Enhancement of tumor cytotoxicity during stress induced immune suppression by administration of an extract from the Chinese Bitter Melon (*Momordica charantia*)

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Microbiology, Immunology and Preventive Medicine
Major: Immunobiology

Iowa State University
Ames, Iowa

1995

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LITERATURE REVIEW

Cell mediated immunity is a key component in the bodys defense against viral and bacterial invaders as well as tumor cells. Various cellular effectors provide essential functions, such as lysis of bacterial or virally infected cells with concomitant production of various anti-bacterial or anti-viral cytokines, and by mediating tumor cell lysis; thereby protecting the body against the spread of infection and the formation of tumors. Cell mediated immunity involves various lymphocyte subsets including cytotoxic T lymphocytes, helper T lymphocytes, and large granular lymphocytes (i.e. natural killer cells). Natural killer (NK) cells were the first type of cell found to be capable of lysis of tumor and virally infected cell independent of prior antigen presentation (i.e. NK activity). Additionally, low NK cell function has been linked to increased incidence of tumor formation. For these reasons, much research has focused on NK cells and other cells capable of NK-like activity.

Natural Killer Cells

Natural killer cells were discovered about 15 years ago during the investigation of cell-mediated cytotoxicity (Herberman et al., 1975; Kiessling et al., 1975a; Kiessling et al., 1976). Investigators were surprised to find that lymphocytes from a normal, healthy individual possessed appreciable cytotoxic activity against a variety of tumor cells, some microorganisms, and against virally infected cells. These lymphocytes, were called natural killer cells due to the spontaneous cytotoxicity exhibited against various target cells. Natural

killer cells represent a subset of lymphocytes (approx. 5%); they differ from T and B lymphocytes by their morphology, cell surface phenotype, and functional ability to spontaneously kill a variety of tumor cells and virally infected cells independent of major histocompatibility complex (MHC) restriction (Jondal et al., 1987; Ortaldo et al., 1987) or prior sensitization by antigens (Henney et al., 1978; Herberman and Ortaldo 1981; Karre et al., 1980). The unique property of NK cells being functionally un-restricted by the MHC which gives them the capacity to kill cells with no detectable MHC antigen, such as occurs with a variety of human solid tumor, leukemic, or virally-infected cells. Additionally, if recognition and binding of processed antigen in the presence of MHC were required, the time allowed for a specific immune response to be initiated could let tumor cells or viruses replicate out of control of such a response. Therefore, the lack of requirement for MHC restriction enables these cells to play a primary role in immune surveillance against tumors and infectious agents.

Morphologically, NK cells are a subpopulation of lymphocytes, large granular lymphocytes (LGLs), that constitute about 5% of peripheral blood lymphocytes and 1-3 % of total mononuclear cells (Timonen and Sakesela, 1980; Timonen et al., 1981, 1982a, b; Reynolds et al., 1981a and b; Reynolds et al., 1982; Kumugai et al., 1982). Additionally, LGLs are non-phagocytic, non-adherent cells that lack surface immunoglobulin and T cell receptor molecules.

Most LGLs have surface receptors for the Fc portion of IgG, and thereby mediate both antibody-dependent cellular cytotoxicity (ADCC) and NK activity (Herberman 1980;

Herberman 1982; Herberman and Ortaldo, 1981). NK cells can mediate either Fc-R dependent cytotoxicity (ADCC) against antibody-coated target cells or direct cytotoxicity against a variety of tumor cells. Cross-linkage of NK cell FcRIII (CD16) with anti-FcRIII (anti-CD16) mAb or direct binding to NK-sensitive tumor targets results in a phospholipase-C mediated release of inositol phosphates, activation of protein kinases and increases in intracellular [Ca^{2+}], which are critical early components of this signal transduction processes in NK cells (Seaman et al., 1987; Windebank et al., 1988; Steele and Brahmi, 1988; Chow et al., 1988; Cassatella et al., 1989; Edwards et al., 1989). This receptor dependent [Ca^{2+}] increase consists of primary calcium release from intracellular stores, and a secondary influx of calcium across the plasma membrane (Leibson et al., 1990). This mobilization of intracellular calcium and subsequent protein kinase-C (PKC) activation is a result of phospholipase C-catalyzed hydrolysis of phosphoinositides which leads to the production of second messengers (Berridge and Irvine, 1989). In fact, pharmacologic reagents that directly increase intracellular calcium and PKC activation (i.e. calcium ionphores and phorbol esters, respectively) directly initiate NK cell activation without exposure to cellular targets (Goldfarb and Herberman, 1981; Seaman et al., 1981; Abrams et al., 1983; Beckner and Farrar, 1988; Cuturi and Murphy, 1987; Ortaldo et al., 1989; Nishimura et al., 1989). These intracellular events ultimately affect changes in specific gene expression, cellular proliferation, cytokine secretion, and cell-mediated cytotoxicity. The mechanism of tumor target cell recognition and spontaneous cytotoxicity in NK cells is still unclear. However, tumor cell lysis is known to require Mg^{2+} ions, can occur at 4°C as well as 37°C and is not inhibited by azide, while

