the marrow. BM is either red marrow containing hematopoietic cells or yellow marrow which is largely adipose tissue. The distribution of hematopoietic marrow is dependent on age, decreasing with age. Moreover, in response to demand, the volume of the marrow cavity occupied by hematopoietic tissue expands.

b) On the other side, the thymus is an organ situated in the superior mediastinum, anterior to the great vessels as they emerge from the heart. It consist of two lobes, arising in the embryo as separate primordia on each side of the midline, but later becoming closely joined by connective tissue. The thymus attains its greatest relative weight at the end of fetal live, but its absolute weight continues to increase till the puberty. It then begins to undergo an involution which progresses rapidly until the organ becomes largely replaced by adipose cells in the adult.

Cells of the immune system are organized in tissues that are called lymphoid system and contain less or more mature lymphocytes.

Lymphopoiesis take place almost entirely in primary lymphoid organs, where stem cell grow and differentiate into specialized lymphocyte. Only T lymphocyte escape from BM to thymus for differentiating. In bird B cells differentiate in a specialized organ call bursa of Fabricius. Moreover, in primary lymphoid organs lymphocytes acquire the capacity for distinguishing within self and non-self antigens.

After leaving these organs, cells circulate in the blood until they reach one of the secondary lymphoid organs, such as lymph nodes, spleen and tonsils. They then exit the bloodstream through specialized blood vessels called high endothelial venules. Although the lymphocytes become rather tightly packed (each gram of lymph node contains a billion of them), they can still move about freely. Consequently, the nodes are excellent sites for lymphocytes to become activated by antigen and antigen-presenting cells entering through the afferent lymphatic vessel.

All the lymphoid organs and tissues collaborate directly or indirectly to maintain the blood composition, and to build up the imunological defense.

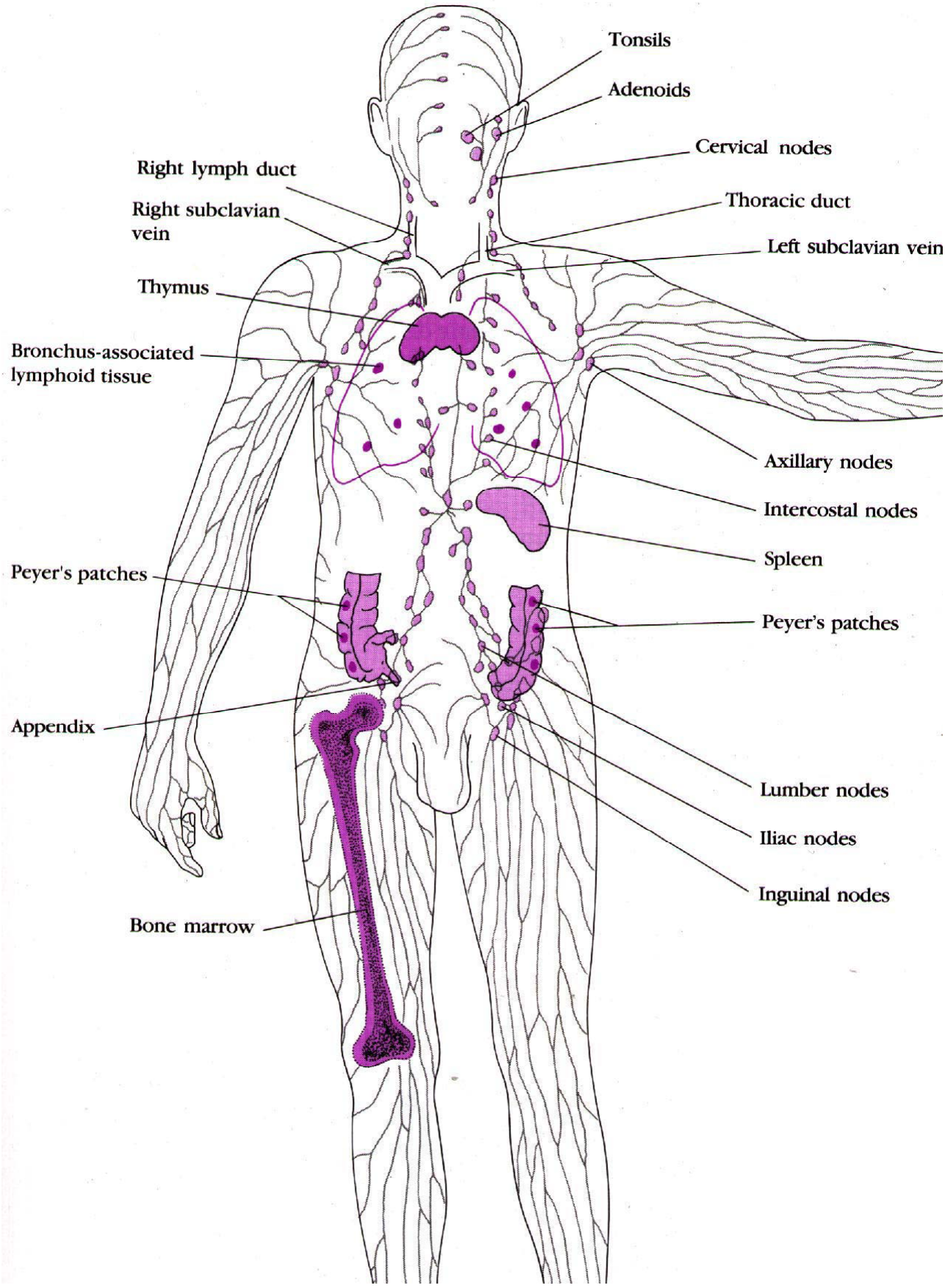


Figure 1: Disposition in the body of primary and secondary lymphoid organs. (from IMMUNOLOGY, second edition, Janis Kuby, Freeman eds)**1.3.2 Bone marrow cells**

Only a small population of stem cells is needed to generate large and different populations of maturing cells. This remarkable phenomenon takes place in the BM. In fact, pluripotent (or multipotent) stem cells are capable of regenerating themselves (self renewal) and, secondly, of generating all types of hematopoietic cells. This process is necessary to maintain the steady state in which the production of mature blood cells equals their loss (principally by cell aging). For example the average erythrocyte has a life span of 120 days before it is phagocytosed and digested by macrophages in the spleen. The various white blood cells have a life span ranging from days for neutrophils to as long as 20-30 years for some T lymphocytes. To maintain a steady-state level, the average human must produce an estimated 3.7×10^{11} cells per day. To better understand this mechanism it is important to define the different components of the primary lymphoid organs (BM and thymus) in which hematopoiesis takes place.

1.3.2.1 Stroma cells

The hematopoietic cells of the BM are embedded in a connective tissue stroma which is composed of fat cells and a meshwork of blood vessel, branching fibroblasts, macrophages, some myelinated and non-myelinated nerve fibers and a small amount of reticulin. Stromal cells are comprised of reticular cells, which include two cell types of different origin: phagocytic reticulum cells (macrophages that originate from a hematopoietic progenitor) and non-phagocytic reticulum cells (closely related to fibroblasts, adventitial cells and probably also osteoblasts and chondrocytes). These cells and their products (humoral factors) constitute the microenvironment, and there is a close interaction between hematopoietic cells and their microenvironment, because each one can modify the other.

1.3.2.2 Multipotent myeloid stem cells

Multipotent myeloid stem cells give rise to all types of myeloid cells: erythrocytes, granulocytes; macrophages, monocytes; mast cells; megakaryocytes and all their precursors. It should be mentioned that the term 'myeloid' can be used with two different meanings. It is used to indicate all cells derived from the common myeloid stem cells and also to indicate only the granulocytic and monocytic lineage as in the expression 'myeloid:erythroid ratio'. It is usually evident from the context which sense is intended but it is important to avoid ambiguity in using this term. The various myeloid lineages differ both morphologically and

in their disposition in the BM. Multipotent myeloid stem cells do not give rise directly to mature cells. For a macrophage, for example, the first step will conduct to a cell that is still capable of differentiating into either a macrophage or a granulocyte (GM-CFU, see figure 2). In a second step, the cell will be definitively committed to become a macrophage (M-CFU), and can no more differentiate into other lineages such as granulocyte, erythrocyte or lymphoid cell. But before a mature macrophage is formed, at least three more steps are necessary. In fact, this M-CFU will first become a monoblast, then a promonocyte, a monocyte and finally a macrophage. These different steps are characterized by the presence of either common cluster of differentiation (CD) at the surface of the cell, for example CD33, or of specific CD (like CD15). CD33 probably functions as cell adhesion molecule and is present from the earlier step of differentiation (CFU-GEMM) till the stage of monocyte, whereas CD15 mediates phagocytosis and chemotaxis, and is present only at the stage of monoblast (see figure 2).

1.3.2.3 Multipotent lymphoid stem cells

As for myeloid stem cells, lymphoid cells also have a common precursor called lymphoid stem cell. Moreover B and T lymphocytes share a common origin with myeloid cells, all of these lineage being derived from a multipotent stem cell. The lymphoid stem cells can differentiate into B lineage (if it stays in the marrow in mammalian, or if it migrates to the bursa of Fabricius in bird) or into T lineage (if it migrates to the thymus). Once more, it does not directly differentiate in mature cells, but undergoes different stages of maturation. In contrast to myeloid cells, that differentiate in the BM and then act in the blood, B and T migrate through three organs. B cells differentiate in the BM, circulate in the blood, but their major concentration is found in the lymph nodes. T cells migrate from BM to thymus where they differentiate and then circulate in the blood and also populate the lymph nodes.

1.3.2.4 Differentiation

Cell differentiation is accomplished via a complex interaction between the different components of the marrow (cells and humoral factors). In certain cases, the first step of differentiation consist in migrating to the specific organ (i.e. thymus for T cells). This migration, as other steps of differentiation, depends on the microenvironment composition. Normally, precursors cells follow a normal pathway of cell cycle (G1/S/G2/M) and reproduce themselves (this self-renewing can also be stimulated, i.e. in the presence of IL-7, pre-B cell

increase the self-renewing rate). Only when the environment contains the right factors, cells begin to differentiate into specific lineages (i.e. the presence of IL-4 will stimulate pre-B cell to mature into B cell). To appreciate the complexity of the differentiation process, we will describe some of the steps that leads to mature B cell. The first step in the development of the stem cell into mature IgM^+IgD^+ B cells is antigen-independent and occurs primarily in the BM. IL-7 is a potent growth factor for B cell progenitors, but it does not appear to act on mature B cells. In vivo, infusion of IL-7 into mice increases the number of lymphoid cells in spleen and lymph nodes and the number of marrow pre-B cells.[42] In contrast, IL-4 induces proliferation of pro-B cells and their differentiation into early pre-B cells and then into mature IgM^+ B cells.[43] However, infusion of anti-IL-4 neutralizing antibodies did not result in an alteration of B cell numbers in lymphoid organs, whereas such treatment profoundly inhibits the increase of serum IgE levels following parasite infection or anti IgD production.[44]

The second step, from mature resting B cell to plasma cells and memory B cells, is antigen-dependent as well as T cell dependent and occurs mostly in secondary lymphoid organs. This is a complex process that requires antigens and the collaboration between B cells, T cells and antigen presenting cells. This process can also be subdivided into two stages: (i) First, B cells specifically bind the thymus-dependent (TD) antigen, by means of their surface Ig receptors. The antigen is then internalized, processed and re-expressed in association with major histocompatibility complex (MHC) class II determinants and finally presented to the T helper (Th) cell. (ii) The second stage permits expansion and differentiation of the antigen specific B cell clones and is essentially under the control of T cell-derived soluble factors.

By this complex mechanism a relatively small cell number can fight a large number of antigens. On the other side, by renewing a small number of progenitor cells one can obtain, in short time, a great number of specialized cells. In fact, the marrow is composed of lymphoid and myeloid cells at different stage of maturation, and when one special population is needed, it will be produced not from the stem cell, but from the closest precursor. Only in a second time the precursors will be substituted by maturation of more undifferentiated cells or by self-renewal. A control of such a system can be achieved only through a large number of soluble factors (interleukines, hormones, neural factors) coupled with cell interaction.

