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Catecholamine secretion in bovine chromaffin cells : influence of immune-derived factors

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CATECHOLAMINE SECRETION IN BOVINE CHROMAFFIN CELLS: INFLUENCE OF IMMUNE-DERIVED FACTORS

BY

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VITA

Zhaohui Wang, daughter of Jingyi Su and Jiabin Wang, was born on May 19, 1967, in Beijing, People's Republic of China.

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INTRODUCTION

Previous *in vivo* studies have demonstrated profound increases in plasma catecholamines (CA) in response to intravenous administration of bacterial endotoxin [1, 2, 3, 4]. Additional evidence suggests that such changes are due to non-neurogenic as well as an enhanced neurogenic release of epinephrine (Epi) from the adrenal medulla [5, 6, 7]. These reports imply that in response to septic insult, mechanisms appear to be activated to cause enhanced release of medullary catecholamines and thus, elevate the functional sympathetic output.

The immune system is at the forefront of whole animal defense mechanisms that are mobilized against septic challenge. One of the key immune responses to endotoxin is the production of cytokines, a class of inducible, water-soluble, heterogeneous proteinaceous mediators with molecular weights above 5000 kD. Cytokines are secreted mainly by cells of the mononuclear phagocyte system. Interleukin-1 (IL-1), Tumor Necrosis Factor (TNF), Interleukin-6 (IL-6) and some others are all known to be produced following the i.v. administration of bacterial endotoxin in experimental animals [8, 9]. Furthermore, the immune and neuroendocrine systems appear to communicate with each other by common signal molecules (i.e. cytokines) and receptors [10]. A growing body of evidence has shown that cytokines can act on neuroendocrine tissues, such as some areas of hypothalamus, pituitary, and adrenal cortex, etc [11, 12, 13].

The present study was designed to examine the possibility that the molecules (e.g. cytokines) produced by activated immunocompetent cells, are capable of modulating catecholamine secretion from chromaffin cells. To test this possibility, *in vitro*

experimental systems were used. Isolated bovine splenocytes were stimulated with mitogen to obtain cytokine-rich media which was then tested on the primary culture of bovine chromaffin cells (BCC). The experimental approach included three steps: 1) characterizing the chromaffin cell model in terms of functional changes (i.e. secretion) over days in culture; 2) using conditioned media, obtained from mitogen-stimulated bovine splenocyte culture, to demonstrate the possible role played by cytokines in modulating chromaffin cell secretion; and 3) examining whether the catecholamine release, induced by the conditioned media, is due to elevated exocytotic activity or damage of the cell membrane.

The results of this study demonstrate that immune-derived products from mitogen-activated splenocytes stimulate CA secretion from cultured BCC and enhance the nicotinic agonist induced release. This CA release does not appear to be related to cell membrane damage.

I. Sympathetic Activation in Septic Shock.

Sympathetic hyperactivity has been implicated in the pathogenesis of endotoxin (ET) shock [14]. In experimental models of ET shock, increased circulating catecholamines (CA) [1, 2, 3, 4], increased norepinephrine (NE) turnover rate in heart and spleen tissues [15] and depletion of CA content in sympathetically innervated organs at the end stage of shock [16, 17, 18] have been demonstrated. These findings are interpreted as evidence for increased CA release from both sympathetic nerve terminals and the adrenal medulla, and are considered to be directly related to increased sympathetic nerve activity from the central nervous system. Recent studies, however, suggest the existence of non-neurogenic stimulation of adrenal CA release following ET administration. In conscious adrenal denervated animals, plasma epinephrine (Epi) was elevated at 90 min post ET, although the amplitude was about one third of that observed in control rats given ET [6]. In addition, other experiments have shown that ganglionic blockade could not prevent the stimulation of adrenomedullary secretion during endotoxicosis [5]. *In vitro* incubation of adrenal chromaffin cells with endotoxin or endotoxin-elaborated agents, such as bradykinin, histamine and prostaglandins, resulted in CA secretion [19, 10, 21, 22]. These findings imply that adrenal CA secretion may be due in part to non-neurogenic mechanism. The cellular mechanisms mediating this non-neurogenic CA efflux remain largely unexplored.

A wide range of responses occur when ET interacts with mammalian host

defenses. In an older view, histamine, arachidonic acid metabolites and vasoactive peptides have been considered to be major mediators responsible for various pathophysiologic responses during endotoxin shock [23]. In addition, it has been generally accepted that circulatory hypodynamic responses to ET activate autonomic reflex adjustments with a concomitant increase in CA release from adrenal glands [23]. This response would tend to compensate for the early hypotensive effects of ET and improve perfusion pressure and circulation to vital organs. The efflux of CA, especially Epi, from adrenal medulla may be caused not only by the acceleration of efferent impulse traffic over sympathetic nerve pathways but also by the direct interaction of certain humoral factors with medullary chromaffin cells. The influence of humoral factors is supported by the presence of various receptors on bovine chromaffin cell membrane. Besides the nicotinic and muscarinic receptors, chromaffin cells possess histamine H1, prostaglandin E-2 (PGE2), vasoactive intestinal peptide (VIP), angiotensin II, atrial natriuretic peptide (ANP), bradykinin, endothelin, insulin-like growth factor 1 (IGF1) and ATP receptors, which have stimulatory effect on CA secretion upon binding to their agonists [24]. Therefore, ET-elaborated factors such as histamine, PG and some vasoactive peptides may play a role in non-neurogenic CA secretion from adrenal medulla in the course of developing septic shock.

II. Cytokines as the Mediators of Septic Shock.

The diverse spectrum of mediators elicited by ET leads to the complexity of biological responses [25]. Many effects of ET apparently result from its interaction with various components of host immune system. In particular, immune-derived cytokines

have attracted much attention in studies of the pathogenesis of gram-negative bacterial infection. Cytokines are a heterogeneous group of peptides that help sustain, amplify and regulate the cellular immune and inflammatory response to local infection [26]. They have endocrine, paracrine and autocrine roles in the inflammatory response and mediate changes that resemble aspects of the sepsis syndrome [27]. Monocytes release cytokines in response to appropriate stimuli, and the hormone-like secretory products diffuse to target cells, affecting their cellular activity. When the target cell is in the immediate vicinity, a paracrine function is invoked, whereas when the target cell is reached via the blood stream, the function becomes endocrine. At certain concentrations cytokine actions are protective, but their overproduction can cause deleterious changes in organs or tissues, in some cases resulting in death. Cytokines function as endogenous mediators of immunological, metabolic and physiological alterations during bacterial infections, which include inflammatory response, fever, metabolic and vascular changes, increased energy expenditure, anorexia and shock. Although the precise knowledge of the initial biochemical events after the bacterial invasion is not well established, much new information has emerged in the past five years.

A. Induction of Cytokine Expression by LPS.

The expression of cytokines appears to be tightly regulated. Little transcription of the cytokine genes takes place under normal conditions. A specific stimulus, bacterial or viral insult for instance, is usually required for induction of cytokine genes. Endotoxin, a component of the gram-negative bacterial cell wall, is composed of a polysaccharide portion and a lipid moiety called lipid A, and is generally referred to as

lipopolysaccharide (LPS). Biological analysis of the synthesized compounds has confirmed that lipid A is the active center of LPS [28]. Due to its amphipathic nature, LPS is able to interact with mammalian cell membrane nonspecifically by imbedding itself into phospholipid bilayer. Although such nonspecific interaction has been documented with a variety of cell types, the actual relevance to the cellular response remains to be determined. There is now strong experimental evidence to support the concept that specific receptors for lipid A do exist and are functional on mammalian lymphoreticular cells. For example, LPS of *E. coli* is able to stimulate TNF secretion from macrophages, while the nontoxic LPS of *Rhodoppseudomonas sphaeroides* was shown to block this production in a concentration-dependent manner [29]. Since the structure of lipid A from the nontoxic LPS is quite similar to that of its counterpart of *E. coli* LPS, the action of the non-toxic LPS is most likely explained by competitive binding to the specific receptors for lipid A. Similar phenomena were also observed in human monocytes [30] and neutrophils [31].

Morrison and colleagues have used a cross-linking approach to identify potential LPS receptors [32]. CD14, a 55-kD glycoprotein held on the mononuclear cells surface by a phosphotidylinostiol glycan anchor, has been identified as a receptor for LPS [33]. In fact, this receptor was able to recognize LPS only if it was complexed to LPS-binding protein (LBP) [33]. LBP is a serum protein synthesized in liver as an acute phase reactant [34]. LBP serves as an opsonin by binding stably to gram-negative bacteria [35]. The ligated CD14 may trigger the synthesis and secretion of cytokines including TNFα and IL-1 [36, 27]. The signal transduction pathway coupling stimulus-secretion

is unclear. It has been suggested that microtubule-associated protein may be involved, because taxol, a microtubule-disturbing agent could mimic cellular responses to LPS [37]. It is conceivable that LPS could affect the cell function by altering the activities of microtubule-associated kinases or phosphatases.

TNFα and IL-1 are produced in the early phases of sepsis [38]. IL-1 can regulate T cells and induces the production of IL-2 [39]. Both TNFα and IL-1 are able to induce the expression of IL-6 in monocytes, endothelial cells and fibroblasts [38, 40]. Despite the fact that TNFα, IL-1 and IL-6 are predominant in sepsis, other cytokines such as IL-8 and interferons (IFN) are also released as a sequel to endotoxemia [41, 42]. IFNγ is produced by lymphocytes following antigenic or mitogenic stimulation. Monocytes/macrophages are involved in the induction of IFNγ synthesis via IL-1. IL-1 probably stimulates T-cell IL-2 secretion, and IL-2 may in turn induce IFNγ release [43]. Mice infected with Gram-negative bacterial exhibited a transient increase if IFNγ in plasma between 2-4 days after infection. Spleen cells explanted from infected mice produced *in vitro* IFNγ spontaneously; this production was enhanced considerably by LPS or concanavalin A [44].