Mg^{2+}, low temperatures and azide all inhibit Tc cell adhesion to targets (Targan and Newman, 1983). Upon recognition, a number of organelles become localized directly between the nucleus of the effector cell and the region of plasma membrane in contact with the target cell. This reorientation of organelles facilitates the movement of cytoplasmic granules and/or secretory vesicles towards the target cell, with the eventual release of their toxic contents via a Ca^{2+} dependent exocytosis (Austyn and Wood, 1994). Soon after the cytoplasm is reorganized the granules, containing perforin and various proteolytic enzymes (specifically, serine proteinases termed granzymes), are released and the target cell dies (Smyth and Trapani, 1995). Perforin polymerizes into transmembrane channels, intercalates into the target cell membrane, and produce pores like those produced by the complement membrane attack complex (MAC). This pore formation alone is capable of lysing some target cells due to osmotic lysis. Internal disintegration of target cells occurs due to granzymes (and possibly other components) penetrating the newly formed pores and triggering pathways leading to apoptosis (programmed cell death) within a few minutes of encountering an appropriate target cell (Smyth and Trapani, 1995). This process is thought to be similar to cytotoxic T cell activity, except for the mechanism of target cell recognition which, characteristically, is mediated by T-cell Receptor molecules (TCR) and a specific peptide-MHC complex. Additionally, Tc cell killing usually only develops after an immune response to the target has been initiated, except under special circumstances to be discussed later. In addition to the pore forming route, NK and CTLs are also capable of target cell lysis mediated through a non-pore forming route facilitated through ligation of the Fas

protein on the target cell by Fas ligand on the effector cell, but the exact pathway remains unclear (Smyth and Trapani, 1995).

Phenotypically, several cell surface antigens have been identified on the surface of NK cells through the use of monoclonal antibodies. Many of these antigens can be found on other blood lymphocytes such as monocytes and various T-cells but NK cells express a characteristic array of surface markers. Generally, NK cells are $CD3^-$ (pan-T-cell marker), $CD56^+$(NKH1$^+$), $CD16^+$(Fc receptor for IgG-also found on granulocytes (PMN)), which is a highly lytic cell type. This is in contrast to $CD3^-$, $CD56^+$ and $CD16^-$ which is an NK cell subset with lower lytic activity (Hatam et al., 1993). More recently, NK cells have been found to be positive for the CD8alpha chain (Mab OX-8), when both the α and β chain are found together on cytotoxic T-cells. Several new monoclonal antibodies specific for cell surface molecules specific for NK cells have been developed. For example, NKR-P1 a 24.5 kD transmembrane glycoprotein found on the surface of rat NK cells, LGL's and polymorphonuclear cells. This molecule has also been implicated in signal transduction (Ryan et al., 1991; Giorda et al., 1990).

NK cells appear to play a key role in immune surveillance against tumors and represent a primary defense against the metastatic spread of blood-borne tumor cells. In animal models the intravenous injection of tumor cells and subsequent development of metastasis is dramatically increased when NK cell populations are decreased; resistance is restored upon reconstitution of the NK cell population (Barlozzari et al., 1983; Pollack and Hollenbeck, 1982). Patients with disseminated cancer, including breast carcinomas or melanomas, have

significantly lower NK function, measured as spontaneous cytotoxicity to tumor targets, than those whose disease remained localized. Several clinical studies have revealed an inverse relationship between spontaneous cytotoxicity and both localized and metastatic disease (Cunningham-Rundles et al., 1981; Pross and Baines, 1976; Steinhauer et al., 1982; Takasugi et al., 1977; Trinchieri and Perussia, 1984). Additionally, the survival time without metastasis in patients treated for metastatic disease correlates well with the levels of spontaneous cytotoxicity (Choe et al., 1987; Hirofuji et al., 1987; Schantz et al., 1987; Son et al., 1982).

In the case of leukemias in relapse, spontaneous cytotoxicity was found to be lower than the activity found in patients who were in complete remission or normal donors (Adler et al., 1988; Matera and Giancotti, 1983). Reasons for this could be reduced numbers of circulating effector cells, defective function, and/or the presence of suppressor factors.