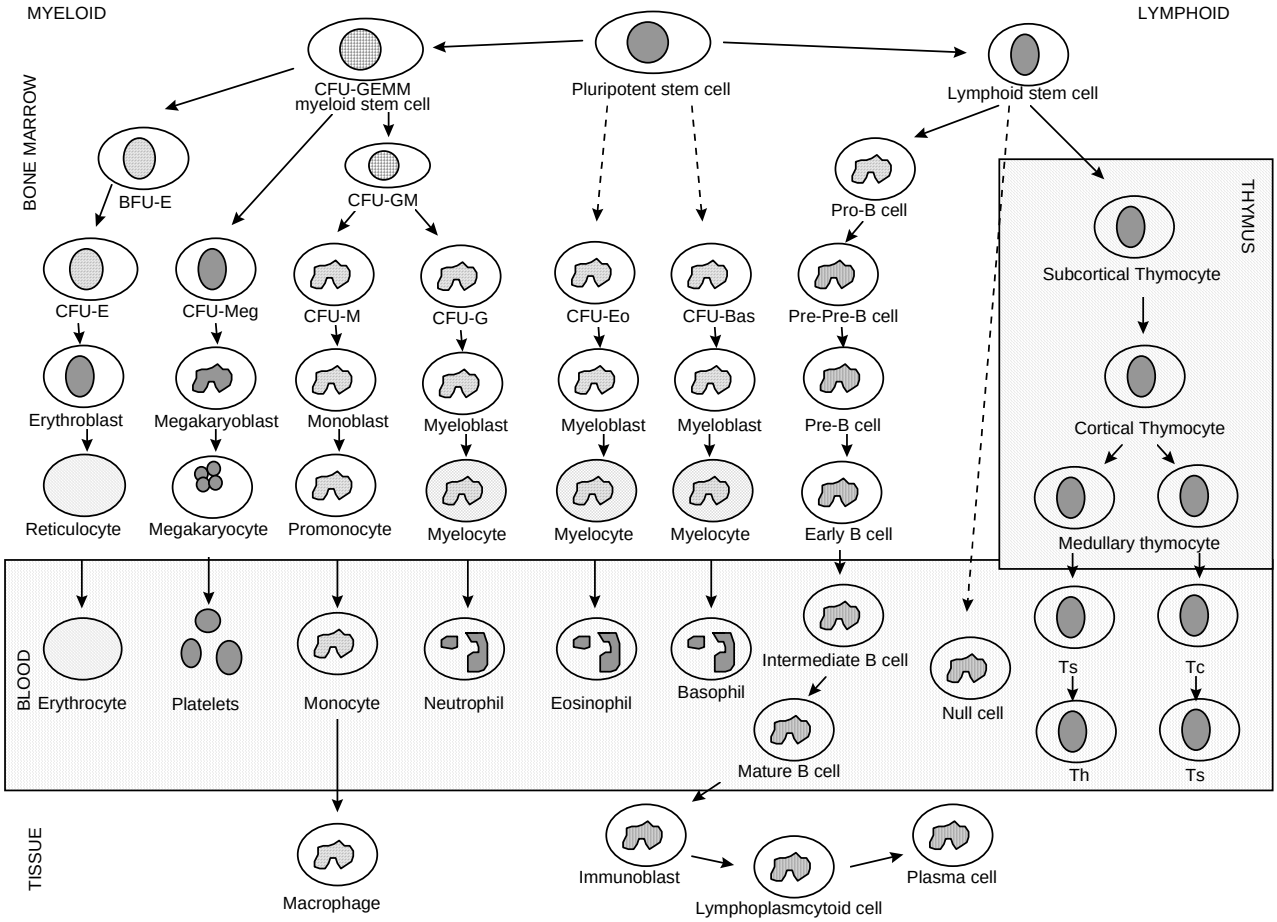


Figure 2: Principal step in differentiation of hematopoietic stem cells to mature blood cells and site of maturation

2 Neural regulation of hematopoiesis

The multiplicity of hematopoietic regulators reflects the need for a subtle physiological control of the complex cell population at any given situations. This poses several problems in our understanding of hematopoiesis, and it poses even more problems from the clinical point of view because of the need to evaluate thousand of combinations of the different hematopoietic regulators. Single hematopoietic regulators are already used to counteract the BM toxicity of cancer chemotherapy compounds or to enhance hematopoietic regeneration after bone marrow transplantation (BMT). However, such procedures remain problematic because of negative side effects and high costs.[41] An endogenous modulation of hematopoietic regulators presents substantial advantages over exogenous administration and circumvents the need for testing thousands of regulators combinations. Working on this line, Maestroni et al[35,45,46] studied whether melatonin, an hormone produced by the pineal gland, was involved in the hematopoietic regeneration in mice after lethal irradiation and syngenic BMT. Knowing that melatonin production is inhibited during the light phase of the day, mice were kept under permanent lighting (24 h light, L24) or were surgically pinealectomized to

investigate whether normal hematopoietic reconstitution was influenced by melatonin. Mice kept under permanent lighting showed decreased peripheral blood leukocytes (PBL) and platelets counts, but, surprisingly, this inhibition was also present in pinealectomized mice. Moreover, supplementation of melatonin in L24 exposed mice did not reverse the negative effect of L24.

This demonstrated that melatonin was not involved in the effect of permanent lighting. It was then hypothesized that the effects observed depend on a neural and not on an hormonal mechanism. The rational of such a hypothesis was the following: beside the main optical system, light activates the accessory optical tract which includes the suprachiasmatic nucleus and the supracervical ganglia (SCG).[47] From the SCG, efferent sympathetic fibers directly innervate the pineal gland and the thymus, other nerve fibers send projections down to the spinal cord, and consequently might reach the BM via paravertebral and prevertebral ganglia.[47,48] Because part of this network regulates melatonin synthesis in pineal gland via release of NE, it was reasonable to think that also in the BM, inhibition of hematopoietic reconstitution after BMT could be mediated by a modulation of NE release from sympathetic terminals in the BM. Maestroni et al.[49] demonstrated that treatment of mice with 6-hydroxydopamine (6-OHDA), a substance that induces a profound, although temporary, NE depletion,[50] counteract the effect of constant lighting after BMT. On the other hand the α1-adrenergic (α1-AR) antagonist prazosin (PRA), but not the β-bloker propanolol (PRO), mimicked and extended the effects of 6-OHDA, also inducing a rapid and significant increase of platelets, marrow GM-CFU, and nucleated spleen cells. Differential count of white blood cells and histological analysis of spleens from PRA-treated mice confirmed that myelopoiesis was greatly enhanced.[49] When PRO was administered in combination with PRA, however, the increase of platelets disappeared, suggesting that this part of the effect was under β-ARs control. Adrenergic agonists and antagonists were effective not only after BMT, but also in normal mice. Consistently, NE and/or adrenergic agonists could inhibit growth of GM-CFU *in vitro*.

3 Adrenergic Receptors

Adrenergic receptors belong to the seven-chains-transmembrane spanning family of receptors. These are normally coupled to a G protein and they respond to the physiological agonists NE or E (see table 4). Adrenoceptors are divided into two types, alpha and beta adrenoceptors. These can be subdivided into many subtypes. The alpha type are subdivided in alpha 1 (which is subdivided in alpha 1A, alpha 1B and alpha 1D) and alpha 2 (which is subdivided in alpha 2A, alpha 2B and alpha 2C). The beta adrenoceptors have been

classified into beta 1, beta 2, beta 3, and beta 4 subgroups. Although these receptors are hystorically related to nervous cells for the propagation of the nervous signal from one neuron to another, or to organs (i.e. muscle), in the last two decades evidence has been produced that these receptor are expressed by a wide number of cell types, and mediate many cell function. In the hematological field, already in 1984 Titinchi et al.[51] reported the presence of adrenoceptors (α2) on human lymphocytes. In the following years many studies reported the presence of different adrenoceptors in different cell types. For example, in 1985 Hellstrand et al.[52] reported that natural killer (NK) activity was increased by E when cells were preincubated with E, while the NK activity was inhibited when E was added directly in the lymphocytes/target mixture. In 1994, Bergquist et al.[11] reported the presence of endogenous catecholamines and their metabolites in single lymphocytes and in extract of T- and B- cell clones. These authors also reported the existence of an uptake mechanism for catecholamines, resulting in a dose-dependent inhibition of lymphocyte proliferation. These results suggested a catecholaminergic regulation of lymphocyte function via an autocrine loop. In summary, Lymphoid cells produce, release and re-uptake catecholamines, and express different adrenergic receptors (AR). The effect of chatecholamines on lymphoid cells may be very different depending on cell type, doses of catecholamine and timing of treatement.

Receptor type	a_{1A}	a_{1B}	a_{1D}
Potency order	NE ≥ E	E = NE	E = NE
Transduction mechanism	activates $G_{p/q}$, ↑ PI turnover, ↑ $[Ca^{2+}]$, activates voltage-gated Ca^{2+} channels		
Receptor type	a2A	a2B	a2C
Potency order	E ≥ NE	E ≥ NE	E ≥ NE
Transduction mechanism	activates G i/o, inhibits adenylyl cyclase, ↓ cAMP, inhibits voltage-gated Ca^{2+} channels, activates Ca^{2+}-dependent K^+ channels		

Receptor type	β1	β2	β3	β4
Potency order	NE ≥ E	E > NE	NE > E	
Transduction mechanism	↑ adenylyl cyclase (via G_S)	↑ adenylyl cyclase (via G_S) ↑ /↓ adenylyl cyclase	↑ adenylyl cyclase ↓ adenylyl cyclase	↑ cAMP levels, ↑ of cAMP-dependent protein kinase (via G_S)

Table 4: Subdivision of adrenergic receptor, and normal transduction pathway

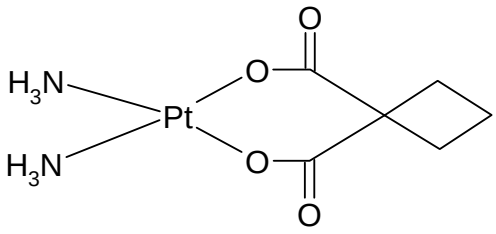
4 Aim of the present study

Maestroni et al., demonstrated that this inhibition is exerted on ARs present on BM cells.[53] Moreover, by functional and pharmacological studies, they showed the presence of two specific binding sites for ^{3}H-PRA which differed in their affinity. Competition studies characterized the high affinity site as an α1b-AR. The remaining site was of less clear characterization and the results obtained were compatible with low affinity α1-AR. Separation of BM cells by counterflow centrifugal elutriation resulted in separation of the two ARs, with the α1b-AR being partially eluted in lymphoid fraction containing no blasts and no assayable GM-CFU.[53] These findings led to the conclusion that hematopoiesis may be modulated via bone-marrow α1-ARs. It is well documented that BM sensitivity to cancer chemotherapeutic compounds is to a great extent related to the cell proliferation rate.[54] The purpose of this study was, therefore, to identify the cell types bearing the α1-ARs and to investigate the feasibility of an adrenergic modulation of the hematological toxicity of anti-cancer chemotherapy compounds or X-rays irradiation.

II. NORADRENERGIC PROTECTION OF BONE MARROW

1. INTRODUCTION

Carboplatin (CBP) is one of a series of platinum coordination complexes which was first reported to have antitumor activity in experimental animal tumors in 1969 by Rosenberg.[55]



$C_6H_{12}N_2O_4Pt$

Figure 3: Structure of CBP

The compound acts via hydratation of platin which is then free to bind with O^6 or N^7 of the DNA guanine. This reaction, with the formation of closed-ring chelate, results in interstrand and intrastrand DNA cross-linking and DNA-protein cross-linking. It is generally thought that the resulting inhibition of DNA synthesis is responsible for the antitumor effect of the compound. The interest of this molecule is that it is active on a wide range of tumors, if administered only once. This was important because the model of NE treatment needs a treatment as short as possible. Moreover CBP, in contrast with other chemotherapeutic substances, is toxic in the BM and in other tissue with high proliferation rate, probably because it acts only on “opened” DNA typical of proliferating cells

The major problem occurring after treatment with CBP is its myeloablative effect. The aim of this study was to find out whether NE could interact in any way with CBP. Because NE inhibits cells growth particularly of GM-CFU, the hypothesis was, that the inhibition results in not “opened” DNA and consequently in protection of the cells.

2 MATERIALS AND METHODS

2.1 Mice

Female 2-3 months old C57BL/6 inbred mice were purchased from Charles River Italia, Calco, Italy. The mice were kept under a 12 h light-dark cycle at 21 ± 1°C with free access to food and water.

2.2 Drugs

L-norepinephrine-HCl (NE), prazosin (PRA) and carboplatin (CBP) were purchased from Sigma Co., St. Louis, USA. Agar is purchased from DIFCO Laboratories, Detroit, USA.

2.3 In vivo experiments

C57BL/6 mice were either treated with one single, lethal intravenous, injection of CBP (200 mg/Kg body weight) or exposed to 300-900 cGy (X ray) TBI using a linear accelerator (15 MV energy equivalent). NE was injected subcutaneously 1 h and immediately before CBP, as well as 2 h and 4 h after CBP. In the irradiation experiments, NE was injected 4 h, 2 h and 30 min before X ray exposure. Control mice were injected with NE or Saline (Phosphate buffered saline, PBS) according to the schedule used in CBP experiments. When PRA was administered to CBP-treated mice, a single injection of 10 mg/kg body weight either alone or with NE was performed. The concentration of PBL (L), platelets (P) and marrow ganulocyte/macrophage colony-forming unit (GM-CFU) was determined 3 days after treatment using a standard method. Briefly peripheral blood was obtained by a little cut of the tail, and leukocytes were simply counted under a microscope with türk coloration (to eliminate erythrocytes). Platelets were counted after diluting in Plaxan (Erne AG, Dällikon, CH).

2.4 In vitro experiments

For GM-CFU experiments we used a modification of the method described by Brandley and Metcalf.[56] Mice were sacrificed by cervical dislocation and BM cells were collected by flushing out the marrow from the bones with a sterile syringe filled with α-minimal essential medium (α-MEM). Cells were washed once and 10^5 cells were resuspended in 1.5 ml 0.3% semisolid agar in α-MEM containing 20 % horse serum (HS), 10% of lung conditioned medium (LCM, as source of GM-CSF) and the different substances (NE, CBP, etc.). LCM was prepared by mincing the lungs from 2 months old C57BL/6 mice. The supernatant was collected after incubation at 37°C in the presence of 5% CO_2 in α-MEM, 20% HS for 3 days. Cultures were then incubated during 7 days at 37°C, in humidified air and 5% CO_2. Colonies containing more that 50 cells were evaluated as GM-CFU by phase contrast microscopy.

3 RESULTS

3.1 NE protects GM-CFU from the toxic effect of CBP

GM-CFU is a method used to evaluate the concentration of a population of precursor cells (GM-precursors) that is normally present at very low concentration in the BM (less than 1%). We therefore incubated BM cells as described in materials and methods in the presence or absence of NE. We found that NE inhibits the GM-CFU in a dose dependent manner. As it is shown in figure 4, NE has no effect until nM concentrations, then it exercises a dose-dependent inhibition, up to over 80% at mM concentration.