B. Cytokine Mediated Responses in Endotoxicosis.

TNFα, originally named cachectin, is a primary mediator in the pathogenesis of infection [27, 45]. Studies of genetic tolerance to endotoxins led to the identification of macrophage products as mediators of the physiological in response to bacterial infection. Monocytes from the C3H/HeJ strain of mice are not responsive to several bacterial endotoxins [46]. Their non-responsiveness results from a defect in LPS-mediated

activation of their macrophages. Macrophages isolated from LPS-resistant mice do not produce TNFα in response to LPS, while those from LPS responders exhibit high levels of TNFα secretion [47]. When serum from an endotoxin-sensitive mouse (C3H/HeN strain) is injected into a resistant mouse, it reproduces the changes induced in the sensitive mouse by endotoxin [48]. This observation led to the identification and later isolation of cachectin [49]. Experimental administration of bacterial endotoxin to human volunteers leads to a marked increase of TNFα in the plasma at 90 minutes [8]. the extent of symptoms, changes in white cells count, and production of ACTH were temporally related to the peak concentration of TNFα. Infusion of recombinant human TNFα into experimental animals produces effects similar to those manifested in Gram-negative sepsis [50]. Elevated plasma catecholamines have been observed as one of the evident responses to i.v. injection of TNFα in rats [51], indicating that the neuroendocrine function of sympathetic nerve and adrenal gland may be affected by TNF or TNF associated events. Passive immunization with various anti-TNFα antibodies protects the host from deleterious effects of both lethal and sublethal doses on endotoxin [52]. These results illustrate that TNFα is an important mediator of pathophysiological alterations characteristic of sepsis syndrome. Although antibodies against TNFα reduce its effect, they do not completely abrogate the alterations induced by endotoxin [52]. This suggests that other endogenous factors may be involved.

TNFα can induce the production of IL-1 and *vice versa*; thus using *in vivo* experimental models, it is impossible to determine whether the actions of TNFα or IL-1 are primary or secondary [53]. IL-1, which has many biological activities similar to

TNFα, has been shown to induce a shock-like state in synergism with TNF in rabbits [54]. The two classic activities associated with IL-1 are fever production and lymphocyte activation, which have been confirmed by using a well-defined recombinant form of human IL-1 [55]. Other activities include promoting coagulation and enhancing the hepatic production of acute phase proteins [25].

Another important inductor of the acute phase protein synthesis is IL-6, which is closely related to the inflammatory response. In the lethal baboon *E. coli* shock model, IL-6 was detectable within two hours post bacterial infusion, and steadily increased during the first 8 hours [56]. In patients with meningococcal infections, IL-6 levels have been associated with fatality [57].

Other cytokines, such as IFNγ and IL-8, were also detected in severe septic states [38, 9], but their exact roles are unclarified. Macrophages from rats pretreated *in vivo* with recombinant IFNγ, released higher amounts of TNFα in response to LPS than the control group did [58]. IL-1 expression induced by ET was also subject to the modification of IFNγ. LPS stimulated about a four-fold increase in IL-1 mRNA in the human monocyte cell line preincubated with IFNγ [59]. These results provide evidence that IFNγ may be involved in the development of septic syndromes at least through its action on macrophages. Collectively, cytokines play a key role in gram-negative sepsis or shock and they are part of the immune response in all vertebrates [38, 6].

C. Cellular Mechanisms of Cytokine Actions.

Cytokines, like hormones, exercise their effects at extremely low concentrations via cell receptors [43]. An individual cytokine interacts in a highly specific manner with

its cell receptor. All cytokine receptors are transmembrane glycoproteins made up of an extracellular amino-terminal ligand binding domain, a short hydrophobic transmembrane region and a carboxy-terminal intracellular domain. Signal transmission across the plasma membrane to activate the functional domain of the receptor is poorly understood. The means by which cytokine-receptor interact with intracellular signalling pathways also remain to be elucidated. However, there is experimental work that strongly implicates the requirement for calcium ions, high-energy nucleotide intermediates (e.g. GTP, cAMP and phospholipid derivatives, etc.) and protein kinases and phosphatases in the transmitting process [61, 62]. At present, at least two signal transduction pathways have been described that appear to be related to hormone- or cytokine-mediated cell responses [60]. One of these involves G-protein, adenylate cyclase, protein kinase A and calmodulin. In the other pathway, the receptor molecules themselves contain a tyrosine kinase which, when activated, subsequently stimulates phospholipase C (PLC), possibly also through G protein intermediates. Plasma membrane inositol phospholipids are then broken down by PLC into inositol triphosphate and diacylglycerol which are active intermediates for intracellular Ca^{2+} release and protein kinase C (PKC) activation, respectively. PKC has a number of potential protein substrates, thus it can alter many intracellular functions.

Intracellular events associated with the binding of TNFα to its receptor include the phosphorylation of tyrosine residues on a stress protein [63] and on the epidermal growth factor receptor [64]. Protein phosphorylation suggests an early effect on protein kinases involved in signal transduction. A transient increase in cAMP after the activation

of both TNFα and IL-1 receptors [65] has been reported. The interaction of IL-6 with its receptor has not been found to induce any known conventional biochemical processes, such as protein phosphorylation, phosphatidylinositol turnover or intracellular Ca^{2+} increase. A novel transducer protein may be involved in the event immediately after ligand-binding [66].

IFNγ receptors are single chain glycoproteins that bind ligand with high affinity in a species specific manner [67]. There appears to be only a single type of IFNγ receptor that is expressed on nearly all cell types. Considerable data indicates that IFNγ induces cellular responses via Ca^{2+} and possible protein kinase C-dependent mechanisms [68, 69, 70]. Exposure of murine and human macrophage populations to the combination of a protein kinase C activator and a calcium ionophore leads to the induction of many of the same cellular responses as are induced by IFNγ. Calcium influx has also been observed on cells exposed to IFNγ with $^{45}CaCl_2$ [70]. An inhibitor of intracellular Ca^{2+} release from endoplasmic reticulum was not able to block the cell response to IFNγ, while depletion of calcium from culture medium by EGTA could. These results suggest an existence of receptor-mediated calcium channel.

III. Neuroendocrine-immune Interactions.

From research conducted over the past 10 years, complex interrelationships among neural, endocrine and immune processes have become apparent [10]. Neuroendocrine and immune systems are intimately linked and communicate with each other through the use of common signal molecules and receptors [71]. During the course of an immune response hormonal changes occur [72]. Conversely, the activation of the

neuroendocrine system during stress leads to changes in immune function, such as inhibition of the production and action of several cytokines [73, 74]. These findings provide the basis for the interesting concept that the immune system may serve as a sensory organ [11, 75], which recognize non-self or altered-self antigens. The recognition of such stimuli is consequently converted into the form of cytokines and other messengers which can be received by neuroendocrine tissues [76] and thus, to induce an integrative reaction.

Corticotropin (ACTH) was the first *de novo* synthesized hormone to be found in the immune system by J.E. Blalock, et al. [77]. ACTH is a 39-amino acid peptide whose production is primarily associated with the pituitary gland and whose classical action is to elicit a glucocorticoid response from the adrenal cortex during times of stress. Human peripheral blood lymphocytes and mouse spleen cells were initially observed to express an ACTH-like peptide following virus infection or interaction with bacterial LPS [42, 78]. Consistent with this observation has been the work of Besedovsky and colleagues. They found that blood levels of glucocorticoids increase at the time of peak immune response to several antigens [72]. Similar to that observed in immunized animals, injection of lymphokine-containing supernatants from concanavalin A-activated spleen cells could mimic the events occurring after antigen injection [79]. Such increases were attributed in part to IL-1 [13, 80] and IL-2 [81] instead of immune-derived ACTH-like peptide. As soon as cytokines became available in pure form, they were tested individually. Intraperitoneal administration of recombinant IL-1 in rats elicited an increased CRF (corticotropin releasing factor) secretion and a slight elevation of plasma

Epi and NE levels [13]. IL-1 may work at any level of the hypothalamo-pituitary-adrenal (HPA) axis. Although it is clear that IL-1 induced HPA activation involves an increased secretion of CRF from hypothalamus [82, 83, 84], effects of IL-1 on hormone release from pituitary [85, 86] and adrenal cortex [87, 88] have also been demonstrated.

Besides IL-1, such cytokines as IL-2, IL-6 and TNF were also found to activate the HPA [89, 90, 91, 92], but they are less potent and less persistent [12]. IL-6 and ACTH have been demonstrated to act synergistically to stimulate the release of corticosterone from adrenal gland cells [93] in culture. These observations clearly illustrate that cytokines play an important role in mediating immune-neuroendocrine communication.

Unlike classical hormones whose receptors are generally restricted in certain tissues, cytokine receptors can be found in virtually all cell types [61]. Specific anti-interleukin receptor monoclonal antibodies can block the behavioral, brain electrical activity and body temperature changes induced by microinfusion of IL-1, IL-2 and IL-3 in the rat brain [94]. Therefore, these interleukin-caused effects are most likely receptor-mediated. Recent studies with radiolabeled IL-1 have shown that there are high-affinity binding sites for IL-1 in certain areas of rodent brain [95, 96, 97]. IL-2 receptors have been identified in human brain under pathological conditions [98]. The expression of IL-2 receptors on pituitary cells was also detected by the monoclonal antibody-binding and by electrophoretic analysis [99]. Structurally, the interferons (IFNs) resemble glycoprotein hormones both in composition and approximate size [100]. Virtually all nucleated cells within a species are sensitive to that species IFN and presumably possess

IFN receptors [101]. Collectively, the presence of interleukins receptors in the CNS and pituitary [102 ,103, 104, 105] is supportive evidence for the communicative role of cytokines between neuroendocrine and immune systems. Since the adrenal medulla is of neural crest origin, expression of cytokine receptors on medullary chromaffin cells is quite likely.

IV. Possible Role of Cytokines in the CA Secretion from Adrenal Medulla.

The existence of signals originating from the immune system to bring about neuroendocrine response has only recently begun to be evaluated. No work has been reported that examines the potential action of cytokines to influence adrenal medullary CA secretion. One single report indicates that IL-1 stimulated corticosterone release occurs through the local CA outflow from the rat adrenal gland (A.R.Gwosdow, et al. Abstract, FASEB meeting 1992). This work suggests that IL-1 can cause CA secretion from chromaffin cells. In contrast, numerous investigations have been conducted to assess the impact of CA on immunological activities [10]. Spengler, et al. (1990) have demonstrated that α-2 adrenergic agonist can augment LPS-stimulated TNF from elicited macrophage [106]. The increase in TNF production was concentration-dependent. The presence of a macrophage α-adrenergic receptor was detected by binding of the specific antagonist ^{3}H-yohimbine. These investigations reveal the role of CA that may regulate cytokine production during an inflammatory response. Conversely, cytokines may carry messages in the opposite direction by acting on the sympathoadrenal system.