The vital function of NK cells, as a defense against cancer, alludes to the severity of disease states that may be caused by a dysfunction or lack of natural killer cells. Patients with Chediak-Higashi syndrome have a high incidence of lymphoproliferative diseases due to a selective and pronounced deficit in spontaneous cytotoxicity (Haliotis et al., 1980; Roder et al., 1980). Moreover, high dose immunosuppressive therapy given to transplant patients to prevent graft rejection, depletes NK cells thereby making these patients at high risk for lymphoproliferative disease and other malignancies (Frizzera et al., 1981; Starzl et al., 1985). These studies of lymphoproliferative disease confirm the possibility that the NK cell system plays a major role in immunosurveillance in the prevention of cancer.

Recently, several types of cells have also been shown to have the ability to exhibit MHC unrestricted killing or natural killer-like activity, where in the past this activity was attributed to NK cells because the most commonly used assay (^{51}Cr release assay, see materials and methods) to detect spontaneous cytotoxicity cannot differentiate between the cell types responsible for the spontaneous cytotoxicity. Scientists are now able to differentiate the lymphocytes capable of spontaneous cytotoxicity through the use of monoclonal antibodies and flow cytometric analysis. Even though some cells involved in the regulation of spontaneous cytotoxicity do comprise a distinct lineage, there can be no question that cells comprising other lineages may acquire such spontaneous cytotoxicity or NK-like activity at one stage or another of their development or activation.

Several types of T cells are capable of MHC-unrestricted cytotoxicity, although certain populations appear to posses an increased ability for spontaneous killing. In particular cytotoxic T-lymphocytes (CTL), which are normally MHC-restricted, can also generate the ability to kill cells that are referred to as targets for NK cells in an MHC-unrestricted manner, particularly after they have been cultured in the presence of interleukin-2 (IL-2) (Acha-Orbea et al., 1983). It is feasible to reason that spontaneous cytotoxicity could be a function of cells other than NK cells in vivo, such as IL-2 induced CTL. Additionally, Matthew and colleagues (1993) have characterized a molecule believed to be associated with non-MHC-restricted killing mediated by both activated natural killer cells and T cells.

Recently, much of the investigation of spontaneous cytotoxicity has centered on T cells possessing the $\gamma\delta$ form of the TCR ($\gamma\delta$ T-cells). This subpopulation of T cells is capable

of MHC-unrestricted killing which can be augmented in the presence of various cytokines including IL-2, Interferon (IFN) and tumor necrosis factor-alpha (TNF-α) (Cesano et al., 1993). In vitro, human $\gamma\delta$ T-cells have been shown to proliferate in response to typical NK cell targets, which leads to cell lysis that is rarely MHC-restricted (Bukowski et al., 1994; Kozbor et al., 1988; Fisch et al., 1992; Okumura et al., 1995). Additionally, Choudhary and colleagues (1995) observed that polyclonal tumor infiltrating lymphocytes (TIL) taken from renal carcinomas develop the ability for nonspecific lysis. Lymphocytes possessing the $\gamma\delta$ TCR were found to be overrepresented in these populations, thereby indicating a potential role in tumor surveillance in vivo.

Many immunocyte cell lines propagated in vitro are often capable of killing tumor cells that they are not thought to be capable of killing in vivo. For example, T-cell clones that have been grown in the presence of interleukin-2 IL-2 have been termed anomalous killer (AK) cells. It is unclear if these cells are $\gamma\delta$ T-cells. Lymphocytes that have been cultured in the presence of IL-2 and have acquired cytotoxic activity in vitro have been termed lymphokine activated killer (LAK) cells (Phillips and Lanier, 1986). The LAK cell phenomenon was first associated with the ability of peripheral blood mononuclear cells (PBMC) which were stimulated by IL-2 to kill primary tumors and tumor cell lines resistant to NK cytotoxicity (Grimm et al., 1982). Later, it was reported that, in fact, NK cells were the predominant IL-2-induced LAK precursor in PBMC (Trinchieri et al., 1984). Lymphokine activated killer cells demonstrate enhanced nonspecific lysis of tumor cells, and because of this are being used frequently in adoptive immunotherapy trials by removing

lymphocytes from the patient, expanding and activating them in the presence of IL-2, and transfusing the activated cells back into the patient. This treatment is generally accompanied by a systemic administration of high doses of IL-2.

In addition to lymphocytes, a main feature of activated macrophages is, by definition, an increased lysis of tumor cells in vitro. The mechanism of this preference for tumor cells is unknown. Additionally, macrophages, like NK cells, have Fc receptors on their surface enabling them to kill tumor cells coated with antibody via ADCC. Tumor killing by macrophages in vitro usually requires 18-22 hours and kills a different array of cells than NK cells. This contrasts with NK cells which kill in 4-6 hours. In addition, some precursors of mononuclear phagocytes may also be able to kill in an MHC-unrestricted manner.

Because there is ambiguity concerning the identity of NK cells, the cell surface phenotype of $CD3^-$, $CD56^+$, $CD16^{+/-}$ must be used to distinguish NK cells from other cells that have cytolytic activity even in the absence of target MHC class I and class II molecules. Due to the inconsistencies concerning true NK activity and NK-like activity, anytime flow cytometry has been performed it will be phenotypically designated throughout the rest of the text.