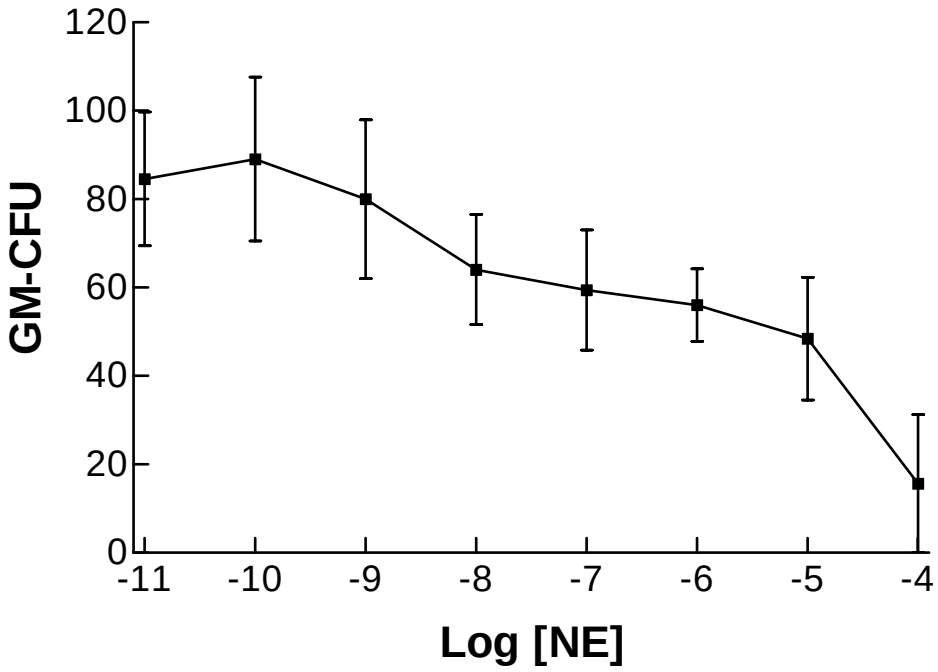


Figure 4: NE inhibition of GM-CFU of BM cells. NE was dissolved in α-MEM containing 20% HS and added at the reported concentrations in the cell suspension before plating. a: $p < 0.01$.

3.2 NE protects mice from lethal dose of CBP and from X-rays exposure

In a first series of experiments we treated mice with CBP with or without NE at different concentrations. Figure 5 shows that NE was highly effective at protecting mice from the toxic effect of CBP at 1, 2 and 3 mg / Kg body weight, while the NE effect decreased at 4 mg / Kg body weight ($p=0.007$) with a bell-shaped dose-response curve. In all cases the most effective dose of NE was 3 mg / kg body weight, and we used this dose for all the other experiments.

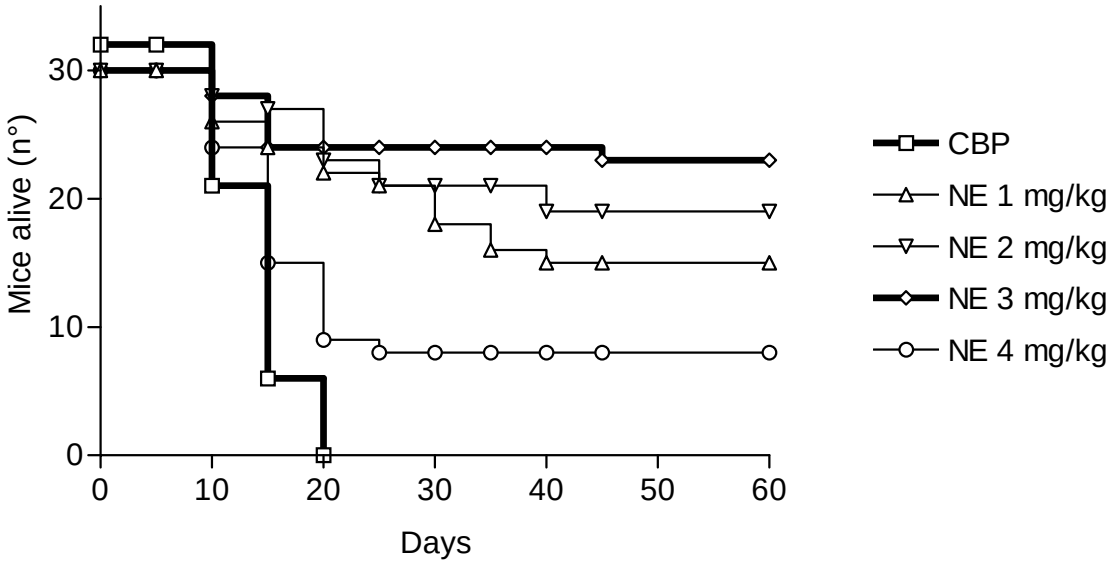


Figure 5: Survival of mice treated with CBP ± NE. The mice were injected with CBP and treated with NE according to the protocole described in materials and methods and then observed daily for survival.

To investigate whether the protective effects exerted by NE depended on a direct hematopoietic protection, we treated mice with NE, either before or after X ray irradiation or CBP injection. We measured the blood concentration of leukocytes (L), platelets (P) and GM-CFU in the BM. As expected, these parameters were increased in NE treated mice when compared with control mice. On the other hand, NE did not affect hematopoiesis in normal mice (not treated with CBP nor irradiated).

	NE			PBS		
	L / µl (x 10^3)	P / µl (x 10^3)	GM-CFU / femur	L / µl (x 10^3)	P / µl (x 10^3)	GM-CFU / femur
TBI 300 cGy	1.9 ± 0.11 [b]	26.5 ± 1.2 [b]	3634 ± 861 [b]	0.9 ± 0.2	18.1 ± 1.6	1659 ± 611
TBI 400 cGy	1.3 ± 0.15 [b]	23.1 ± 0.9 [b]	4680 ± 979 [b]	0.7 ± 0.1	17.3 ± 0.6	1749 ± 627
TBI 500 cGy	1.02 ± 0.2	19 ± 0.35	1029 ± 208 [c]	1.1 ± 0.3	17.8 ± 1.4	423 ± 249
CBP	5.8 ± 1.6 [a]	54.3 ± 21.9 [a]	3630 ± 1721 [a]	2.5 ± 0.7	26.1 ± 13	995 ± 535
CONTROL	**10.6 ± 2.1**	**232 ± 37**	**7695 ± 1356**	**9.6 ± 2.6**	**225 ± 27**	**8870 ± 1235**

Table 5: Protection of blood (leukocytes; L; and Platelets; P) and bone marrow (GM-CFU) parameters by NE (3 mg / kg body weight) in mice treated with CBP (200 mg/ kg body weight) or exposed to TBI. a: $p< 0.001$; b: $p< 0.005$; c: $p< 0.05$

In other experiments, lethally irradiated mice (900 cGy) were treated with NE, but in this case NE was not able to protect mice.

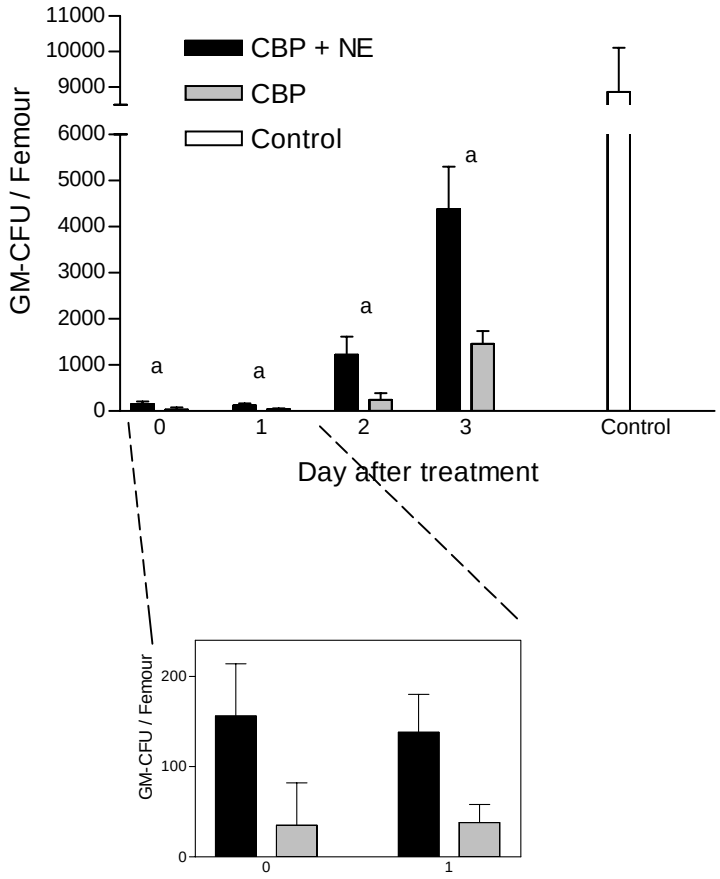


Figure 6: Time curse of the hematopoietic response to NE in mice treated with CBP. The mice were injected with CBP and treated with saline or NE (3 mg/kg). The number of GM-CFUs was evaluated after treatment at time reported. Day 0 means 1 hour after the last NE injection and 5 hours after CBP inoculation. The mean value ± the standard deviation of 10 mice per group is reported. a: $p < 0.001$

As shown in figure 6, in a time-curse study of hematopoietic response, NE was effective in protecting myeloid precursors as early as 5 hours after CBP administration and 1 hour after the final NE injection.

3.3 Prazosin abolished the effects of NE

To investigate whether NE protection was mediated by α1-adrenergic receptors (α1-ARs) we repeated the rescue experiments *in vivo* (figure 5) by the addition of the α1-adrenergic antagonist PRA. As expected, PRA neutralized 80% of the rescue effects in mice injected with CBP and treated with NE. Again, this suggests that NE may protect BM via α1-ARs.

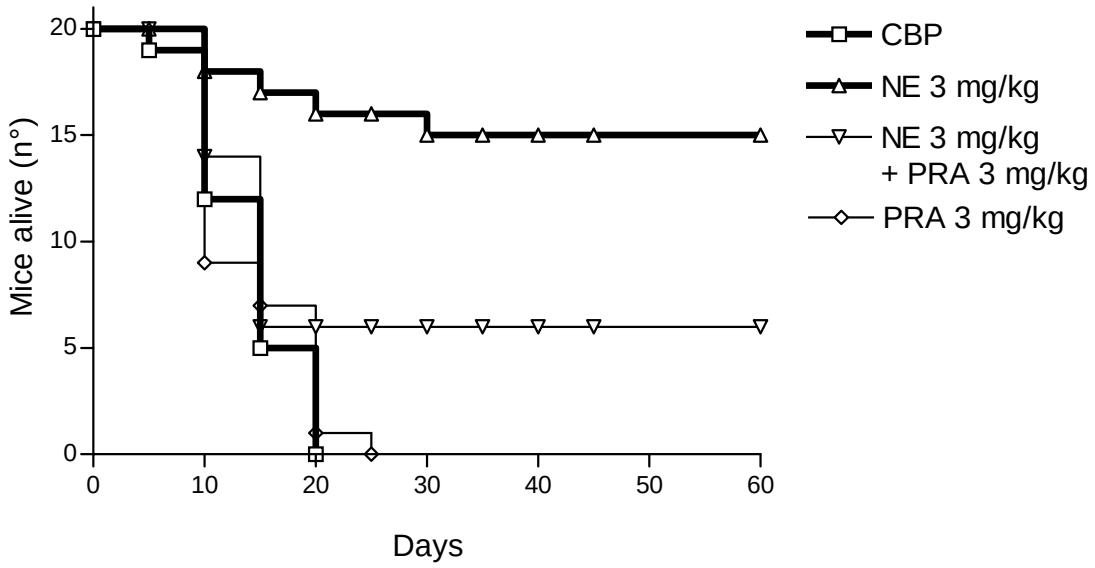


Figure 7: Effect of PRA on survival of mice treated with CBP and NE. All mice were injected with CBP.

3.4 NE protects bone marrow cells in vitro

The GM-CFU in the presence of CBP results in no colony formation, probably because the method involves a 6 day incubation of a small number of cells, and, under these conditions, CBP may kill all hematopoietic progenitors. For this reason, in our experiments $3x10^6$ cells/ml were pre-incubated for 7 h with the various substances (NE, CBP, etc.). After incubation the cells were washed trice and plated in a standard GM-CFU assay. Under these conditions, NE alone did not inhibit GM-CFU colonies (see figure 8). However, NE protected GM-precursors from the toxic effects of CBP. Moreover, when PRA was added, the NE-induced protection was completely abolished, suggesting that the protection was mediated by α1-ARs. In addition, these effects were very specific, with a bell-shaped dose response curve. In fact, low PRA concentrations (ca. 10^{-11} M) abolished 100% of the protective effect of NE. Higher or lower concentrations of PRA counteracted only partially the NE effects.