Receptors for growth hormone share large degree of homology with cytokine receptors, especially within the extracellular sequence [107]. IGF1 (Insulin-like growth

factor 1) receptor is present on bovine chromaffin cells [108]. It is possible that cytokines may influence the secretion of adrenal chromaffin cells through either IGF1 receptors or more specifically through cytokine receptors if they were actually there. The complete signaling loop between immune and sympathoadrenal systems is anticipated. There is evidence that the immune-HPA axis regulatory loop is not the only one involving cross system interactions [12]. The communicating circuit between the two systems might possibly be complemented by immune-derived products, cytokines for instance, acting on the sympathoadrenal end.

Under physiological conditions, CA is contained in the membrane-bound vesicles beneath the plasma membrane and is released through exocytosis following nicotinic and/or muscarinic activation. Nicotinic stimulation results in the influx of cation through ion pore within the receptor itself which then depolarizes the cell. Voltage-dependent Na^+ and Ca^{2+} channels are opened by depolarization. Exocytosis occurs immediately after the rise of $[CA^{2+}]_i$ due to Ca^{2+} entry and calcium-induced calcium release from internal stores. Muscarinic stimulation leads to the production of IP_3 which mobilizes Ca^{2+} from intracellular Ca^{2+} pools. Calcium from IP_3-sensitive pool does not appear to trigger exocytosis in most species. In resting cells, chromaffin granules are bound within a cytoskeletal matrix and are unable to reach the secretory sites. Cortical actin cytoskeleton plays a role in the control of exocytosis by acting as a barrier to secretory granule movement. Disassembly of the cytoskeleton net is necessary, but not sufficient, for a full secretory response. Calcium and/or protein kinase C are involved in bringing about actin disassembly/reorganization, but the exact mechanism is unclear. Several

actin-regulatory proteins that are possible targets for the action of Ca^{2+} and PKC are present in chromaffin cells. Calcium entry and the subsequent rise of $[Ca^{2+}]_i$ is essential and sufficient for exocytosis, whereas PKC is able to modulate this process by controlling Ca^{2+}-sensitivity [24]. As aforementioned, the activation of cytokine receptors may be associated with increases of intracellular calcium levels and protein kinase C activity. Cytokines would be capable of activating the intracellular signals that lead to exocytosis if cytokine receptors existed on chromaffin cells.

Cytokine production is a dramatic whole-body response when immune components are confronted with bacterial insult. Non-neurogenic CA output from adrenal medulla may be an important component of the sympathetic response to sepsis but mediators causing this release are unknown. Based on the knowledge reviewed above, cytokines may play a role in stimulating CA secretion of adrenal chromaffin cells. This thesis project examines this possibility through an initial set of experiments involving immune-derived factors that are applied to chromaffin cells *in vitro*.

I. Bovine Chromaffin Cell Isolation and Culture.

Adult bovine adrenal glands obtained from a local supplier are kept in room temperature buffer during transportation to the laboratory. Glands are trimmed of fat and rinsed through the adrenal vein with Ca^{2+}- and Mg^{2+}-free buffer to remove all blood. Cell isolation is achieved by 0.17% collagenase P (Boehringer Mannheim Biochemicals) digestion (basic procedure by Fenwick et al [109], modified by Greenberg and Zinder [110]). Three to five milliliters of freshly prepared collagenase solution is infused through the adrenal vein followed by incubation of the gland in additional collagenase at 37 °C for 15 min. This step is repeated. The gland is then cut in half sagittally and the medullary contents are removed with forceps, minced and rinsed through 200 μm nylon mesh with additional buffer. The isolated cells are rinsed to remove collagenase and saved in buffer at 37 °C. Tissue remaining on the mesh is incubated for 30 min with additional collagenase containing DNAse followed by filtration through clean mesh and removal of collagenase. All chromaffin cells are combined and layered onto a 1:1 mixture of complete Lockes solution and fetal calf serum for density gradient centrifugation [111]. Following low speed (50 x g) centrifugation, cells are resuspended in complete Lockes solution and the density purification repeated. Determination of viability and purity is achieved by using trypan blue exclusion and neutral red staining, respectively. Cells are counted in a hemacytometer.

Cells are plated on collagen-coated 24-well plates at a density of 6 x 10^5 cells per

2 cm^2 in 1 ml culture media. The media contains a 1:1 mixture of Dulbecco's modified Eagle medium and F12 supplemented by 10% fetal calf serum, 9 mM HEPES, 3.2 mM glucose, 2 mM glutamine, 100 μg/ml penicillin G, 20 μg/ml gentamicin, 50 U/ml nystatin, 2.4 μg/ml 5-FDU and 2.8 μg/ml Ara-C. The media is changed the next day and then every other day. Cells are maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

II. Preparation of Conditioned Media.

1) Isolation of bovine spleen cells:

Bovine spleen is obtained from a local supplier and brought to the laboratory at room temperature. Several tissue pieces are excised from interior of the organ. Cells are dispersed by pushing them through a 520 μm stainless steel mesh in Hank's solution with a plunger from 30 ml syringe. The cell suspension is diluted with Hank's, layered over a Lymphocyte Separation Medium (LSM, Ficoll, Organon Teknika), and centrifuged at 400 x g for 30 min. Lymphocytes and other mononuclear cells (platelets and monocytes) move to the Hank's-LSM interface, and are then recovered by aspirating the layer. The recovered cells are washed twice in Hank's media and centrifuged at 210 x g for 10 min. The cells are finally resuspended in 10 ml RPMI 1640 culture media.

2) PHA conditioned culture:

The lymphocytes are cultured at a concentration of 1-2 x 10^6cells/ml in RPMI 1640 supplemented with 10% FCS (heat inactivated), MEM non-essential amino acids, 4 mM Glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. They are incubated with or without 0.5% phytohemagglutinin (PHA) for 24 hr in 5% CO_2 atmosphere at 37

°C. The conditioned media is then separated from the cells and saved in -20 °C freezer for future experiments.

III. Experimental Protocol.

1) Functional Characterization:

Chromaffin cells are maintained for 3 different periods (1, 4 and 8 days) prior to secretion experiments. Typically, serum containing media is discarded and replaced by Earle's balanced salts solution (EBSS) which is supplemented with 25 mM HEPES, 0.1 mM EDTA and 1.8 mM Ca^{2+}. Incubation in EBSS (0.5 ml/well) is maintained for 30 min. at 37 °C, then the media is removed, chilled, centrifuged and acidified with $HClO_4$ to a final concentration of 0.4 M and 0.1 mM EDTA. The samples are kept at -70 °C for later determination of catecholamines. Catecholamine content in these samples represents spontaneous (corresponding to 'non-neurogenic' *in vivo*) release during the 30 min. incubation period. Immediately following removal of EBSS, the cells are exposed to nicotinic agonist DMPP (dimethylphenylpiperazinium) at 0, 1, 5 or 10 μM in EBSS. After 10 min., the secretagogue-containing media is removed and processed as before. Each well is then flooded with 1.0 M $HClO_4$ with 0.1 mM EDTA and this is used to determine the remaining catecholamine content of the cells.

2) Experiments with PHA conditioned media:

The experiments are carried out 3 or 4 days after chromaffin cell isolation.

(i) Effect of PHA conditioned media on CA secretion from BCC: BCC are incubated with supplemented RPMI 1640 either with or without addition of PHA, or splenocyte culture media either with or without PHA stimulation. After 90-min incubation, the

supernatants are removed and saved, and chromaffin cells are then exposed to 3 μM DMPP for 10 minutes in the presence of corresponding media used during previous incubation.

(ii) Dose-dependent effect of PHA conditioned media: After removal of BCC culture media, the cells are incubated with different dilutions of conditioned media (1:99, 1:9, 1:1 in supplemented RPMI 1640, and full strength) at 37 °C for 90 min. Samples are then collected and saved. Immediately after this incubation, cells are exposed to 3 μM DMPP for 10 min. DMPP is dissolved in the corresponding preincubation media.

(iii) Time-dependent effect of PHA conditioned media: Cells are incubated with full strength conditioned extract for different times (10, 30, 60, 90, 150, 210 min) followed by 10 min DMPP stimulation at 3 μM. Supernatants are collected before and after DMPP stimulation.

3) Test of Exocytotic Activity vs Leakiness:

Chromaffin cells are incubated with PHA conditioned media, splenocyte culture media without PHA stimulation, fresh RPMI 1640 media with or without addition of PHA, DMPP (in RPMI 1640), or 20 μM Digitonin at 37 °C for 90 min. The supernatants are then collected and saved for LDH assay. Samples digitonin-permeabilized cells which leak LDH into the media are used as positive experimental control.

All experiments of this project are conducted using triplicate samples for each condition, and repeated on at least 3 different chromaffin cell preparations.

IV. Catecholamine Assay.

Secretion of epinephrine and norepinephrine is determined by HPLC with electrochemical detection. HPLC mobile phase consists of 0.1 M monochloroacetic acid, 1 mM sodium octyl sulfate, 0.5 mM Na_2-EDTA, and 1.5% acetonitrile at pH 3.0. The column is a 10 cm OSD Biophase II (BAS, West Lafayette, IN), and the working electrode is glassy carbon with a model LC-4B amperometric detector (BAS). Before the assay, the samples are filtered through nylon membrane with 0.22 μm pore size.

V. Lactate Dehydrogenase Assay.

LDH is measured by monitoring the formation of NADH from NAD at 340 nm with spectrophotometer. LDH was determined with the generous cooperation of Clinical Chemistry of LUMC.

VI. Statistics.

The basic test statistic used was an one-way analysis of variances (ANOVA). Significant differences indicated by ANOVA were followed by Newman-Kuels test for individual comparisons.

Data from BCC characterization underwent transformation and was shown to be normally distributed. The effect of dose and time was tested with two-way ANOVA followed by Tucky's test for individual comparison.