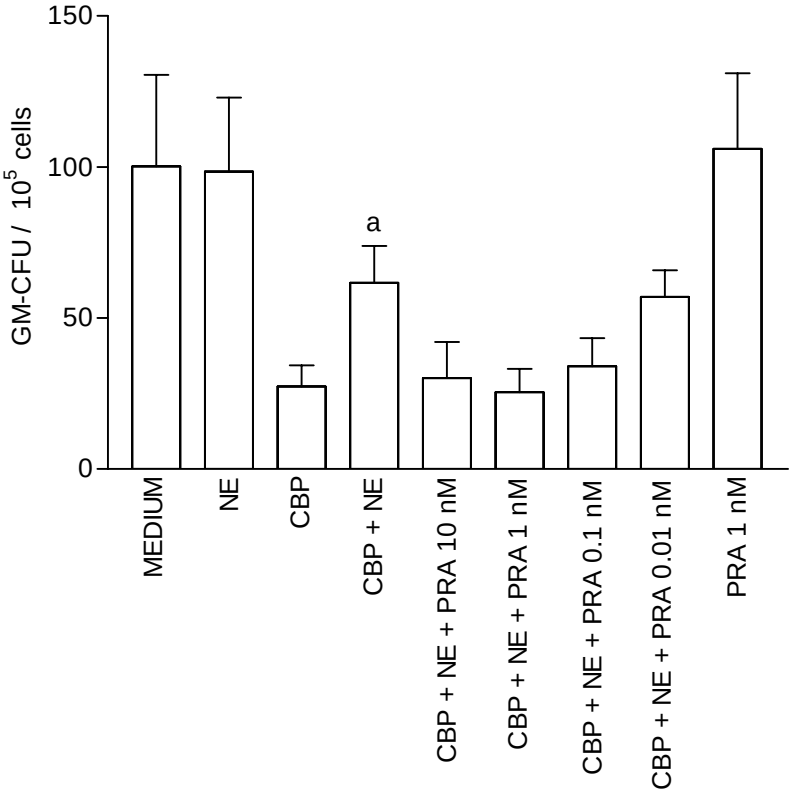


Figure 8: α1b-adrenergic receptor-mediated rescue of GM-CFU in CBP-treated BM cells. BM cells were incubated at 37°C for 7h with CBP (25 μM), in the presence or absence of NE and with the reported concentrations of the α1b-adrenergic receptor antagonist PRA. Control cultures were incubated with tissue culture medium (MEDIUM), PRA or NE alone. a: $p<0.01$

4 DISCUSSION

In this study we showed that NE may protect, either *in vivo* or *in vitro*, BM cells from the toxic effects of CBP or X-ray TBI. However NE does not protect lethally irradiated mice. The reason for this is unclear and needs further studies. We report a dose-response curve of NE *in vivo* that shows a decreased protection from 3 to 4 mg / kg body weight. This might be explained by a non-specific toxicity of high doses of NE, or by a real bell-shaped biological response. *In vivo*, the differences between mice treated with NE and PBS are reflected on blood parameters only three days after treatment. When blood parameters are evaluated just after the treatment no significant difference is apparent. However, on the bone marrow GM-CFU concentration the effect was already measurable after few hours. This suggests that NE protection was exerted on hematopoietic progenitors. This protection is probably mediated via specific α1-ARs because the specific α1-adrenergic antagonist PRA neutralized the effect. The fact that this is abolished only partially by PRA may be explained by the presence of other ARs on hematopoietic cells. This is consistent with the observation of Maestroni et al.[49,53] that addition of the β-adrenergic antagonist PRO counteracted PRA-induced enhancement of platelet formation. This suggested that a β-adrenergic dependent mechanism is involved in platelet generation. On the other hand, it has been previously shown that BM cells express two α1-adrenoceptors, with high and low affinity for ^{3}H-PRA respectively. Since only 1 nM PRA is required for neutralizing the NE effect *in vitro*, probably tehe receptor involved is the high affinity one (Kd = 1 nM). In conclusion our results suggest that NE participates actively in the regulation of hematopoiesis via specific ARs present on BM cells.

III. IDENTIFICATION OF BONE MARROW CELL POPULATION BEARING THE HIGH AFFINITY α1-ADRENERGIC RECEPTOR

1. INTRODUCTION

In the previous chapter we have demonstrated that the NE-induced modulation of hematopoiesis via α1-AR may result in rescue mechanism when mice are treated with lethal doses of CBP. Moreover, we showed that PRA counteracts at least part of this rescue, both *in vivo* (when PRA is injected *in vivo* to counteract the rescue effect of NE against CBP) and *in vitro* (when protection is measured on GM-CFU). The concentration of PRA needed to counteract such protection *in vitro*, suggested that this effect was mediated by the high affinity AR. For this reason, we were interested to know which cell type bears the α1b-AR. This is fundamental for understanding if the mechanism is mediated via the release of a soluble factor (cytokine) or if the AR bearing cells modulate directly BM cell proliferation.

2. MATERIALS AND METHODS

2.1 Drugs

L-norepinephrine (NE), prazosin (PRA), phentolamine-HCl (PHE) were purchased from Sigma (St. Louis, MO). ^{3}H-PRA is purchased from NEN Du Pont de Nemours, Regensdorf, Switzerland.

2.2 Antibodies

Rat anti B220 monoclonal antibody (IgG2a) was purchased from PharMingen, San Diego, USA. Rat anti-Mac1 monoclonal antibody (IgG2b) and FITC-conjugated mouse anti-rat-IgG2a or-IgG2b monoclonal antibody were purchased from Readysystem, AG, Bad Zurzach, Switzerland. FITC-conjugated goat anti-mouse IgM (μ-chain specific) was purchased from Sigma Immuno-chemicals, St. Louis, MO, USA. Goat anti-rat IgG antibody coupled to paramagnetic micro-beads was purchased from Miltenyi Biotec GmbH, Berghish Gladbach, Germany.

2.3 ^{3}H-Prazosin binding

BM cells were suspended at 2x10^{6} cell/ml in α-MEM with 1% HS in the presence of various concentrations (0.05 to 20 nM) of ^{3}H-PRA at room temperature for 1 hour. Non specific binding was assessed by addition of 100 μM PHE. The assay was terminated by filtration trough Titertek-TM glass fibre filters, and the activity was counted by liquid scintillation in Ultima Gold MW and with a β-counter type 1900 of Canberra Packard, Meriden, CT, USA.

2.4 Separation of bone marrow cells by adherence

BM cells obtained as described in the previous chapter, were suspended in α-MEM with 5% HS at concentration of 3.5x10^{6} cells/ml and incubated overnight (16-18 h) at 37°C in 5% CO_2 in tissue culture flask (Costar, Cambridge, MA, USA). After incubation, non-adherent cells (n-ADH) were obtained by harvesting the supernatant. Adherent and loosely-adherent cells (ADH) were harvested after addition of PBS with 5 mM EDTA for 10 min at room temperature. To optimize cell recovery, culture flasks were washed with PBS three time.

2.5 Magnetic cell sorting

The magnetic cell separator MACS, was purchased by Myltenyi Biotec GmbH, Bergish Gladbach, Germany. Adherent BM cells were suspended at 10^{7} cells/0.1 ml and incubated in PBS with 2% HS for 30 min at 4°C

with 30 μl rat-anti mouse Mac1 or B220 monoclonal antibody. After washing, cells were incubated again for 30min at 4°C with goat anti rat IgG conjugated with paramagnetic micro-beads. The secondary antibody was used only in the case of $Mac1^+$ antibodies, while anti-B220 primary antibodies was already coupled to magnetic micro-beads. Cells suspended at 10^7 /0.1 ml were incubated 30 min at 4°C with anti B220 micro-beads coupled antibody. After 3 washings, cells were loaded on to a steel-wool column where labeled and unlabeled cells were magnetically separated. Enrichment was obtained by eluting cells using an A2 column with a G24 needle for flow regulation. For depletion, the flow was regulated using a G27 needle. Efficiency of separation was evaluated by flow cytometry.

2.6 Long-term cultures of B-cell precursors

Amplification of B-cell precursors was obtained by a modification of a method previously described.[57] Briefly, $4x10^6$ BM/ml were incubated overnight in α-MEM, 10 μM 2-mercaptoetanol (2-ME) and 10% HS. The culture medium was then discarded with n-ADH and replaced with an equal amount of fresh α-MEM containing 10% fetal calf serum (FCS) and human recombinant interleukin 7 (IL-7, 500 U/ml) kindly provided by Sterling Winthrop Inc., Collegesville, PA, USA. Twice a week, half of the culture medium was replaced with fresh medium containing 1000 U IL-7/ml. By 28 days virtually all non-adherent and loosely adherent cells appeared to be $Mac1^-$, $sIgM^-$, $B220^+$ lymphocytes.

2.7 Flow cytometry

Depleted, enriched or cultured cell populations were washed trice and 10^6 cells/sample were incubated for 30 min at 4°C with saturating concentrations of anti Mac1, anti B220 or FITC-conjugated goat anti-mouse IgM. The fractions which were already $B220^+$ or $Mac1^+$ labeled, were then washed twice and incubated with FITC-conjugated mouse anti-rat-IgG. After further washing the cells were analyzed by flow cytometry (FACScan, Beckton Dickinson, Mountain View, CA, USA). 10^5 cells were analyzed per sample. Negative controls included unstained cells and cells stained with the secondary antibody only. The percentage of positively staining cells was calculated on a channel-by-channel subtraction of the control data (secondary antibody alone) from experimental data.

2.8 Incubation with anti-TGF-β

BM cells were processed as described in point 2.3 of chapter 2 for the “direct” GM-CFU. For an “indirect” GM-CFU, 3 x 10^6 cells / ml were suspended in α-MEM with 5% HS and incubated for 8h in triplicates in the

presence of CBP with or without NE and/or anti-mouse transforming growth factor-β (TGF-β) monoclonal antibody. After incubation at 37° C in 5% CO_2 in 24 wells tissue culture plates (Becton Dickinson, Oxnard, CA, U.S.A.), the cells were washed trice and processed for the GM-CFU assay.

2.9 Umbilical cord blood cells

10 ml of umbilical cord blood were diluted in 25 ml RPMI 1640 (BioConcept, Allschwill, CH) and loaded on 15 ml Ficoll 1.077. After 30 min centrifugation at 400 x g at 20°C, the cells on the interface between Ficoll and RPMI were harvested, washed three times and diluted at $2x10^6$ cells/ml for binding studies.

2.10 Cell line cultures

NALM-6 was kindly provided by Dr Seckinger, Centre d'immunologie INSERM-CNRS, Marseille, France. KM-3 was kindly provided by Prof. Trubiani, Istituto di Citomorfologia Normale e Patologica del CNR, Università di Chieti, Italy. 70Z/3 was purchased from ATCC. Cells were maintained at concentration between 0.1 and 1 x 10^6 cells / ml in RPMI 1640 with 10% FCS and, for 70Z/3, 5 mM 2-ME.

3 Results

3.1 Distribution of α1-adrenergic receptors in adherent vs. non-adherent cells

In previous works Maestroni and co-workers[49,53] demonstrated that the two ARs-bearing cell populations could be separated into two distinct CCE cell fractions. Unfortunately, both fractions were heterogeneous in their cell composition and it was impossible to determine which cell population expressed α1-ARs. However, we found that the fraction which expresses only the low affinity receptor included most of the GM precursors. In contrast, in the fraction showing the high affinity receptor, no GM-precursors were found. This suggested the existence of two cell populations expressing different α1-ARs. By searching a way to separate these cell populations we found a simple adherence procedure (see materials and methods) to be effective. In the adherent fraction (ADH) both receptors were still present, while in the non-adherent one (n-ADH) the high affinity receptor seems to be absent (Table 6). In the latter fraction (n-ADH) an assessable number of GM-precursors was present. A GM-CFU assay demonstrated that NE was not active. This was in contrast to what happened in unseparated BM cells, were NE shows a significant growth inhibition of such precursors (figure 4, chapter 2). This strongly suggested that GM-CFU inhibition occurred via the high affinity receptor which seems to be absent in the n-ADH fraction (Figure 11). As shown in Figure 11 high concentrations of NE (10^{-4} M) are necessary to inhibit GM-CFU in n-ADH cells in contrast with unseparated BM cells where 10 nM NE exerted a significant GM-CFU inhibition (figure 4, chapter 2).

	Kd_{high} nM	$Bmax_{high}$ fM/10^6 cells
Adherent cells	1.14 ± 0.64	5.2 ± 2.3
Non-adherent cells	—	—

Table 6: Distribution of α1-AR in adherent vs. non-adherent BM cells. BM cells were collected from C57Bl/6 mice and incubated as described in materials and methods to separate adherent from n-ADH. The cells were then washed and adjusted at concentration of $2x10^6$ cell/ml to perform binding study. The values represent the mean of 4 experiments ± SE and have been obtained by a computer-assisted non-linear regression analysis (Ligand).[58]

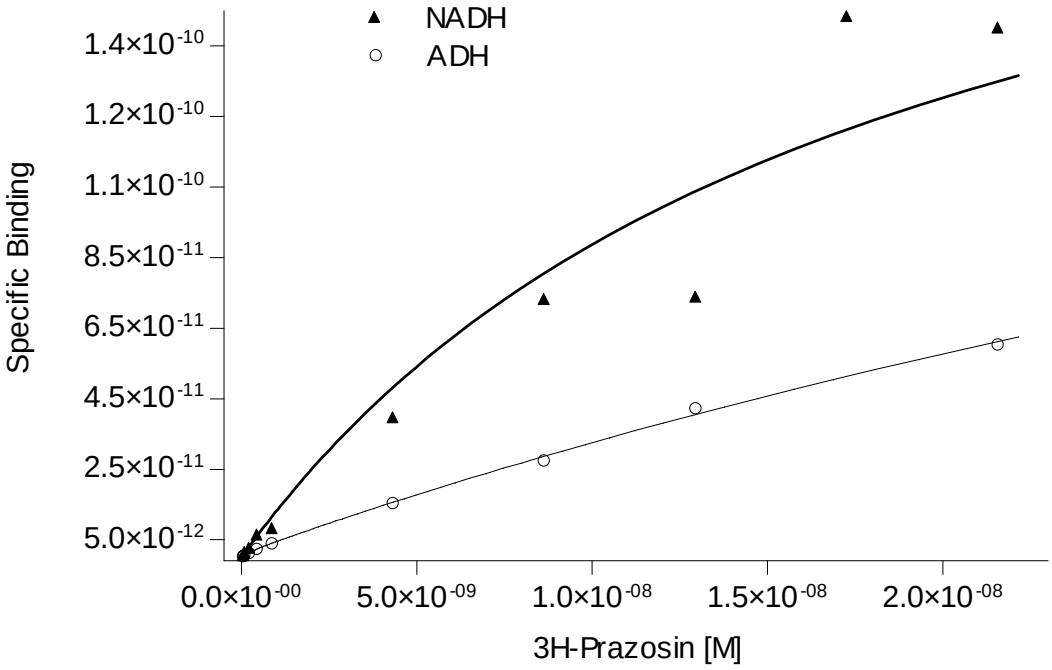


Figure 9: Plot of saturation isotherm of ^{3}H-PRA binding in adherent (ADH) and non-adherent (NADH)cells. Non linear regression analysis gave two binding sites for the ADH and on for the NADH fraction as reported in Table 6. The result are obtained by the mean of tree different experiments.