I. Characterization of Isolated Bovine Chromaffin Cells in Primary Culture.

The quality of bovine chromaffin cell (BCC) preparations was indicated by the viability and purity of the isolated cells (Table 1), which were detected with trypan blue exclusion and neutral red stain respectively. Typically, 50-100 million cells were harvested from one adrenal gland with the variation most likely linked to the gland size. The percentage of viable cells was consistently high (93 $\pm$ 2 %), while the purity ranged at 90-95% in 15 out of 18 preparations.

In order to characterize functional changes of cultured BCC over time, cultures were maintained for 1, 4 or 8 days before the secretion response to DMPP (nicotinic agonist) was tested. Cells were stimulated with 1, 5 and 10 μM DMPP for 10 minutes. In Figure 1, catecholamine (CA) secretion was expressed as the absolute amount from each well, containing 600,000 cells. Between 1-5 μM, there is over a five fold increase in response to the agonist. There was no significant increase of secretion when the dose was raised from 5 to 10 μM. At the level of 10 μM DMPP, saturation started to occur and was confirmed with higher concentrations (data not shown). There was significant interaction between dose (DMPP) and time (days in culture) effects for both Epi and NE secretion. This suggests that the culture duration influences the dose-response relation of nicotinic-mediated secretion. It appeared that the longer the cells had been cultured, the more responsive they were to nicotinic stimulation. The differences were apparent when comparisons were made between 8- or 4-day vs 1-day groups. Statistical analysis

confirmed the differences with different culture durations at 1 μM DMPP for both Epi and NE secretion. The overall effect of culture duration on combined Epi and NE secretion irrespective to DMPP concentration were significant between 8- or 4-day vs 1-day, though there was no interaction. This indicates that the curves of the combined Epi and NE secretion are shifted parallel to the left.

To correct for the possible variations between BCC preparations and differences in the number of cells in different wells, catecholamine secretion from each well was normalized to its own total cellular content. In Figure 2, the percentage of total Epi, NE or both, released in response to DMPP, is displayed. The pattern of dose-response curves is very similar to that in Figure 1, while the apparent change across time appears more obvious. The overall effect of culture duration on percent CA secretion is significant between 8- or 4-day vs 1-day regardless of DMPP concentration. After BCC had been cultured for several days, 5 μM DMPP induced up to 17% secretion of NE and 12% of Epi, whereas only 5-6% was induced by 5 μM one day after isolation. The cells underwent a bigger change during the first four days in culture than they did in the second four days. Within the dose range examined, the maximal release evoked by 10 μM DMPP was around 15% for epi and 20% for NE.

Total cellular CA content are also compared between cells maintained in culture for varying durations (Figure 3). The total catecholamine content stores in each well decreased over time. Epi content counted for roughly 75% of CA storage in chromaffin cells. The greatest decrease of Epi was seen during the initial four days (15% of initial content) and there was a 20% drop after eight days in culture. Statistical differences in

TABLE 1

SUMMARY OF BCC PREPARATIONS

DATE	HARVEST (10^6 cells)	VIABILITY (%)	PURITY (%)
5/7/92	60	93.5	90
	48	96	94
4/14/92	75	95	85
3/30/92	80	95	91
3/23/92	48.5	95.5	91.5
3/9/92	85	92	91.5
2/27/92	100	91.6	73
2/13/92	93.5	90.5	92.7
2/3/92	78	92	94.4
1/23/92	33.4	93.5	90.6
	88.5	95	92
1/16/92	41	91	91
12/19/91	90	91.2	91
12/5/91	61.8	94	83
11/21/91	61.6	89	89.6
11/14/91	20	93	92
11/11/91	110	98	94
10/31/91	90	90	95
MEAN		93.3	91.7
SD		2.28	4.18

BCC: Bovine Chromaffin Cells

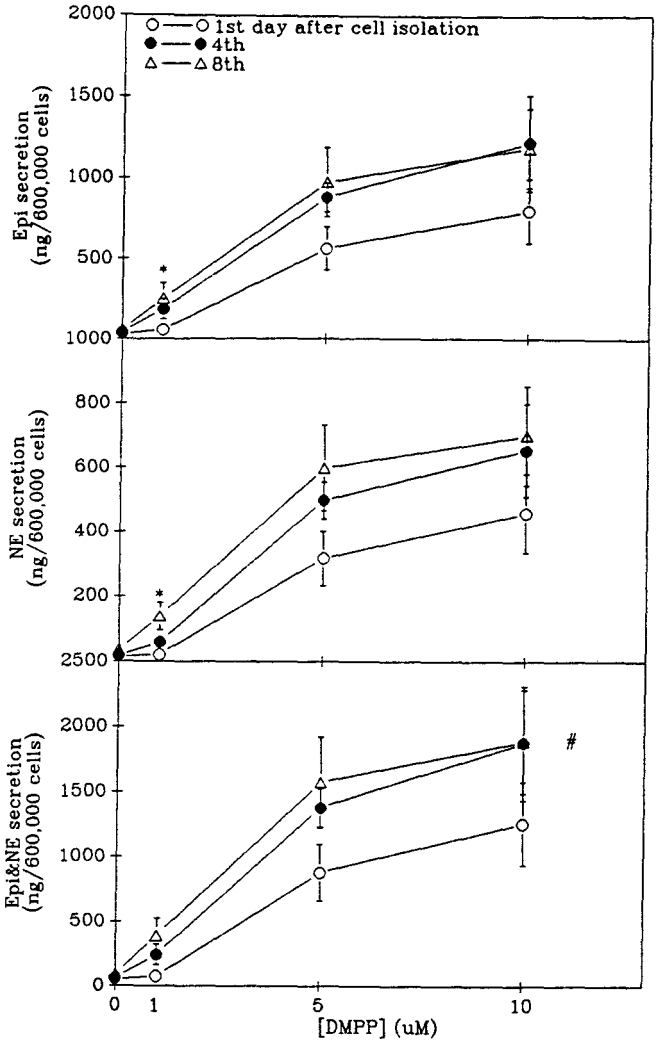

Figure 1. Changes in DMPP-mediated CA secretion over time in culture. BCC were exposed to 0, 1, 5 and 10 μM DMPP after 1, 4 or 8 days in culture. For each dosage, secretion was determined in triplicate wells. The same protocol was repeated with four different adrenal glands (N=4). The data points are means $\pm$ SEM. Significant differences exist between 10 or 5 vs 1 μM DMPP in all plots (not marked). *, $P < 0.05$ for 8- or 4-day vs 1-day at that dose. The overall means of Epi&NE secretion at 8- or 4-day are significantly different from 1-day irrespective of DMPP concentration ($P < 0.05$, not marked).

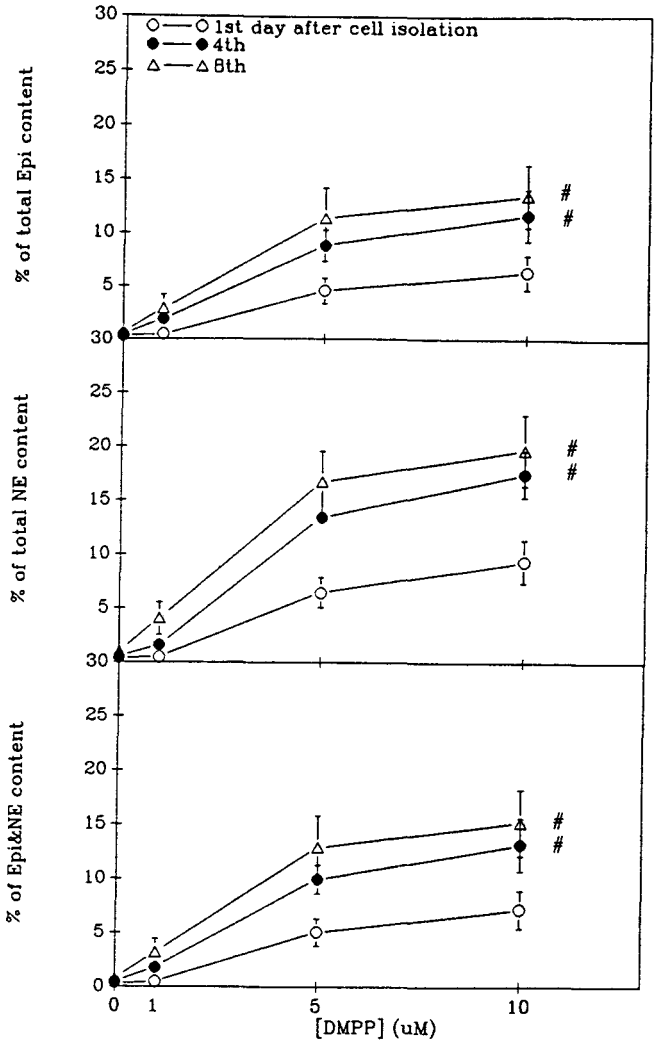

Figure 2. Changes in DMPP-mediated CA secretion over culture time. The secretion data shown in Fig-1 was normalized to the corresponding total cellular content, *i.e.*, secretion was expressed as % of initial total. The overall means of 10 or 5 μM are significantly different from 1 μM irrespective of time ($P < 0.05$, not marked). The overall effect of culture duration is significant betwee 8- or 4-day vs 1-day irrespective of dose (#, $P < 0.05$).

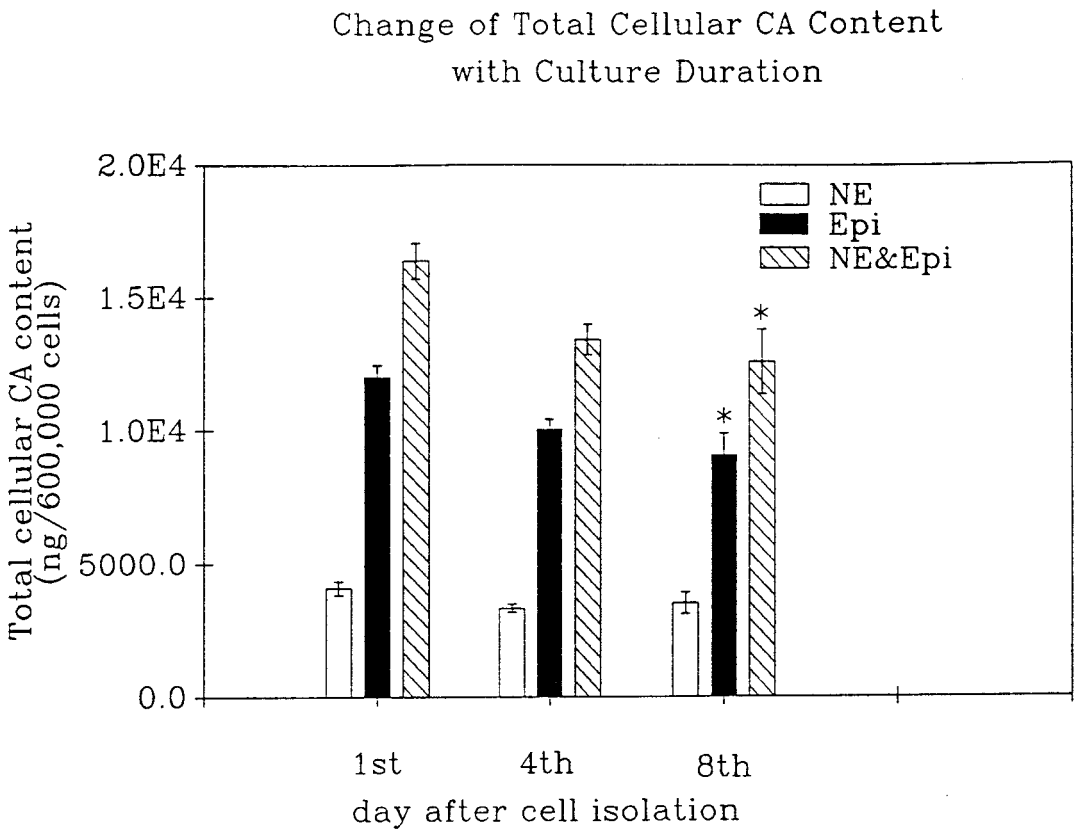

Figure 3. Changes of cellular catecholamine content over time in culture. Total NE, Epi and combined NE&Epi contents were measured 1, 4 or 8 days after cell isolation. Values are Means ± SEM. Four triplicate determinations were made in each of four BCC preparations (N=4).

Epi or combined Epi and NE could only be detected between 8- and 1-day. In general, total CA content was 13,000 - 16,000 ng/0.6 x 10^6 cells. Based on changes in the functional characteristics, all the following experiments were conducted on 3-5 days old BCC.

II. Effect of the Culture Media from PHA-stimulated Splenocytes on CA Secretion

Mononuclear cells, which include B and T lymphocytes, monocytes/macrophages and platelets, were isolated from bovine spleen and cultured in the presence (Stim-Extract) or absence (NonStim-Extract) of 0.5% (v/v) PHA. After 24 hours, the culture supernatants were collected. Chromaffin cells, incubated with the PHA-conditioned media for 90 minutes, released over 20% each of their Epi or NE content which is 4-5 fold more than that of the control groups (Figure 4). In contrast to the secretory response to nicotinic agonist, this conditioned media induced slightly more Epi release than NE in terms of percent secretion. The PHA-conditioned media exerted its effect in a concentration-dependent manner (Figure 5). One to one dilution of the original supernatant induced a secretion response at about half of the maximal magnitude, which was still a statistically significant increase as compared to that of the group treated with non-stimulated extract.

PHA-conditioned media not only caused the CA release of chromaffin cells, but also enhanced the secretion resulting from nicotinic activation. Immediately after the first 90-minute incubation with different media, the cells were exposed to DMPP. As demonstrated in Figure 6, 3 μM DMPP in control media brought about 3-4% secretion of Epi or NE during a 10-minute incubation, while the same concentration of DMPP in

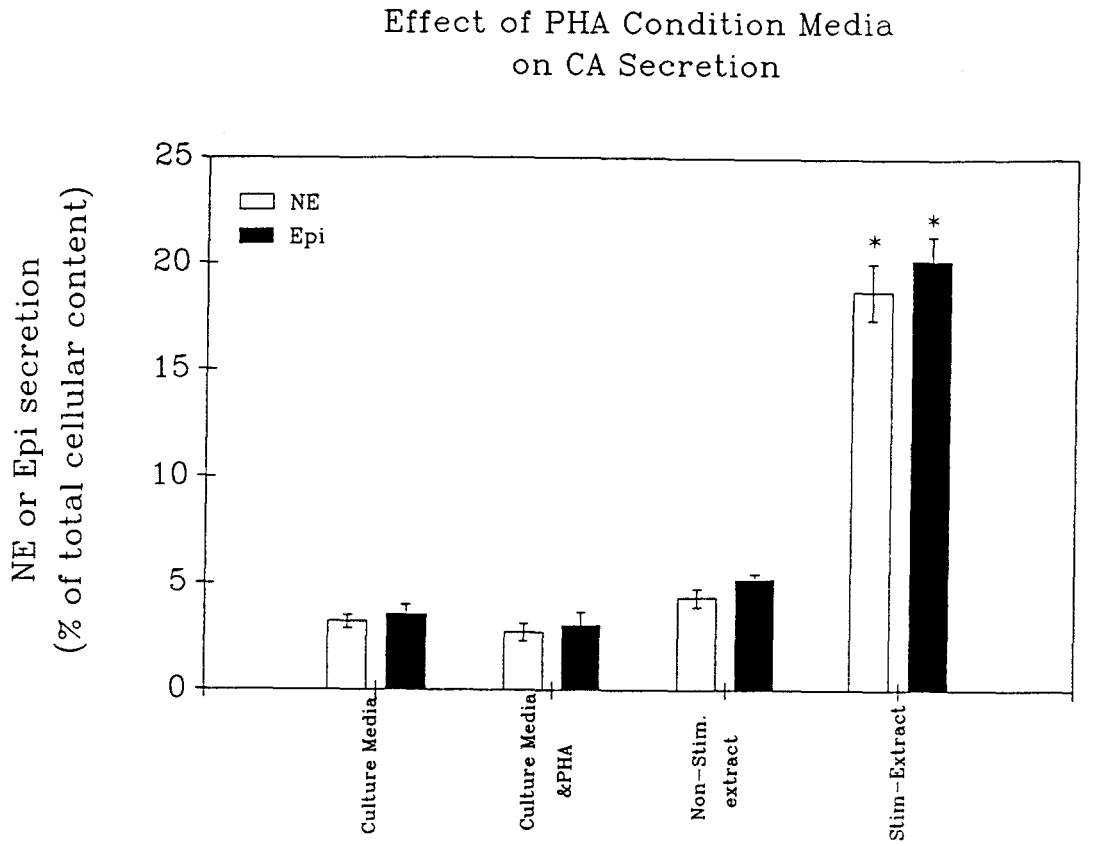

Figure 4. Effect of conditioned-media on CA secretion. After 3-5 days in culture, chromaffin cells were treated with supernatants from 24-hour splenocyte cultures with or without PHA stimulation, and fresh RPMI 1640 with or without addition of PHA. After 90 minutes, media was collected and saved for NE and Epi assay. Two spleen preparations (N=2) were tested with four BCC preparations (N=4). Each condition was performed in a triplicate. *, $P < 0.05$ compared to all the other groups.

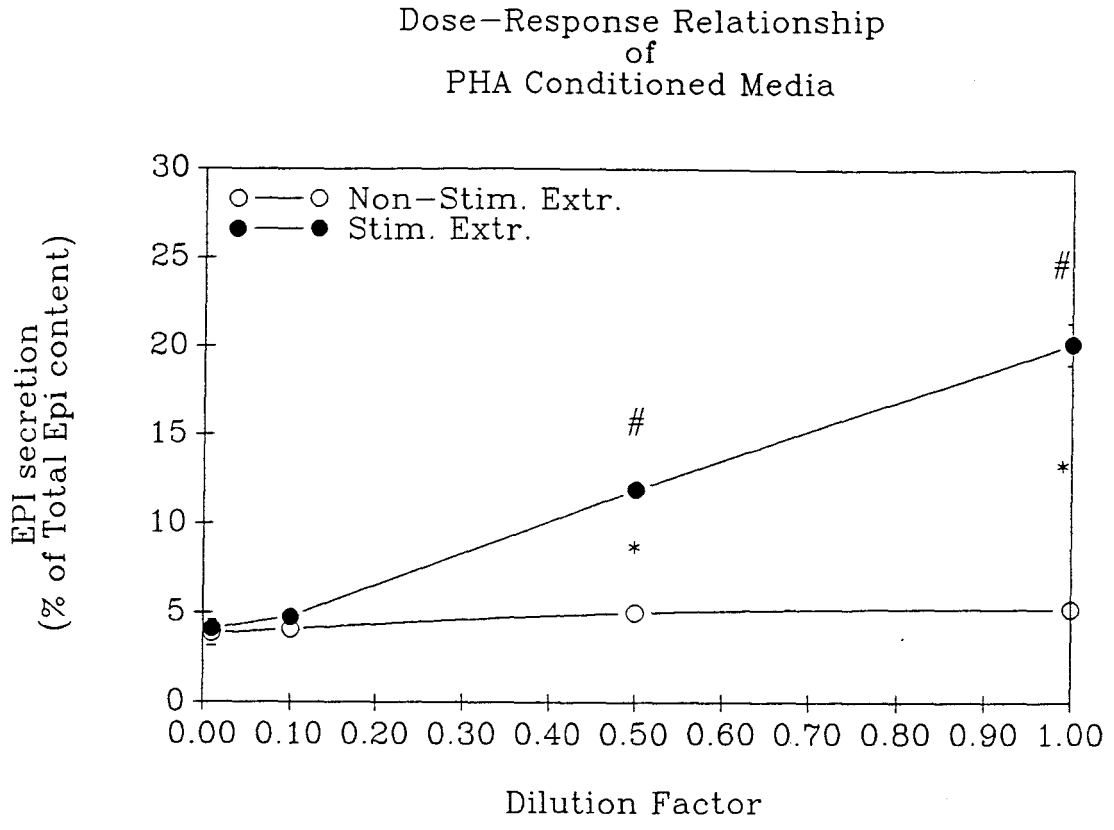

Figure 5. Concentration-dependent effect of conditioned media on Epi secretion. Different dilutions of PHA-conditioned media were tested on chromaffin cells with 90-min incubation. The concentrations were 1:99 (0.01), 1:9 (0.1), 1:1 (0.5) and full strength (1.0). *, $P < 0.05$ for t-test comparison between Stim- vs NonStim- Extract. #, $P < 0.05$ for individual comparisons following ANOVA of Stim-Extr across dilutions; marked points are significantly different from those at any other dilutions. Two splenocyte (N=2) and four BCC (N=4) preparations were used. Triplicate determinations for each condition.