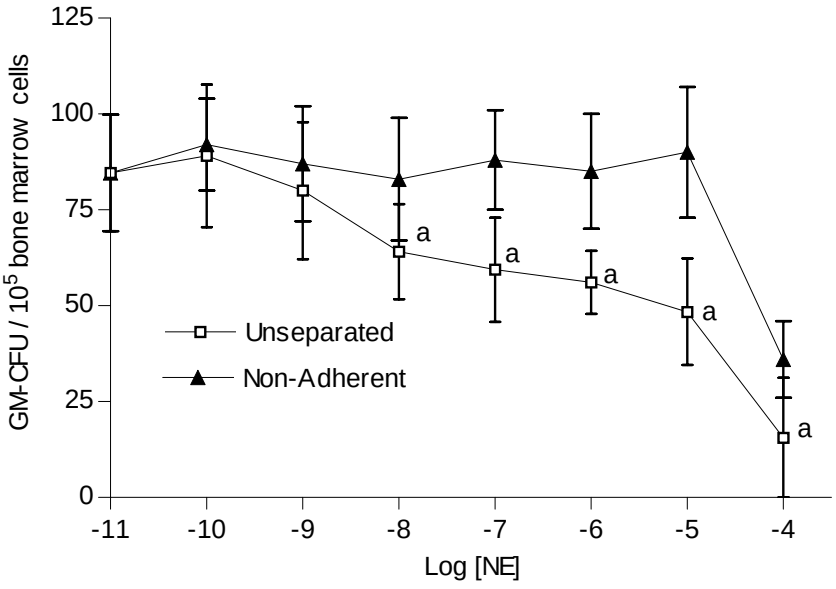


Figure 10: Noradrenergic inhibition of GM-CFU in unseparated vs. non-adherent BM cells. For each concentration the mean value from 5 experiments ± SD is shown. a: $p < 0.01$.

NE sensitivity of ADH cells was not tested because, as previously reported,[59] GM-CFU precursors are practically lacking in this fraction. In this fraction only 10-15 cells over 100'000 cells are GM precursor, and this prevents a proper evaluation of possible differences.

3.2 Distribution of α1-adrenergic receptors

Flow cytometry analysis of ADH cells revealed that two specific antigenic determinants were most represented: B220 and Mac1 both at ~ 40%. To separate these two cell populations, Magnetic Cell Separation (MACS, described in materials and methods) allows over 75% enrichment or depletion of the desired cell population. Binding studies of such populations gave the results reported in Table 7. These results suggest that the high affinity receptor is expressed on adherent $B220^+$ $Mac1^-$ cells whereas the low affinity one is found in this fraction on $B220^-$ $Mac1^+$ cells.

Adherent cells	% Mac 1^+	% $B220^+$	Kd_{high} nM	$Bmax_{high}$ fM/10^6 cells	Kd_{low} nM	$Bmax_{low}$ fM/10^6 cells
Mac1 depleted	6 ± 4	63 ± 6.9	2 ± 1	14 ± 6.3	—	—
B220 depleted	67.9 ± 7.8	10.7 ± 10.6	—	—	46 ± 19	200 ± 80
Mac1 enriched	81 ± 4.3	12 ± 5	—	—	54 ± 12	189 ± 36
B220 enriched	13.4 ± 1.3	74.4 ± 14.1	1.5 ± 0.9	56.4 ± 5.4	—	—

Table 7: Characterization of the α1-ARs positive adherent BM cells. The mean values of 5 experiments are shown. Variation are SD of percentage and SE of Kd and Bmax. The number of cells per tube in the ^{3}H-PRA saturation studies was of $250x10^3$ in the enriched populations and 10^6 in the depleted ones. The Kd and Bmax values were obtained by analyzing together the isotherm saturation curves by Ligand.[58]

3.3 High affinity α1-adrenergic receptor on pre-B cells

Cells of the B lineage (bearing the B220 antigen) are heterogeneously represented in the BM (pre-B cells, B mature cells, plasma cells, etc.). Therefore, we aimed at identifying the B lymphocyte sub-population cells which bears the high affinity receptor.

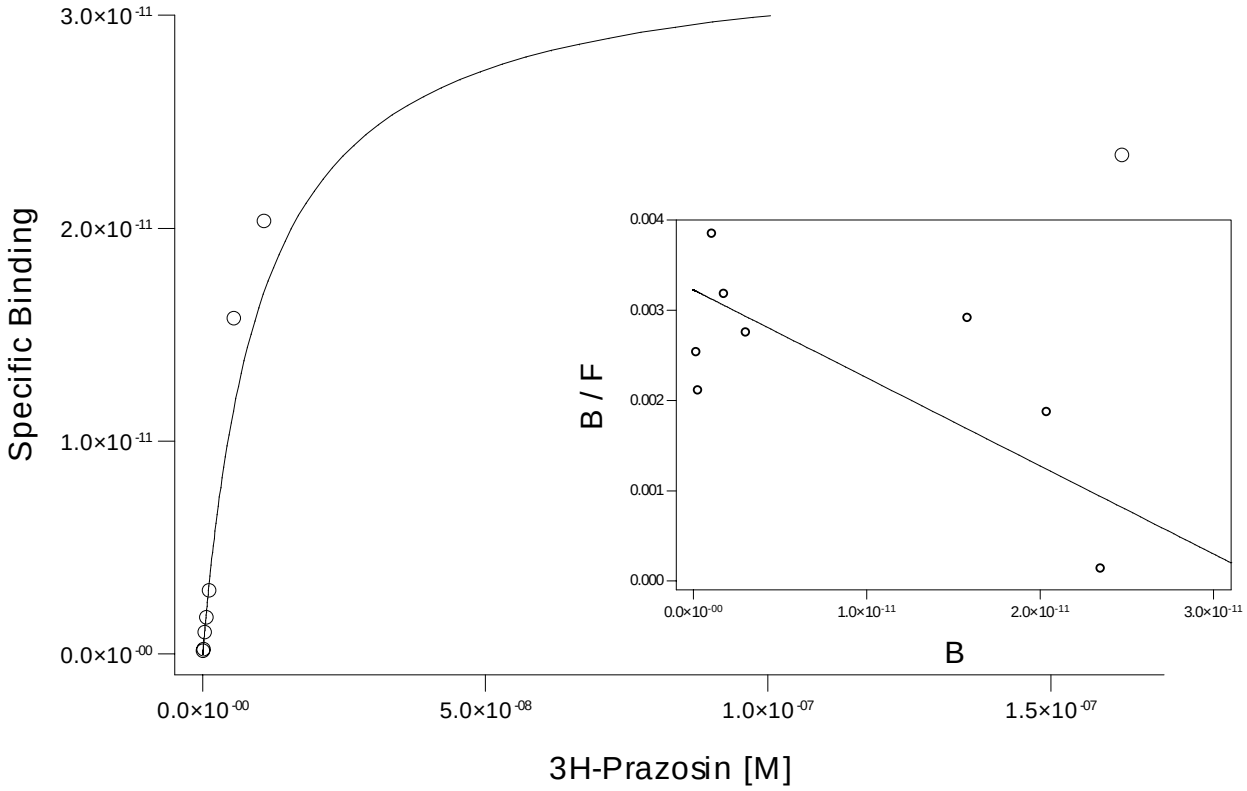


Figure 11: Plot of saturation isotherm of ^{3}H-PRA binding in intact spleen cells. The results are obtained from two different experiments. Non linear regression analysis gave the scatchard plot reported (inset), which describes a single binding site with Kd 50.8 ± 34.2 nM, and B max 177 ± 109 fmol/2x10^{6} cells.

To answer this question we initially tested whether mononucleated spleen cells, which consist only of mature B cells, express the high affinity AR. However, as it appears in Figure 11, mononucleated spleen cells contain only the low affinity receptor. This suggests that the high affinity adrenoceptor is expressed on more primitive cells. By means of the MACS system it was impossible to obtain a large number of purified primitive B cells. Therefore we tried to grow pre-B cells by setting up long term BM cultures by adapting a method previously described (see material and methods).[57] ^{3}H-PRA isotherm saturation studies performed on cells obtained after 28 days of culture gave the results reported in Table 8 which demonstrate that Mac1^{-}, sIgM^{-}, B220^{+} B precursors (pre-B cells) indeed express the high affinity α1-adrenergic receptor.

%Mac1^{+}	%B220^{+}	%sIgM^{+}	Kd nM	Bmax fmol/106 cells
0	97 ± 1.5	0	0.83 ± 0.76	12.5 ± 9.6

Table 8: The mean values ± SD of 3 experiments are shown. The Kd and Bmax values were obtained by analyzing the isotherm saturation curves by Ligand.[58]

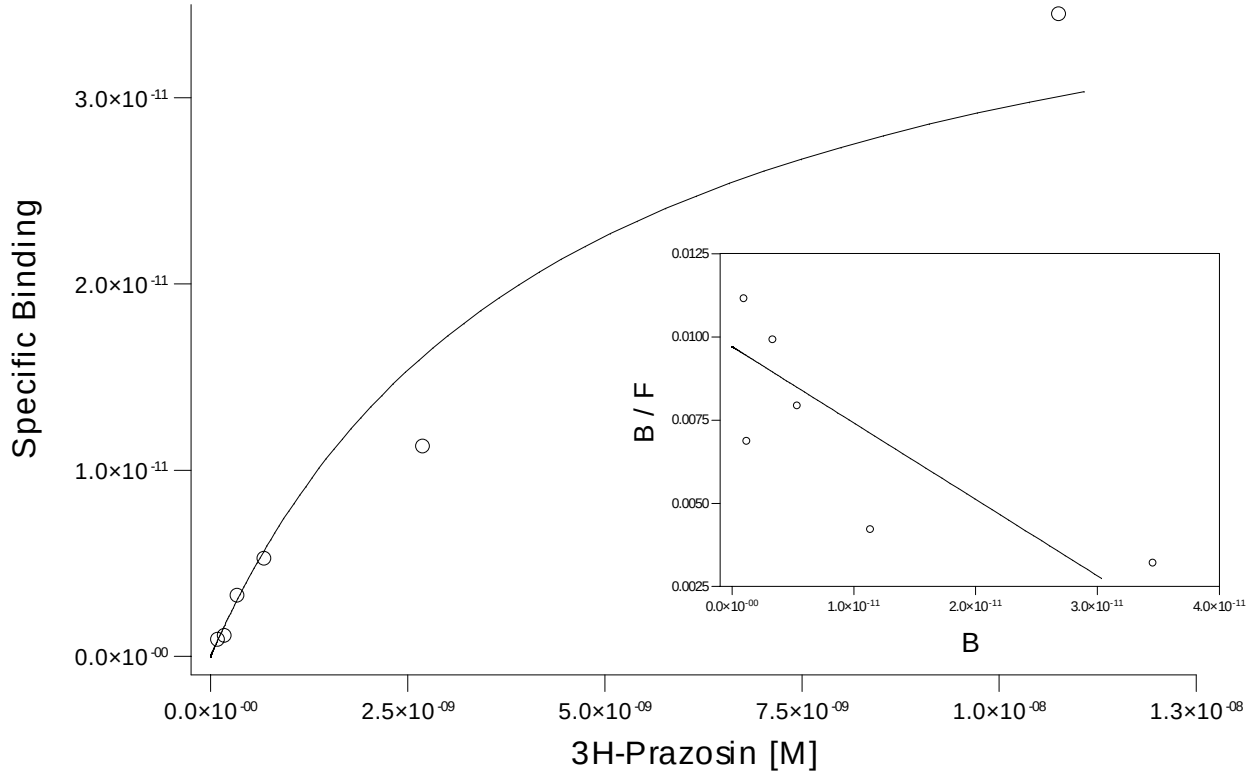


Figure 12: Plot of saturation isotherm of ^{3}H-PRA binding in non-adherent cells from lymphoid LTBMC. The results are obtained from two different experiments. Non linear regression analysis gave the scatchard plot reported (inset), which describes one single binding site as reported in Table 8.

3.4 Effect of anti-mouse TGF-β monoclonal antibody on the hematopoietic rescue induced by NE

Cells of the B lineage can produce inhibitory cytokines such as tumor necrosis factor-α (TNF-α) and TGF-β.[60] Both cytokines have been reported to rescue hematopoiesis from myeloablative treatments.[61,62] TGF-β has been also proposed to be a physiological regulator of B cell growth and differentiation.[63] We, therefore, investigated whether anti-TNF-α or anti-TGF-β antibodies could neutralize the inhibitory effects exerted by NE on GM-CFU and, eventually, the NE-induced hematopoietic rescue. As shown in figures 11 and 12, anti-mouse TGF-β but not anti-TNF-α antibodies were indeed able to counteract the effect of NE both in GM-CFU cultures (Figure 13) or in the rescue experiments (Figure 14). These results suggest that activation of the high affinity α1-AR present in B cell precursors may induce the production or activation of TGF-β.

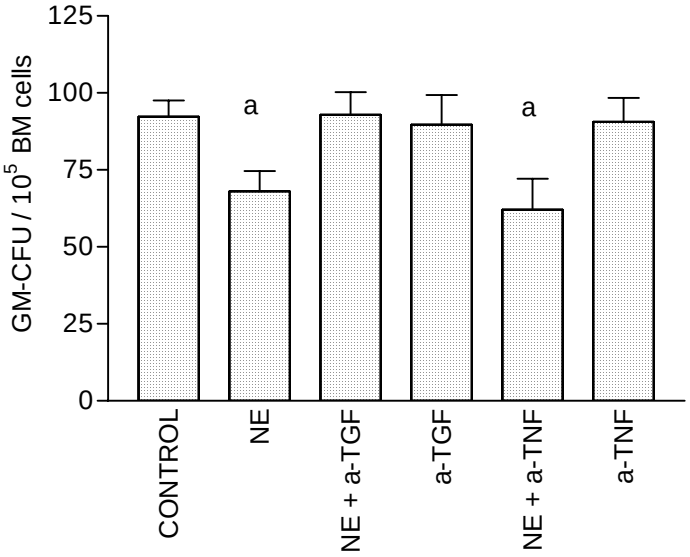


Figure 13: Noradrenergic inhibition of GM-CFU in unseparated BM cells in presence or in absence of anti-TGF β monoclonal antibody. Value are the mean of 3 experiments ± SD. a: $p < 0.01$, NE vs Control, NE+a-TNF vs control and vs a-TNF alone.

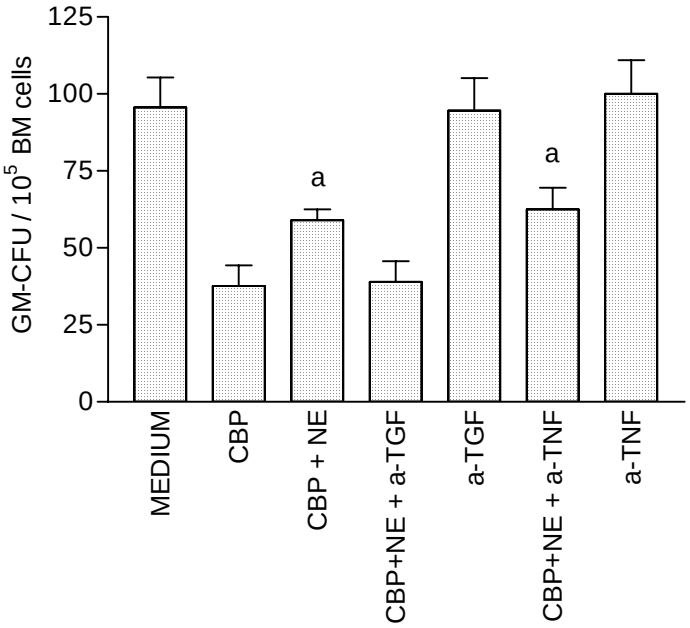


Figure 14: Rescue of GM-CFU in BM cells incubated with CBP via α1b-AR. BM cells were incubated at 37°C for 7h with CBP (25 μM) in the presence or absence of NE and anti-TGF-β or anti-TNFα monoclonal antibody. a: $p < 0.01$, CBP+NE vs CBP alone, and for CBP+NE+a-TNF vs CBP and vs a-TNF.

3.5 High affinity receptor on pre-B cell lines

Tacking advantage of the results obtained with the long-term cultured normal pre-B cells, we also investigated the ^{3}H-PRA isotherm saturation binding on murine and human pre-B cell lines. Two human cell lines and one murine cell line have been used. These three cell lines express the high affinity adrenoceptor, as shown in Table 9. This confirms the presence of the high affinity α1-adrenergic receptor on pre-B cells, both of murine and human origin.

CELL LINE	NALM-6 human	KM3 human	70Z/3 murine
Kd nM	0.36 ± 0.05	2.25 ± 0.87	0.98 ± 0.49
B max fmol /10^6 cells	1.13 ± 0.08	0.66 ± 0.19	4.08 ± 1.87

Table 9: ^{3}H-PRA isotherm saturation binding performed on cell lines and analyzed with Ligand.

3.6 Adrenergic receptor on umbilical cord blood cells

Because we found the high affinity α1-AR to be present on human pre-B cell lines, we thought that, in analogy with mice, also normal human pre-B cells should express this receptor. As it is rather difficult to obtain human BM cells we used umbilical cord blood cells which contain enough hematopoietic progenitors.

^{3}H-PRA binding	Kd	Bmax
high affinity	0.1 ± 0.25 nM	0.61 ± 0.75 fmol / 10^6 cell
low affinity	21.7 ± 10.54 nM	8.2 ± 2.62 fmol / 10^6 cell

Table 10: ^{3}H-PRA isotherm saturation bindings performed on cells harvested from umbilical cord blood, and analyzed with Ligand. The results are the value obtained with the cells of one donor.

The results of the binding studies reported in Table 10 also indicate the presence of two different AR in umbilical cord blood cells. Moreover the Kd of the high affinity receptor is consistent with our previous results obtained on murine BM cells and on pre-B cell lines.

4. DISCUSSION

We demonstrated that the high affinity α1-AR is expressed on pre-B cells ($B220^+Mac1^-sIgM^-$) whereas the low affinity receptor seems to be present on ADH and n-ADH $Mac1^+B220^-$ cells. We did not characterize better the low affinity AR because all our experiments suggested that the effects of NE were mediated by the high affinity receptor. In fact, we have demonstrated previously that the inhibition of the GM-CFU by NE can be completely abolished by addition of 10-100 pM PRA. This is consistent with the Kd measured on BM cells for the high affinity receptor (0.98 nM), and suggests that the inhibitory effect was mediated by the high affinity α1-adrenergic receptor. In addition, when mice treated with CBP and NE were also treated with PRA (10 mg /kg body weight) part of the protection exerted by NE was neutralized. Similarly, when 1 nM PRA was added in BM cells cultures incubated for a short period (7h) with CBP ± NE the effects of NE, i.e. protection of GM-precursors against CBP, were neutralized. These observations are consistent with NE being effective on the high affinity α1-adrenergic receptor. The next obvious question was, then, the identification of the cell type bearing the high affinity receptor. By separating BM cells by adherence we confirmed that the GM-CFU inhibition exerted by NE on GM-CFU was performed via the high affinity adrenoceptor. Indeed in n-ADH cells, where this receptor was not expressed, NE was effective only at higher concentration (0.1 mM). Taking advantage of such distribution we concentrated our efforts on ADH cells and we found that two phenotypic markers were most represented in this fraction, namely $Mac1^+$ and $B220^+$. To separate these two cell populations, we used the MACS system because it uses small beads (~ 30 nm) that do not interfere with cytofluorimetric measurement and binding studies. By this system the efficiency of enrichment or depletion was relatively poor but could be improved by a second elution in the column. Our problem was to obtain pure cells in sufficient amount to perform binding studies. For this reason we also cultivated cells in long term cultures. The results obtained were very clear: pure pre-B cells ($B220^+Mac1^-$ $sIgM^-$) bear the high affinity α1-AR. The same receptor (same affinity for PRA) is also present in human cells, in umbilical cord blood cells, and in pre-B cell lines. This indicates that the expression of α1-AR on pre-B cells is a common feature and suggests the possibility for a general physiological role of this receptor. The mechanism of the inhibition of GM-CFU by NE is still unclear and needs further investigation. For this purpose we tested the possible production of a soluble factor but the results were negative. In these preliminary experiments, we analyzed the supernatant from normal cells stimulated with NE. The supernatant was then dialyzed with a membrane with a cut-off of 4000 Da (to get rid of NE), but it turned out not to contain any inhibitory activity. Of course, the hypothetical soluble factor could be smaller than 4000 Da. Trying to clarify this point we have tested different antibodies against known cytokines and found that anti- TGF-β monoclonal antibody was capable to abolish the inhibition of NE. This suggests that a soluble factor is indeed synthesized and/or released by the pre-B cells after NE stimulation. Another possible mechanism not involving soluble factors is a cell-to-cell interaction between GM-CFU and pre-B cells.

IV. NOREPINEPHRINE IN BONE MARROW CELLS

1. INTRODUCTION

We described above the presence and certain biological effects mediated by adrenoceptors in BM cells. These findings imply that hematopoiesis may be physiologically regulated by catecholamines. This is also suggested by the fact that BM is innervated by both afferent and efferent sympathetic fibers.[47] Moreover, it has been reported that some blood cells are influenced by catecholamines,[11,64] and that lymphoid cells may also produce catecholamines.[12] In this chapter we demonstrate that murine BM contains substantial amount of catecholamines which are not only of neuronal origin but also seems to be produced by BM cells. In particular NE and dopamine (DA) showed a circadian rhythmicity with peak values during the night. Finally we investigated the possibility of an association of this rhythm with the proportion of cells in the various phases of the cell cycle.

2 MATERIALS AND METHODS

2.1 Mice

Female, 2-4 months old C57BL/6 mice were purchased from Charles River, Como, Italy and kept at 21 ± 1°C, with free access to food and water under a 12 h light:dark photoperiod for at least 15 days before the experiments. Light intensity at the level of the cage floor was 800 lux.

2.2 Reagents

(-)-norepinephrine bitartrate (NE), (+)-epinephrine bitartrate (E), dopamine hydrochloride (DA), 6-hydroxydopamine (6-OHDA), 3,4 dihydroxybenzylamine hydrochloride, sodium pyrosulfite, TRIZMA base, vanillylmandelic acid (VMA), 3,4-dihydroxyphenylglycol (DHPG), 3,4-dihydroxyphenylacetic acid (DOPAC), Homovanillic acid (HVA) were purchased from Sigma ST. Louis, MO, USA. All other chromatography reagents and solvents were purchased from Merck, Darmstadt, Germany.

2.3 Chemical sympathectomy

The mice were injected intraperitoneally with 100 mg/kg body weight of 6-OHDA 2 days before sacrifice. In adult mice, 6-OHDA produces a profound, although temporary, depletion of peripheral NE, inducing a rapid degeneration of sympathetic neurones.[50]

2.4 Bone marrow collection

The mice were sacrificed by cervical dislocation. During the dark phase of the photoperiod this operation was performed under a dim red light. The marrow was flushed out from the long bones either with culture medium (α-MEM with 10% HS) for cell cycle analysis or with 2 ml of $HClO_4$ 0.4 N containing 0.84 mg/ml disodium EDTA, 25 μl of a 4% sodium pyrosulfite solution, for measurement of the catecholamines. For this purpose the samples were then centrifuged (14'000 x g, 4°C, 10 min), the supernatant was frozen and stored at -80°C until assayed

2.5 Bone marrow cultures

BM cells ($3x10^6$ cells/ml) were incubated at 37°C in α-MEM, 10% HS. In certain experiments the cells were harvested after 24 h incubation (short term cultures). In others, half culture medium was replaced with fresh

medium once a week and the culture was continued for 3 weeks (long term cultures). At the end of the cultures, cells were harvested, centrifuged and the pellet processed for catecholamine determination.

2.6 Cell line cultures

The human pro-B and pre-B cell lines MO2058, NALM-6 and the murine pre-B cell line 70Z3 which express α1-adrenoceptors were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 200 mM Glutamine, 50 µM 2-ME, 100 U/ml penicillin / 50 µg/ml streptomycin at 37°C in a moist atmosphere of 5% CO2.

2.7 Catecholamine assay

BM catecholamines were assayed by high performance liquid chromatography (HPLC) with electrochemical detection in supernatants which were aluminia extracted as previously described.[65] An analytical Beckman C18 ultrasphere-XL ODS 3 µm (70 x 4.6 mm) equipped with XL ODS 3 µm (5 x 4.6 mm) guard cartridge was used. The mobile phase was composed of ultrapure water : acetonitrile (82 : 18 v/v), 20 mM K_2HPO_4, 0.69 mM EDTA, 0.27 mM SDS. The pH was adjusted to 3.0 with H_3PO_4 and the solution was filtered (Millipore, 0.22 µm). Flow rate was set to 0.9 ml/min. An ESA Coluchem II electrochemical detector with 5011 analytical cell was used for detection of catecholamines. The first cell potential was set to + 300 mV and the second one to - 300 mV. Chromatograms were processed with a computerized integrator.

2.8 Cell cycle analysis

Mice were injected i.p. with 5'- bromo -2'-deoxyuridine (50 mg / kg body weight, BrdU, Sigma Co., St. Louis, U.S.A.) 1 hour prior to sacrifice. BM cells were collected from the femurs, washed in PBS, 1% HS and resuspended in 100 µl of PBS at 4° C. The cell suspension was added in drops into ethanol at - 20°C under continuous stirring and then incubated on ice for 30 min. The cells were then centrifuged, the supernatant removed and the cells resuspended again. After addition of 1 ml HCl 2.5 N in 0.5 % Triton X-100 the cells were incubated for 20 min at room temperature. Cells were centrifuged, the pellet was resuspended in 1 ml $Na_2B_4O_7$ 0.1 M, pH 8.5, and centrifuged again, resuspended, counted and aliquoted at a concentration of 10^6 cells / 50 µl in Tween 20, 1 % HS. 20 µl of anti-BrdU-FITC antibody (Beckton Dickinson, Basel, Switzerland) were added and the cells were incubated for 30 min at room temperature. Finally the cells were resuspended after centrifugation in 1 ml of PBS containing 5 mg / ml of propidium iodide (Sigma, Co., St.

Louis, U.S.A.) and analyzed by cytofluorimetry (FACscan, Beckton Dickinson). The analysis was performed by Lysis 2 on 10^5 cells per gated region. An example of the linear fluorescence intensity plots obtained is shown in Figure 15.

2.9 Evaluation of catecholamine concentration and statistical analysis

The amount of catecholamines in each sample was calculated using peak area ratio relative to 3,4 dihydroxybenzylamine hydrobromide, the internal standard, to correct for incomplete extraction. The concentration of catecholamines was expressed per g of tissue. The statistical tests used were the two-tailed Student's t-test for unpaired data, ANOVA and linear regression analysis after log transformation of the data in order to homogenize the variance. A cosinor analysis to assess the existence of circadian rhythmicity was performed by a computer assisted plot of a least squares fit of a cosine function to the data (Chronolab 3.0)

2.10 Cell line cultures

NALM-6 was kindly provided by Dr Seckinger, Centre d'immunologie INSERM-CNRS, Marseille, France. KM-3 was kindly provided by Prof. Trubiani, Istituto di Citomorfologia Normale e Patologica del CNR, Università di Chieti, Italy. MO2058 was kindly rpovided by Dr. Gianni Soldati, Ospedale Regionale "La Carità", Locarno, Switzerland. 70Z/3 was purchased from ATCC. Cells were maintained at concentration between 0.1 and 1 x 10^6 cells / ml in RPMI 1640 with 10% FCS and, for 70Z/3, 5 mM 2-ME.