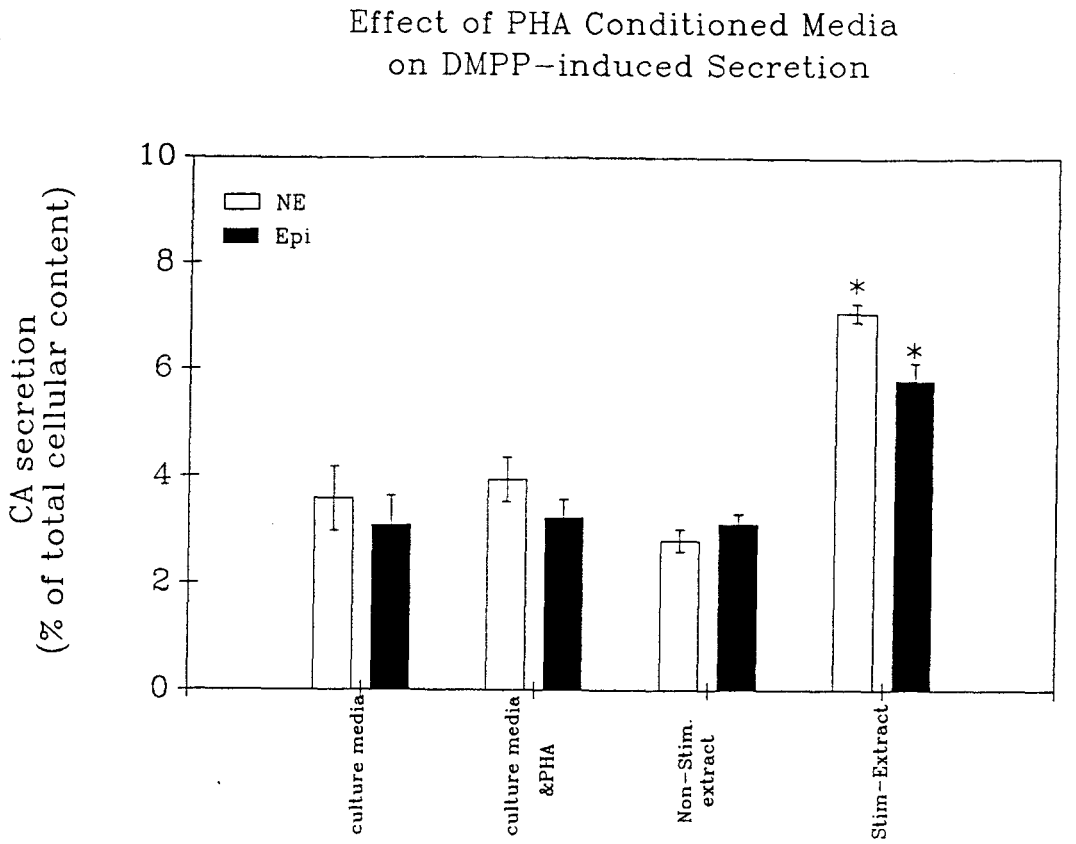

Figure 6. Effect of conditioned media on DMPP-mediated CA secretion. Immediately following the 90-min incubation with various media, chromaffin cells were stimulated with 3 μM DMPP in the presence of corresponding incubation media for 10 minutes. CA secretion from cells treated with PHA conditioned media was significantly enhanced in comparison to all the others (*$P < 0.05$).

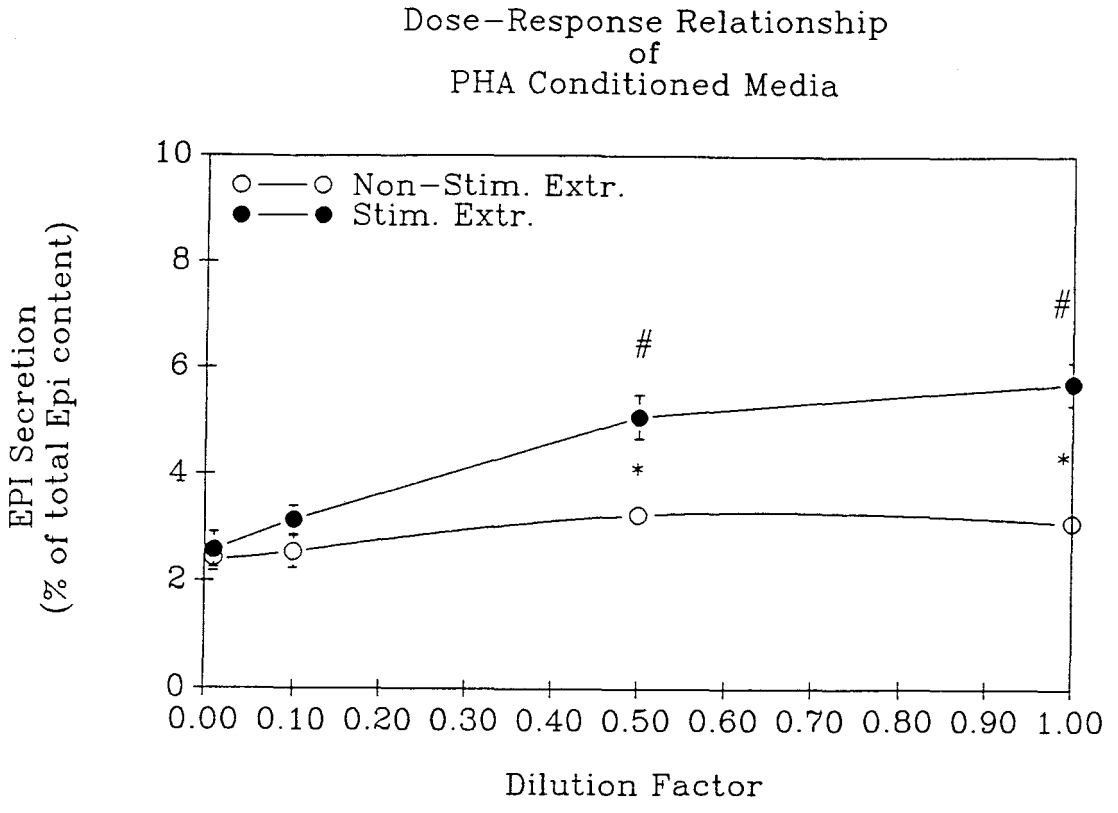

Figure 7. Concentration-dependent effect of conditioned media on DMPP-induced Epi secretion. Following 90-min exposure to different dilutions of conditioned media, Epi secretion in response to 3 μM DMPP were examined in the presence of corresponding different dilutions of conditioned media. *, $P < 0.05$, t-test comparison between Non-Stim and Stim Extracts at each dilution. #, $P < 0.05$ for individual comparison following ANOVA of Stim-Extr across dilutions; significant differences are present between 1.0 or 0.5 vs 0.1 or 0.01.

the presence of PHA-conditioned media doubled the release. This enhancement of DMPP-stimulated secretion was also concentration-dependent (Figure 7). Statistical differences across different dilutions of conditioned media occurred between any two concentrations except between 0.1 vs 0.01.

Figure 8 contains the results of the time course of Epi secretion in response to PHA-conditioned extract. Six different incubation times ranging from 10 to 210 minutes were examined. Within the first 60 minutes, Epi was released to the media at a higher rate than that during the later phase. There was a transient plateau between 60 to 90 minutes, then chromaffin cells continued to secrete at a relatively constant rate during the 2 hours after the initial 90-minute period. NE secretion also followed the similar pattern (data not shown). At the end of 210-minute, up to 30% of the cellular Epi storage was secreted. To examine if pre-incubation duration effects the DMPP evoked secretion, the chromaffin cells were stimulated with 3 μM DMPP in the presence of conditioned media after they had been incubated for various times as described above. No matter how long the cells were pretreated with the extract of PHA activated splenocytes, the following DMPP stimulation was not affected.

Results of the nine splenocyte preparations are summarized in Table 2. The potency of the conditioned media from these mitogen-stimulated cells was variable. Four preparations were relatively more potent than the others in terms of their ability to cause CA release. The difference in CA response between extracts from the cultures with or without PHA stimulation was demonstrated in only one of the spleen preparations.

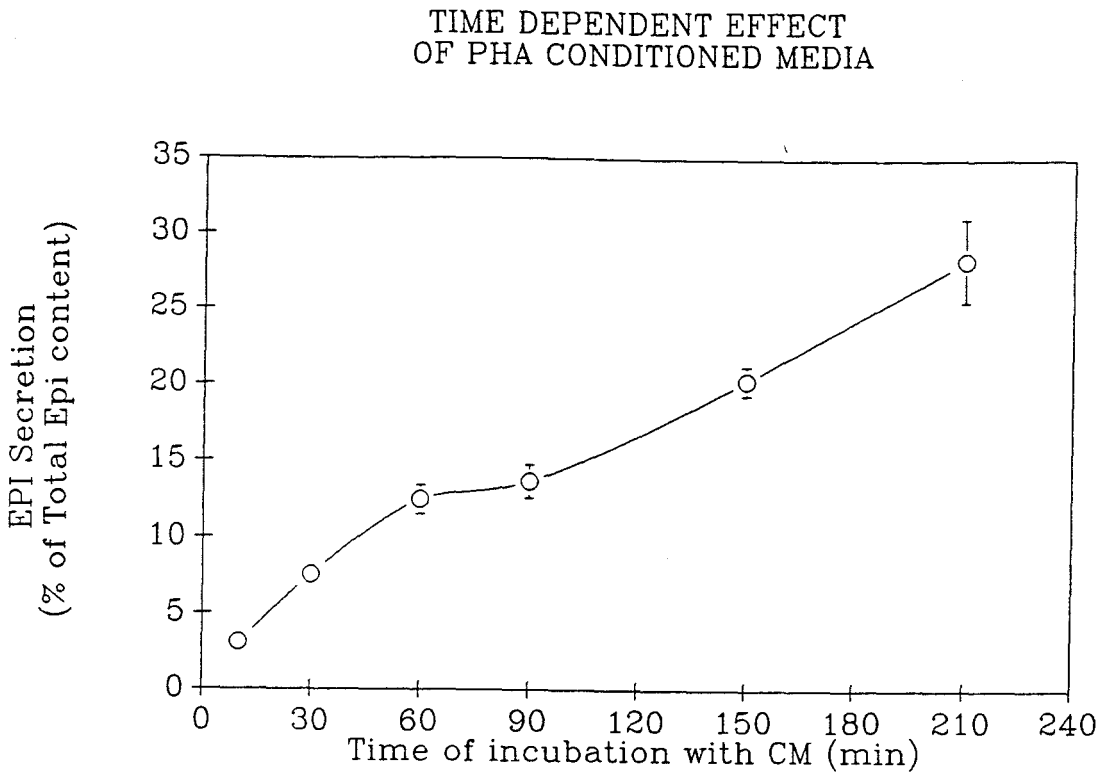

Figure 8. Time course of the effect of PHA CM on Epi secretion. Different times of chromaffin cell incubation with PHA CM were followed by 3 μM DMPP stimulation for 10-min. Statistical differences exist between any two data points except between 90' and 60' ($P < 0.05$, not marked). Triplicate determinations. BCC, N=5; spleen, N=4.