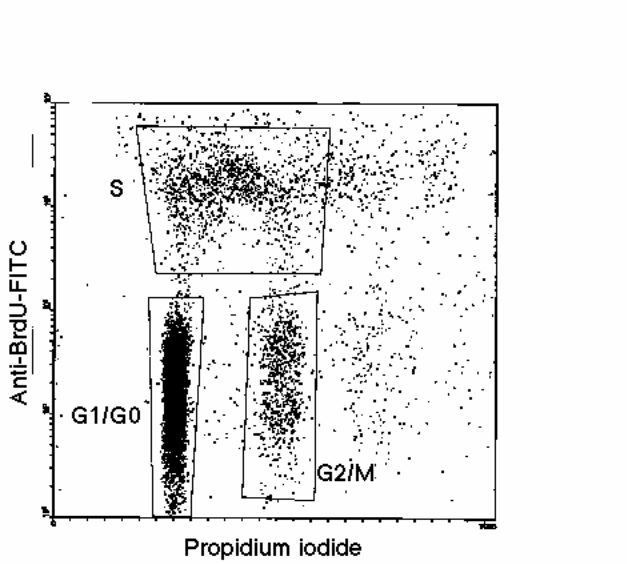


Figure 15: Two color cell cyle analysis using anti-BrdU and propidium iodide. Laser excitation at 488 nm and standard FITC/PI emission filters were used on FACScan flow cytometer. (Becton Dickinson).

3.1 Bone marrow catecholamines

We found that catecholamines are present in substantial amount in the BM. Figure 16 shows the concentration of NE (panel A), DA (panel B) and E (panel C) which are present at various times of the day in the BM from both normal and chemically sympathectomized mice. It seems evident that NE and E are most represented while DA is present at lower concentration. Unlike E, the mean concentration of NE and DA seems to be higher in the marrow sampled during the dark phase than during the light phase of the photoperiod. To get an index of catecholamine input in the BM over a given time, the data were also expressed as area under the curve (AUC). Table 10 reports the AUC relative figures which show that the difference in NE and DA mean AUC between the dark and the light phase of the photoperiod is significant. Chemical sympathectomy reduced the marrow concentration of NE and DA during the dark phase to levels which were similar to those found in the light phase (Table 11). The difference in NE and DA concentration between the dark and light phase of the photoperiod suggested the existence of a daily rhythmicity. Cosinor analysis of the data revealed the presence of a significant daily rhythmicity of NE and DA in both normal and sympathectomized mice ($p < 0.005$ for NE and $p < 0.05$ for DA, Figure 17 A, B). The daily rhythm of NE was apparently reversed by chemical sympathectomy (Figure 17A) while that of DA was shifted with the

acrophase occurring at the transition between the light and dark phase (Figure 17B). E did not show any significant daily rhythm (data not shown).

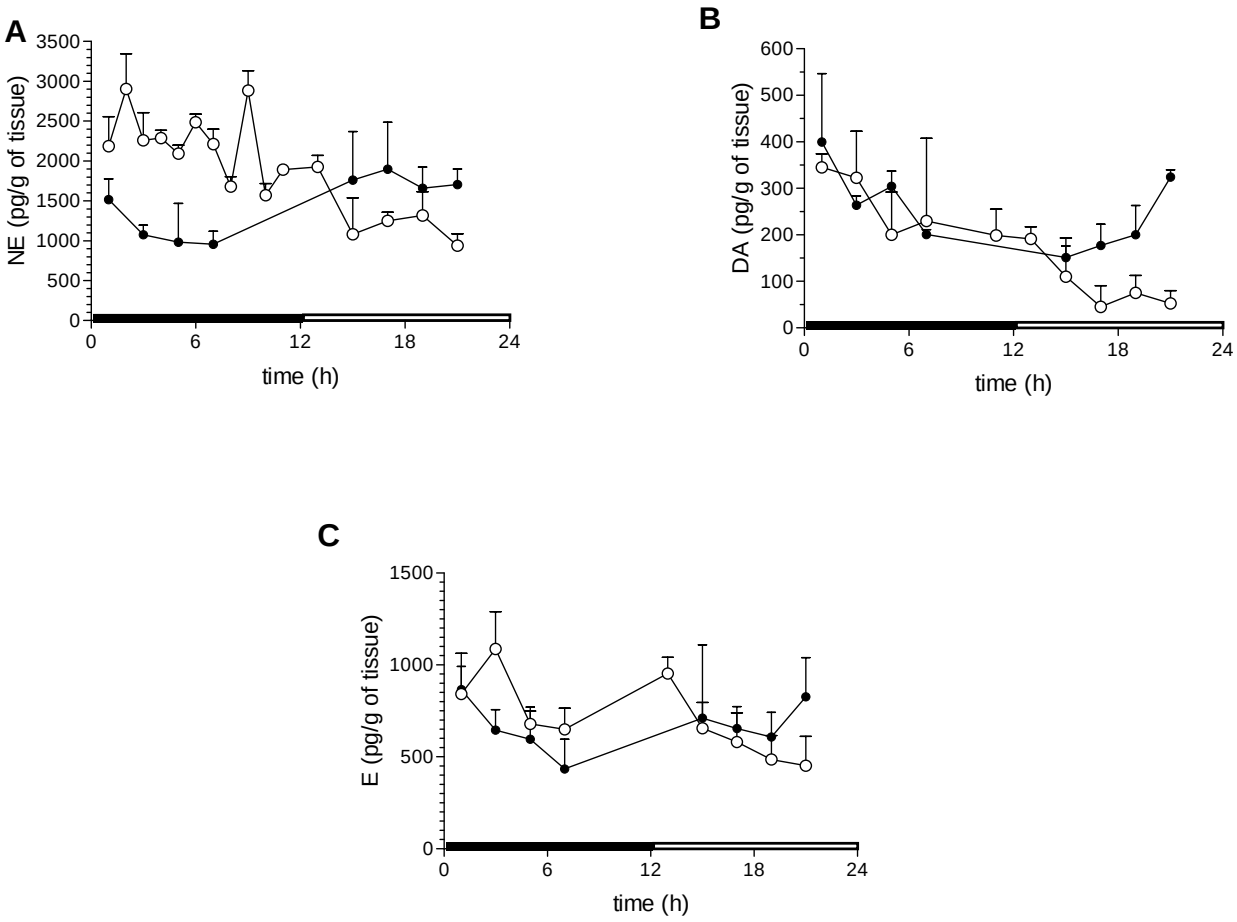


Figure 16: Measurement of NE (A), DA (B) and E (C) concentration in the BM of normal (open symbols) and 6-OHDA-treated (closed symbols) mice exposed to a 12h dark:light photoperiod. Each point is the mean of at least 9 animals. Vertical bars indicate the standard error. The black segment of the X axis indicates the dark period.

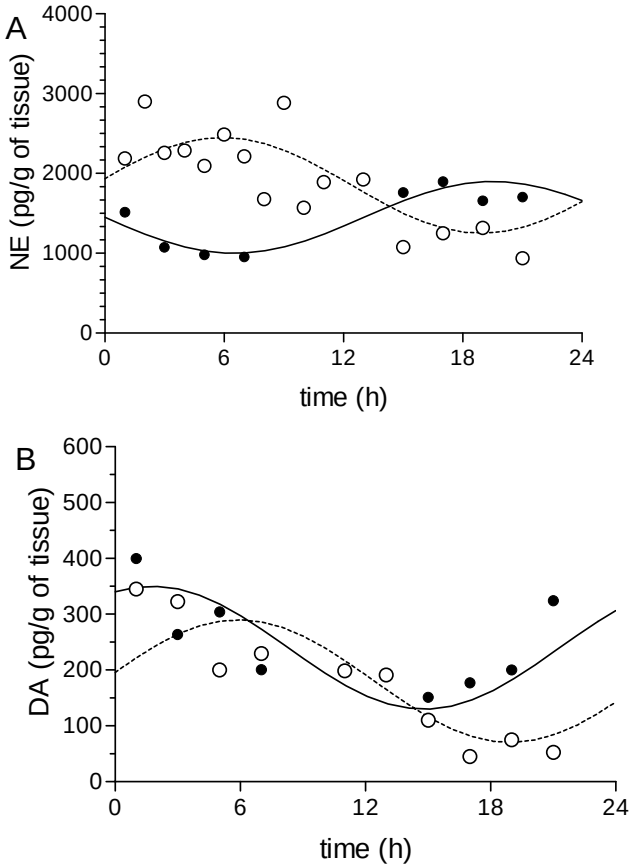


Figure 17: Cosinor analysis of NE (A) and DA (B) circadian periodicity in the BM of normal (open symbols) and 6-OHDA-treated (closed symbols) mice exposed to a 12 h dark: light photoperiod. The black segment of the X axis represents the dark period. Data points are the same as those reported in Fig 14. NE (normal mice): rhythm-adjusted-mean (MESOR) = 1823.3 ± 104.3 pg/g of tissue; amplitude (A)=609.0 ± 144.8 pg/g of tissue; acrophase (Φ) = 4h 51 min. NE (6-OHDA-treated mice): MESOR =1413.1 ± 39.0 pg/g of tissue; A= 426.1 ± 61.2 pg/g of tissue; Φ = 18h 16 min. DA (normal mice): MESOR =172.8 ± 23.5 pg/g of tissue; A = 11.0 ± 33.4 pg/g of tissue; Φ =5h 53 min. DA(6-OHDA-treated mice): MESOR = 236.5 ± 15.2 pg/g of tissue; A:106.6 ± 20.4 pg/g of tissue; Φ = 0h 46 min.

	Normal ($pgxg^{-1}xh^{-1}$)	**light vs dark**	**6-OHDA ($pgxg^{-1}xh^{-1}$)**	**light vs dark**	**normal vs 6-OHDA**
NE light	17409 ± 3082 (n=9)		20223 ± 4641 (n=9)		ns
NE dark	26507 ± 2418 (n=11)	p=0.02	13916 ± 3252 (n=9)	ns	P=0.005
DA light	1326 ±355(n=9)		2770 ±518 (n=9)		P=0.01
DA dark	3902 ± 259 (n=9)	p<0.001	2312 ± 1210 (n=9)	ns	ns
E light	5176 ± 3332 (n=9)		8563 ± 2718 (n=9)		ns
E dark	9623 ± 1572 (n=9)	ns	7243 ± 2176 (n=9)	ns	ns

Table 11. Comparison of AUC relative to BM NE, E and DA in the light and dark phase of the photoperiod. The AUC obtained by plotting the concentration of catecholamines against time was calculated using the trapezoidal rule and taken as an index of catecholamine input into the BM over a given time interval. The values represent the mean pg per g of tissue per hour. n figures stand for number of mice per each time point considered.

3.2 Catecholamine metabolites

To investigate whether changes in catecholamine concentrations reflected a change in their turnover, we measured the BM concentrations of NE and DA metabolites at two time points during the dark and the light phase. The results obtained which are shown in Figure 18A (NE metabolites) and 16B (DA metabolites) show that changes of catecholamine concentrations correspond to changes of their metabolites. This suggests that the daily rhythm in the BM catecholamine concentration reflects a rhythmic catecholamine release and function.

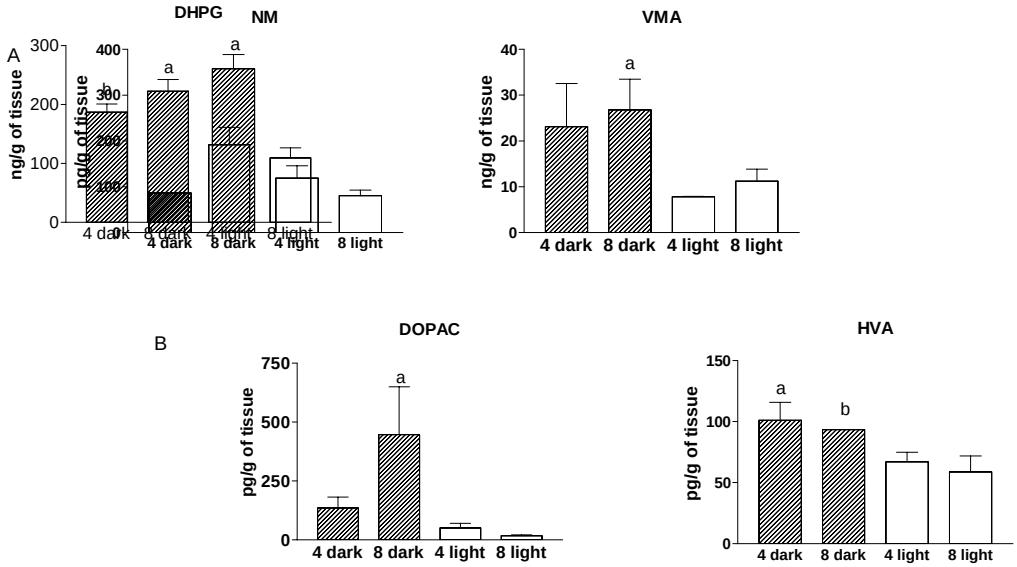


Figure 18: NE **(A)** and DA **(B)** metabolite concentration in BM sampled during the dark and light phase of the photoperiod. Six mice per group were killed at 4 and 8 hour of the dark and light period. The values represent the mean of individual values ± standard error of the mean. NE metabolites: NM = normetanephrine; VMA = vanillylmandelic acid; DHPG = 3,4-dihydroxyphenylglycol. DA metabolites: DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovanilic acid. a: $p<0.01$; b: $p<0.05$

3.3 Association with bone marrow cell cycle.

BM functions show a circadian periodicity both in human and mice.[66-68] Furthermore, our previous findings showed that NE is able to affect hematopoietic functions directly.[49,53] Therefore we wondered whether the marrow concentration of NE and DA could be associated with any particular change in the proportion of cells in the various phases of the cell cycle. The BM from groups of normal and 6-OHDA pretreated mice was taken at various times either in dark or light phase of the photoperiod. At each time, the mice were randomized in two subgroups as donors of BM cells to be evaluated for catecholamine content and cell cycle analysis, respectively. Regression analysis of data show that NE was associated positively ($r = 0.39$, $p = 0.01$) with the percentage of cells in the G2/M phase (Figure 19 A) and with the percentage of cells in the S phase

(r= 0.37, p= 0.02) of the cell cycle (Figure 19 B). When the percentage was substituted for the number of cells per femur, the positive association between NE and number of cells in S or G2/M phase remained significant (data not shown). No other significant association was found.