TABLE 2

DATE (spleen preparation)	RESPONSE OF BCC TO CONDITIONED MEDIA						
	Viability (%)	Purity (%)	Age (day)	90' secretion		10' 3uM DMPP	
				Non-Stim.	PHA-Stim.	Non-Stim.	PHA-Stim.
1st.	98	94	1		11.2		5.5*
			4		10.4		5.2*
2nd.	89	89.6	1	7.8	8.4	4.7*	4.3*
3rd.	94	83.5	1	13	10		
			4	16.3	15.3		
4th. 12/13/91	90	91	1	8.5	7.6	4.5*	4.7*
			4	11	11.3	7.3*	5.9*
5th. 1/2/92	91	91	4	5.7	20.1	3.3	6.1
	93.5	91	3	5.1	18.3	3.3	4.5
	95	92	4	5.9	18.9	3.4	6.3
	92	94.4	4		16.6		6.7
	90.5	92.7	4	4.1	12.1	3.3	5.4
6th. 2/20/92	91.6	73	3	8.0	7.1	3.6	3.5
			4	7.0	6.3	4.3	4.0
7th.	92	91.5	3	6.5	5.0	4.6	4.4
8th. 3/13/92			5	16.9	16.7(M)	4.4	4.0(M)
					21.8(W)		4.5(W)
9th. 3/30/92	95	91	3	12.4(W)	14.2	4.2	4.0
				14.2	13.2	3.5	3.6
				13.8	14.3	8.1	6.1

* : stimulation with 10 μM DMPP.

M and W stand for two different preparations of supplimented RPMI 1640 medium, i.e., made by Dr. Matthews and Z. Wang respectively. 2-ME is 2-mercaptoethanol. 1-2FCS is the fetal calf serum used on Jan 2, 1992.

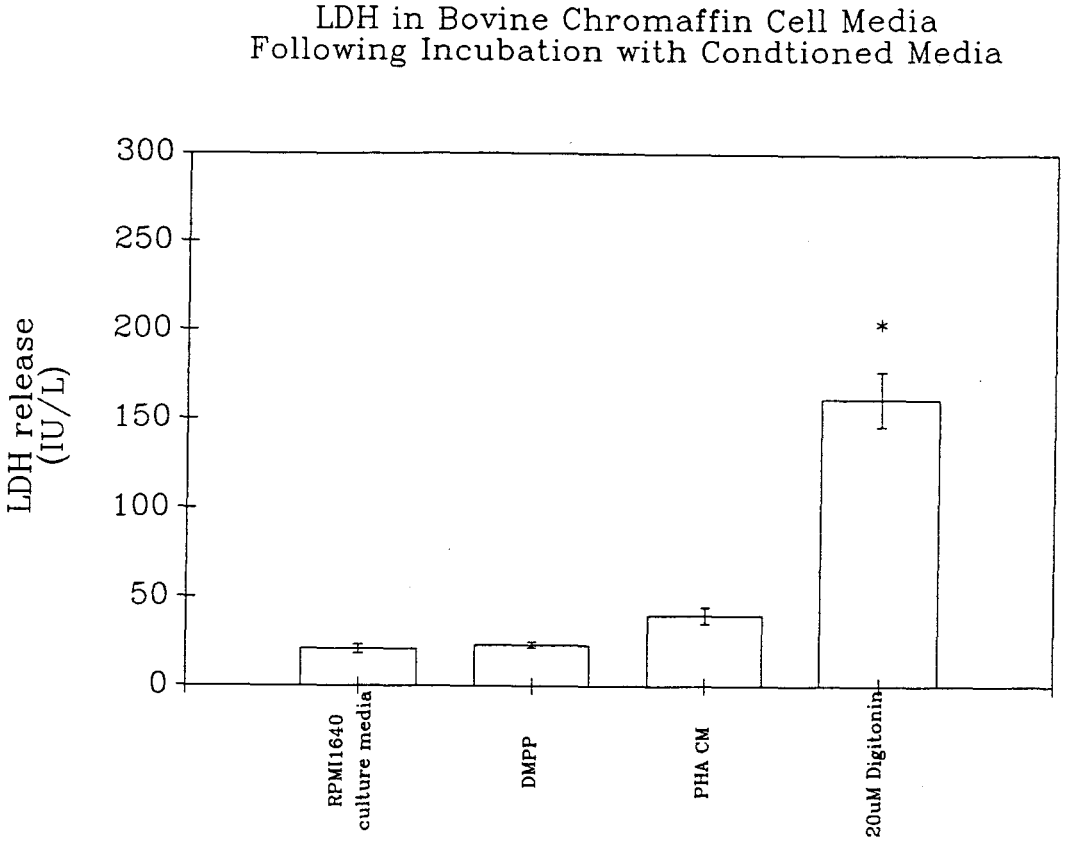

Figure 9. LDH in BCC media following incubation with splenocyte conditioned media. Chromaffin cells were incubated with PHA CM, fresh RPMI 1640 culture medium, 3 or 10 μM DMPP, and 20 μM Digitonin for 90 minutes. The supernatants were then collected for LDH assay. Each bar is mean $\pm$ SE. Triplicate determination for each treatment. PHA CM from four spleen preparations were tested on three BCC isolations. Six different combinations (N=6) of PHA CM and BCC were used for the group of PHA CM; N=3 for all the contols. *, significant difference compared to all the other groups at $P < 0.05$.

III. Detection of Cell Membrane Integrity by LDH Assay.

Lactate dehydrogenase (LDH) normally exists in cytoplasm. LDH concentration in culture supernatant was estimated to confirm that the cells maintained their membrane integrity when they were exposed to different testing media. There was no real difference between the groups treated with lymphocyte culture medium, 3 or 10 μM DMPP and PHA conditioned splenocyte medium (Figure 9). Digitonin is a detergent that can render the cell membrane leaky to LDH and is applied as a positive control agent. Digitonin-treated cells released much more LDH than all the other groups.

The chromaffin cells of bovine origin (BCC) have been extensively used in the studies of endocrine mechanisms, neuronal function and basic cell biology of the secretory process [112, 113]. It is a particular advantage that they can be isolated in large quantities to provide a relatively homogeneous cell population. The primary culture of BCC can usually be maintained for up to 10 days, but the cells change morphologically and functionally during time in culture. Owing to this concern, functional characterization, especially of the secretory change with culture age, must be established prior to experiments with this *in vitro* model.

In bovine species, nicotinic activation of chromaffin cells is the physiological stimulus leading to CA secretion, whereas muscarinic activation is not sufficient to trigger exocytosis [24, 114]. DMPP (Dimethylphynylpiperazinium), a synthetic nicotinic agonist, was used to mimic "neurogenic" secretion *in vitro*, because adrenal medullary CA output *in vivo* is mediated by nicotinic activation by acetylcholine, released from preganglionic nerve endings. The dose-response relationship was examined on BCC of four different adrenal glands. As the culture duration gets longer (1, 4 and 8 days), the secretory response seems to become larger for the same concentration range. Changes were more obvious in the first four days (Figures 1, 2). There are at least three possible explanations for this phenomenon. One is that membrane proteins of the chromaffin cells may be damaged by the collagenase digestion procedure of cell isolation. The initial increase in response to DMPP could be due to the recovery of nicotinic receptors on cell

membrane. The second explanation is that there is a functional shift from muscarinic- to nicotinic- cholinergic receptors during the primary culture of BCC. This finding has been reported by Nakaki *et al* [115] who observed that in less than 2 days of primary culture, muscarinic but not nicotinic stimulation leads to IP_3 (inositol triphosphate) formation and calcium mobilization, whereas at periods longer than 4 days, the opposite occurs. Thirdly, the sensitivity to nicotinic agonist may be increased after a period of time of culture. This could be the result of an alteration in any event of stimulus-secretion coupling. Therefore, it is important that chromaffin cells be cultured for a consistent duration before secretion experiments are conducted, to ensure appropriate comparison.

The CA content was indicated to decline over time in culture (Figure 3). Fifteen to 20 % reduction was observed after 4-8 days in culture. Epi content accounted for about 75 % of the total CA, and the Epi drop reflected a loss of apparent storage without any significant changes of NE content. Similar observations have been reported by other groups [114, 116]. This result may be interpreted in two ways: either by a selective death of Epi-storing cells or by a decrease of PNMT (phenylethanolamine N-methyltransferase) activity in former Epi synthesizing cells. Since the activity of PNMT is regulated by the pituitary-adrenocortical axis, it has been proposed that the lack of functioning adrenal cortical tissue in BCC cultures accounts for the decrease in Epi-forming capacity [114]. However, BCC treated with dexamethasone failed to alter cellular CA levels [117]. Glucocorticoids may have a permissive effect rather than a positive one, but the mechanism and the relevance of this phenomenon are poorly understood. In general, the characteristics of *in vitro* BCC model revealed in the present experiments are in good

agreement with the observations by other laboratories. These include the high viability and purity of the BCC preparations, the percentage of secretion in response to nicotinic agonist, and the change in expression of nicotinic *vs* muscarinic receptors with culture time.

Studies with adrenal chromaffin cells in culture have been particularly valuable in elucidating postsynaptic events involved in stimulus-secretion coupling and in suggesting mechanisms that may operate *in vivo* to control secretion from adrenal medulla. Previous findings in whole animal models demonstrate the existence of non-neurogenic CA release in endotoxic states [5, 6]. Elucidation of potential mediators responsible for stimulating chromaffin cell secretion would be facilitated by using *in vitro* model systems.

The objective of this project was to assess the role of immune-derived factors, such as cytokines, in mediating the CA secretion from adrenal chromaffin cells. The experimental approach to accomplish this goal involved the following:

1) A potent inducer of cytokine production is needed to obtain a broad spectrum of cytokine. Phytohaemagglutinin (PHA), a mitogenic lectin from red kidney beans, is a widely used polyclonal stimulant of lymphocytes. PHA exerts its mitogenic effect through the induction of cytokines. In contrast to stimulation by antigens, in which specific clones of lymphocytes are activated, PHA can motivate multiple clones irrespective of their antigen specificity, so that the percentage of responding cells is 70-80 % of the lymphocyte population [118]. The activated cells release a variety of cytokines which have pleiotropic activity and are able to regulate cell growth and differentiation. IL-2

(originally known as T-cell growth factor) and IFNγ are the best characterized among them. IL-2 is required for the proliferation of activated T cells [119]. Meanwhile, IL-2 can result in the production of IFNγ in spleen cells [120]. TNFα is the main cytokine produced by IFNγ-activated macrophages [121]. In addition, activated-macrophages secrete IL-1, which is necessary for the production and release of IL-2 [122].

2) One easily obtainable source of immune cells from the same species as chromaffin cells is the bovine spleen. A mononuclear cell population, predominantly including B and T lymphocytes, monocytes/macrophages, and some platelets, was prepared from bovine spleen. After isolation, they were then stimulated with PHA and cultured for 24 hours. Cell proliferation is an indication of cytokine release because most of the cytokines are pleiotropic. The cell-free supernatant of this culture (referred as PHA-conditioned media) is considered to contain various cytokines and other soluble factors released by PHA-activated splenocytes.

3) To determine the potential role of immune-derived factors, presumably cytokines, on CA secretion from adrenal medulla, the cytokine-rich supernatant was placed on bovine chromaffin cells in primary culture and CA secretion was measured.

As illustrated in Figure 4, the PHA-conditioned media did affect CA secretion remarkably, with a 4-5 fold increase over the controls after 90 minutes. Not only did it stimulate CA release by its own, it also enhanced the nicotinic secretion (Figure 6). Both responses exhibited concentration-dependency (Figure 5, 7). However, the potency of the conditioned media was variable. Four out of nine splenocyte preparations were relatively more potent than the others in terms of their ability to cause CA release. The difference

in BCC secretary response between extracts from the cultures with or without PHA stimulation was demonstrated in only one of the spleen preparations. Several components of the lymphocytes culture medium, such as fetal calf serum, 2-mercaptoethanol and L-glutamine, which might have big influence on splenocyte culture, were ruled out. The other possibility is that these spleen-originated mononuclear cells may have been already activated if they have exposed to certain antigen *in vivo*. For instance, domestic cattle (which the spleens were obtained from) are usually given double stranded RNA (structurally similar to RNA virus) to prevent them from viral infection. The strategy of this treatment is to induce the production of IFN which has antiviral activity. In addition, the cell isolation procedure itself and the serum-containing culture medium may also serve as stimuli to these immune-participating cells.

In the course of 210-minute incubation with PHA-conditioned media, almost one-third of CA store was released into the media (Figure 8). It seems that this elevation of secretory response is sustained and lasts for as long as four hours. If these results reflected the situations *in vivo*, it would provide an explanation to the non-neurogenic CA outflow from adrenal medulla following immune response to LPS.

To examine the nature of this CA release, LDH (lactate dehydrogenase) activity in the media was estimated. Because LDH is a cytosolic enzyme, its release is an indication of cell membrane disruption. Some detergents, such as digitonin, can make the cell membrane permeable to cytosolic molecules with MW less than 1000 kD including LDH. Digitonin has therefore been used as a positive control of plasma membrane leakiness [123]. Although the LDH level of PHA-conditioned media-treated group

appeared slightly higher than the negative controls, it was much lower than that of detergent-treated groups. There was no significant release of LDH above basal level when the cells were exposed to either DMPP or PHA-conditioned media. The integrity of BCC membrane appeared intact, while CA release through exocytosis instead of cell lysis is implied. Ion analysis of the splenocyte supernatant excluded the possibility of potassium elevation. The direct effect of PHA by itself was eliminated by measuring CA released in the presence of PHA alone. Taken together these findings imply that the soluble products derived from the activated immune cells may serve as stimuli to cause exocytosis in chromaffin cells.

Despite the fact that the precise contents in this conditioned media has not been clarified, information about cytokine receptors and the cellular mechanisms of cytokine action supports the possibility that cytokines may influence chromaffin cell secretion. As previously reviewed, cytokines transmit their biological signals to specific cells within hematopoietic, lymphoid as well as other organ systems via interaction with specific cell-surface receptors. Cytokines and their receptors play a key role in the communication between neuroendocrine and immune systems [71, 75, 76]. For instance, IL-1 and IL-2 receptors were expressed on pituitary cells and other areas of the brain in animals and human [95, 96, 97, 98, 99]. Virtually all nucleated cells within a species are sensitive to IFN of that species and presumably possess IFN receptors [101]. It is unknown if certain cytokine receptors exist on chromaffin cell membranes. To date, there is only one report indicating that IL-1 stimulates corticosterone release indirectly through the action of local CA outflow which is also induced by IL-1 in rat (A.R. Gwosdow, et al.

Abstract, FASEB meeting 1992). Nonetheless, BCC was revealed to express a number of different receptors including IGF1 receptor in addition to the nicotinic and muscarinic. IGF1 has been shown to have a stimulatory effect on CA release from chromaffin cells [108]. Growth hormone receptors share a large degree of homology with cytokine receptors, especially in the extracellular sequence [107]. It is possible that cytokines effect CA secretion via binding to the IGF1 receptors.

The intracellular events associated with cytokine receptor activation usually include calcium elevation and increased protein kinase activity. For example, calcium influx has been observed on cells exposed to IFNγ, and the combination of a PKC activator and a calcium ionophore induced many of the same cellular responses as IFNγ [68, 69, 70]. Protein kinase may also be involved in signal transduction of TNFα, IL-1 and IL-6 [63, 64, 65]. Coincidentally, calcium entry and the subsequent $[Ca^{2+}]_i$ rise is essential and sufficient for exocytosis in chromaffin cells, whereas PKC is able to modulate stimulus-secretion coupling by controlling Ca^{2+}-sensitivity [24]. If the BCC express such cytokine receptors as IFNγ receptor, it is likely predicted that IFNγ can alter CA secretion since it shares the same intracellular messengers as those normally triggering exocytosis in chromaffin cells. CA release caused by the cytokine-rich media could possibly result from the calcium entry through an unidentified channel which might be activated by ligand-receptor binding.

The enhancement of nicotinic secretion may be explained by the involvement of PKC. Calcium and/or PKC can in some way disassemble/reorganize the actin network and consequently potentiate the secretory response by freeing more NE or Epi granules,

which are normally fixed within an actin cytoskeleton matrix.

Although results of the present study do not examine specific effect of cytokines, cytokine action on CA secretion from adrenal medullary cells is inferred. The physiological implication of these findings lies in the interrelationship of immune response and catecholamine action. Numerous studies have been conducted to assess the impact of CA on immune function. NE and Epi may alter immune response through modulation of such functions as cellular migration, lymphocyte proliferation, antibody secretion, or cell lysis [124]. Both α- and β-adrenergic receptors are found on lymphocytes and accessory cells in human and other species. In particular, IL-2 synthesis by human T-cell line were shown to be inhibited following stimulation of β-adrenoceptors [125]. The presence of primarily β_2-adrenoceptors on lymphocytes suggests that Epi may be more influential than NE in altering lymphocyte function [126, 127, 128]. The major source of Epi is adrenal medulla of which the hormonal output delivers messages to the body immune section.

In a physiological model, potential communication from the immune system to the sympathoadrenal system is likely to occur. Such information flow could be accomplished by soluble mediators (e.g. cytokines) secreted from immunocompetent cells, which influence local or distal neural or endocrine targets. Based on previous studies and present results, a feedback circuit can be pictured for *in vivo* reciprocal regulation of immune-sympathoadrenal function. If immune system is viewed as a sensor of foreign invasions, it then likely passes the signal to all of its cooperative organs via molecules produced by the activated immune cells. The observations of the present

investigation demonstrate that the adrenal medullary chromaffin cells respond to these immune-derived signal molecules by releasing more CA into circulation. Since the immunocompetent cells bear adrenoceptors, CA may exert their cooperative effort to fine tune the immune response to any insults. As a feedback signal, CA is capable of down regulating lymphocyte activity after earlier activation. For example, later events in an immune response such as continued cell proliferation, antibody secretion, and cytotoxic T lymphocyte-mediated lysis are inhibited by β-adrenoceptor stimulation and cAMP elevation [129]. Consequently, the modulation by CA ensures that the magnitude of immune response is roughly proportional to the amount of antigen encountered and the ongoing response is appropriately terminated. At this point, the regulatory loop between immune organs and adrenal medullum is completed.

SUMMARY

I. Immune-derived products from mitogen-treated splenocytes stimulate EPI and NE secretion from cultured bovine chromaffin cells, and enhance the nicotinic agonist induced release.

II. The CA release induced by immune-derived factors is not due to the damage of cell membrane. Alternatively, these results suggest that exocytosis is triggered when cells are exposed to the media of mitogen-stimulated splenocyte culture.

III. Specific factors that are responsible for stimulating CA secretion from BCC remain to be investigated.

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[SAMPLE THESIS/DISSERTATION APPROVAL SHEET]

The thesis submitted by Zhaohui Wang has been read and approved by the following committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is, therefore, accepted in partial fulfillment of the requirements for the degree of M.S.

9/2/92
Date

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