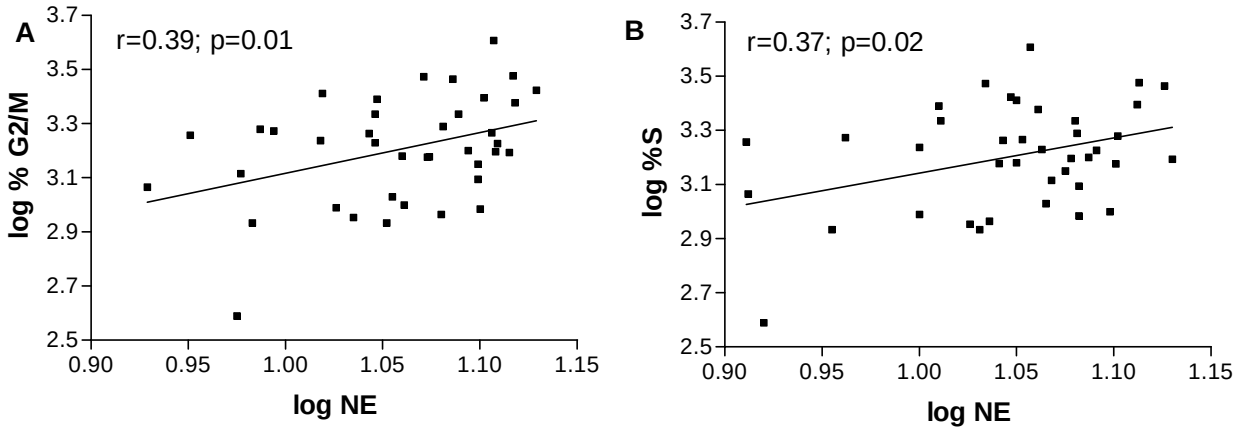


Figure 19: Positive relationship between NE and the percent of BM cells in G2/M (A) and S (B) phases of the cell cycle. At least 6 normal mice per group were sacrificed at 3, 5, 7 and 9 hours of the dark and light period. The BM sampled from individual mice from two subgroups of 3 mice then served for cell cycle and catecholamine analysis, respectively. At 5, 7 and 9 hours of the dark and the light period, 6-OHDA-pretreated mice were also sacrificed and the relative values included in the regression analysis.

3.4 Catecholamines in bone marrow cultures and lymphoid cell lines

Catecholamines were present either in short term or in long term BM cultures (Table 12). The amount of catecholamines contained in the culture medium was undetectable. This suggests that endogenous catecholamines in the BM are produced in part by BM cells.

Our previous studies demonstrated that $\alpha 1$-adrenoceptors are expressed on normal pre-B lymphocytes and on both human and murine pro- and pre-B cell lines.[69] In the attempt to get a preliminary indication of the BM cell type which may produce catecholamines we measured the catecholamine content in MO2058, NALM-6 and 70Z3 cell lines and found that these cell lines seem indeed to produce catecholamines (Table 11).

	NE (pg/g)	DA (pg/g)	E (pg/g)
STBMC	342 ± 184	677 ± 288	uq
LTBMC	96 ± 36	113 ± 7	91 ± 47
Medium	uq	uq	uq
70Z/3 (m)	153 ± 67	10’380 ± 2937	4018 ± 3829
MO2058 (h)	1034 ± 541	11’000 ± 4804	4952 ± 1765
NALM-6 (h)	436 ± 67	8000 ± 4283	5805 ± 1292
Cell medium	uq	uq	uq

Table 12: The values represent the mean ± SD in pg x g of pelleted cells from 3 experiments. STBMC: short-term BM cultures; LTBMC: long-term BM cultures; BM Medium: BM culture medium constituted by α–MEM, 10% HS; Cell Medium: cell lines culture medium constituted by RPMI 1640 medium 10% heat-inactivated FCS, 200 mM Glutamine, 50 µM mercaptoethanol, 100 U/ml penicillin/streptomycin. The catecholamine concentration in culture media are relative to freshly prepared media. Cell cultures were harvested in the esponential growth phase. uq: unquantifiable; m: murine; h: human.

4 DISCUSSION

In this study we show that BM contains NE, DA and E. To our knowledge this is the first study showing the presence of substantial amounts of catecholamines in the BM. Previous studies were focused mainly on peripheral lymphoid organs in rats in which catecholamine concentration was found to be 10-100 fold higher than those here reported in murine BM.[70-72] The concentration of marrow NE and DA shows a significant circadian periodicity with peak values during the night. Chemical sympathectomy seems to reverse the circadian rhythm of NE and to advance the acrophase of DA from the middle of the dark phase to the transition zone between light and dark phases. However, when the difference in NE and DA between night and day was analyzed taking in consideration the intra-group variability, the effect of chemical sympathectomy appeared to be just that of abolishing the difference. This might mean that the circadian rhythm of NE and DA is due to a circadian periodicity in the activity of the BM sympathetic innervation which might well reflect the general sympathetic periodicity in a nocturnal species such as Mus Musculus. On the contrary, E did not show any rhythmicity and sensitivity to 6-OHDA , a finding consonant with a non neural origin. In our previous work we got evidence that NE exerts an inhibitory effect on hematopoiesis.[49,69,73,74] However, the positive association found is rather surprising as all our previous results indicate that NE exert inhibitory action on hematopoiesis. Clearly further studies are needed to clarify this point. The fact that BM cells seem to be able to produce catecholamines is also indicative for a physiological role of these substances in hematopoiesis. The cell type which may produce catecholamines are unknown. It has been recently reported that catecholamines and their metabolites are present in single lymphocytes and extracts of T-and B-cell clones and down-regulate lymphocyte function via an autocrine loop.[11] Therefore, BM catecholamines might derive, in part, from BM lymphocytes or from their precursors. Both for lymphocyte function and hematopoiesis the role of catecholamines seems to be inhibitory.[11,49,69] In the case of hematopoiesis, this inhibition might be exploited in modulating the BM sensitivity to myelotoxic anti-cancer drugs.[73]

These and our results emphasize the importance of the functional role of catecholamines in hematopoiesis.

VI CONCLUSION AND PERSPECTIVES

This work constitutes the continuation of investigations performed previously in our laboratory.[49,53] Those observations showed the functional presence of ARs in hematopoietic cells. One of the most relevant finding of our work was that administration of NE in mice treated with chemotherapeutic substances, could neutralize the hematopoietic toxicity of the treatment. We also found that hematopoietic cells express two adrenoceptors. It was already reported that mature blood cells express AR, and that these receptors play a role in the mobilization of blood cells. In fact, in 1995 Madden et al.[72] reported that *in vitro*, adrenergic agonists modulate all aspects of an immune response (initiative, proliferative, and effector phases), altering functions as cytokine production, lymphocyte proliferation, and antibody secretion. *In vivo*, chemical sympathectomy suppresses cell-mediated (T helper-1) responses, and may enhance antibody (T helper-2) responses. Noradrenergic innervation of spleen and lymph nodes diminish progressively during aging, a time when cell-mediated immune function also is suppressed. In animal models of autoimmune disease, sympathetic innervation is reduced prior to onset of disease symptoms, and chemical sympathectomy can exacerbate disease severity. These findings illustrate the importance of the sympathetic nervous system in modulating immune function at the level of mature cells. Nevertheless , to our knowledge, this is the first time that such a receptor is reported to be expressed in BM cells precursors. One of these receptors was found to be a low affinity α-adrenoceptor. It was not better characterized because our data suggested that it is not involved in the hematopoietic effect exerted by NE. The other receptor was characterized as an α1b-adrenoceptor. At the beginning we had some problems because the receptor was apparently expressed by a rare cell population (<1% of the hematopoietic cells). A two steps cell separation resulted in a splitting of the adrenoceptors in two cell populations. In one fraction only the low affinity receptor was present, in contrast with the other fraction which contained both adrenoceptors. The fact that only the low affinity receptor was present in one fraction, allowed us to demonstrate that the absence of the high affinity receptor coincided with the absence of the protective effect of NE. By this method we indirectly demonstrated, that the receptor responsible for the protection of BM cells was the high affinity one. In addition we showed that the receptor was expressed on lymphoid cells, in particular on pre-B cells. The fact that an adrenoceptor expressed on a rare cell population like pre-B cells, could protect the entire animal against substances like CBP, is remarkable. We tried, therefore, to understand the mechanism through which this protection was exerted. To study the mechanism we needed large numbers of cells. For this reason, we decided to use cell lines. Cell lines have, in fact, the advantage that they can be easily cultured and in large amounts. On the other side, they are immortalized, i.e. have a deranged cell cycle control. This should be kept in mind, especially in our experiments in which, apparently we activated a cell mechanism responsible for the cell growth checking.

We showed that NE acts on hematopoietic cells inhibiting their growth, and in some circumstances may induce apoptosis. We demonstrated that NE inhibits the growth of hematopoietic precursors. This seems true in the cells that express the high affinity adrenoceptor (pre-B), and also in other hematopoietic progenitors (GM-CFU). Our study leads to the conclusion that this growth inhibition is responsible for the protection of the cells from the toxic effect of CBP. In fact it is known that when cells are resting, their DNA is packed (chromatin). In this situation, alchylating substances like CBP, cannot gain access to DNA. We tried to demonstrate this mechanism using cell lines, but we encountered many difficulties. The major problem was that NE exerted the inhibitory effect on cell line growth only at high concentration (10^{-4} M). In addition, at this elevated concentration NE not only inhibited cell growth but induced cell death. We wondered whether cell death was due to apoptosis or to necrosis. We got only a preliminary evidence that NE effect was due to apoptosis. We measured Ca^{2+} release, p53 expression, and lysophosphatidylserine (LPS) on the cell membrane (data not show), but we found that only a small amount of cells (~ 20%), was apoptotic. With regard to the mechanism of action, autooxidation of catecholamines is considered unlikely because the addition of antioxidants (MEL, Vit C, Vit E) could not influence the effect of NE. The fact, that also PRA could not counteract the effect of NE further complicated the interpretation of our results. As the effect of 10^{-4} M NE on cell lines was very similar to that obtained with 10^{-6} M on normal cells, it can be hypothesized that the mechanism of action is similar. We demonstrated by pharmacological methods that the receptor in cell lines, shows the same affinity for PRA as in normal cells. The work performed is certainly not exhaustive, but it elucidates some important points and holds the potential for possible clinical application. If we suppose that the effect observed in mice will be similar in humans, we might find a schedule for NE and/or other adrenergic agonists treatment. This will allow the use of more effective doses of chemotherapeutic substances. Today, a serious problem in chemotherapeutic treatments is their secondary toxicity (myeloablatives effects that induces immunodeficiency states). The use of limited doses of drugs to avoid toxicity implies low efficiency of anti-cancer treatments. The possibility given by a BM protection with NE would allow to increase the dose of the chemotherapeutic compounds without (or at least reducing) the secondary adverse effects. Another interesting and new aspect emerging from this work, is the evidence that catecholamines may be produced in BM cells. If one can be surprised that cells of the hematopoietic system express catecholamine receptors, it seems even more astonishing that BM cells are capable to produce catecholamines. However, if these substances have to act as cytokines, it seems reasonable that hematopoietic cells can produce them. We investigated not only the presence of catecholamines in these cells, but also the presence of their metabolites, and as expected, we found them. In contrast, we were surprised to find a positive correlation between the amount of NE in the BM and the percentage of cells that were in the G2/M and S phases of the cell cycle. This might depend on a synchronization between NE

rhythmicity and the circadian rhythm of hematopoiesis, or be related by a delayed cause-effect mechanism.[75]

In conclusion all the results previously obtained and the fact that hematopoietic cells are able to produce catecholamines suggest that these substances have a physiological role in hematopoiesis. The cells that produce catecholamines seem to belong to the lymphoid lineage: high concentrations of NE were measured in lymphoid cell lines and in short term cultures, while lower amounts were found in Dexter type long term cultures (myeloid lineage). This is consistent with reports about the presence of NE and its metabolites in single lymphocytes and in T and B cell extract, and with the fact that catecholamines and their metabolites regulate in a negative fashion lymphocytes function via an autocrine loop.[76,77] These results underline the importance of the functional role of catecholamines on the hematopoietic system. It is clear that other studies are needed to understand the mechanism of these phenomena. On the other hand, data not reported showed that NE cannot rescue BM cells against etoposide (ETO). To understand this fact one should consider that the mechanism of action of ETO differ to that of CBP because it is cell cycle specific. This is because ETO acts only in the G2 phase of the cell cycle, in particular when the enzyme Topoisomerase II is active.[78] We showed that NE positively correlated with G2/M phase of the cell cycle. The fact that NE apparently induced apoptosis (p53, LPS) only in a small percentage of cells (~20%) but, on the other hand, killed 100% of cells after 24h, suggests that only cells in a particular phase of cell cycle may be sensitive to NE. A possible explanation of the fact that these cells cannot be rescued from ETO, is that they are sensitive to NE only when they are in G2 phase, i.e. when ETO is also active. This hypothesis deserves to be confirmed in further studies.

The message of this work is that, in analogy with the immune system,[47,79-82] neural and neuroendocrine factors should be considered as important hematopoietic regulators. It is probable that beside catecholamines many other factors may affect hematopoiesis. For example, it has been reported that substance P and neurokinin-A may induce both positive and negative hematopoietic regulators in bone marrow stroma.[83,84] These findings indicate, in fact, that the endogenous release of multiple hematopoietic regulators may be controlled by neural or neuroendocrine factors. We might be before the tip of an iceberg representing a mechanism of hematopoietic regulation capable of transducing environmental information to the blood-forming system. This open new frontiers in the immunological field. A central question is whether the neural regulation of hematopoieisis plays any role in aplastic anemia, leukemia, immune-based diseases or during emergencies such as acute infections and/or stress events. Any positive answer to this question might provide a conceptual framework in which new pharmacological strategies to prevent or correct pathological situations could be devised.

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