Upon discovery that NK and NK-like activity (spontaneous cytotoxicity) can be stimulated or enhanced in a variety of cells by treatment of the cells with cytokines, such as IL-2, there has been much research in the area of NK cell function and regulation and the effect of cytokines on such function and regulation. Now that a portion of this spontaneous cytotoxic activity has been attributed to other cell types; researchers are beginning to identify ways to get maximal stimulation of tumor cytotoxicity through the use of cytokine therapy.

Activators of Cytolytic Activity

Cytokines are proteins that serve as intracellular messengers like hormones although their function is usually autocrine or paracrine in nature whereas hormones are endocrine in nature. These regulatory peptides can be produced by nearly all nucleated cells in the body and induce a wide range of regulatory effects, ranging from maintaining bodily homeostasis, enhanced host responses to invading organisms, tumors or trauma (Austyn and Wood, 1994). More recently, it has been discovered that various cytokines can control, and more notably, increase NK cell function and replication that is stimulated during infection, inflammation, or other immune challenges (Cortesina et al., 1988; Grimm et al., 1982; Henney et al., 1981). Recent research has focused on cytokines and other compounds that up-regulate immune function (known as biological response modifiers) thereby stimulating spontaneous cytotoxicity. The use of cytokine therapy is being studied in a wide variety of disease states such as autoimmune disorders, neoplasias and immunocompromised patients with AIDS.

Resting peripheral blood NK cells can respond directly to recombinant cytokines. A key cytokine in regulation of NK activity is IL-2 and its effects have been studied extensively. Henny et al. (1981) provided the first evidence for a direct stimulatory role of IL-2 in NK cell function in a murine model. Since that time, treatment with IL-2 alone has been shown to stimulate growth and increase the activity of NK cells, increase the cytotoxic granule content of NK cells and T cells including CTL and $\gamma\delta$ T-cells (Cesano et al., 1993), enhance adhesion molecule expression, and induce LAK activity and proliferative activity in human NK cells (Phillips et al., 1986; Trinchieri et al., 1984; Smyth and Trapani, 1994).

Additionally, IL-2 has also been shown to increase ADCC activity, and induce mRNA transcription and secretion of cytokines in NK cells and LAK cells (Cuturi et al., 1987; Cuturi et al., 1989; Kasid et al., 1988; Robertson et al., 1992; Smyth et al., 1991). IL-2 also stimulates synthesis of other T cell-derived cytokines, such as IFN-γ and TNF-β, found to be key mediators of cytolytic activity in NK cells, $\gamma\delta$ T-cells and macrophage activation.

The systemic administration of IL-2 alone resulted in the partial or complete destruction of tumor mass in some patients with solid tumors, particularly melanoma and renal cell carcinoma (Rosenberg et al., 1987; West et al., 1987). Additionally, local (perilesional) administration of IL-2 can produce partial and complete regressions of tumors in the head and neck in humans (Cortesina et al., 1988). Cancer therapy in humans utilizing autologous, IL-2 expanded, lymphokine-activated killer cells in combination with rIL-2, demonstrated increased antitumor effector function in vivo (Phillips, J. H., 1987). This combined treatment of LAK and IL-2 has been reported to yield increased frequency of complete regression than treatment with just IL-2 alone in several types of cancer (Phillips et al, 1987; Cortesina et al., 1988; Austyn and Wood, 1994).

Natural killer cell function can be rapidly activated in vivo by IL-2 as demonstrated by augmented cytotoxic activity, increased expression of activation antigens, cellular proliferation (Trinchieri and Perussia, 1984) and lymphokine production (Henney et al., 1981; Anegon et al., 1988). The process by which this activation occurs has not been completely elucidated. Generally, upon binding of IL-2, a target cell, or the Fc receptor, a rapid increase in tyrosine phosphorylation of multiple proteins in NK cells occurs in

concert with calcium influx (Einspahr et al., 1990; Einspahr et al., 1991; Ferris et al., 1989; Sharon et al., 1989; Khattri et al, 1994). The majority of NK cells enter the cell cycle after exposure to rIL-2, in contrast to resting peripheral blood T cells which require additional signals such as IL-1 or activators of protein kinase C to induce proliferation (London et al., 1986). Binding of IL-2 to either the intermediate or high affinity receptor (Kd ~ 1 nM, Kd ~ 10 nM respectively) induces signal transduction and cellular activation in NK cells and T-cells (Waldman 1991; Farrar et al., 1990; Hatakeyama et al., 1989).

Although IL-2 is a potent stimulator of spontaneous cytotoxicity, many cytokines exhibit a synergistic effect when administered along with IL-2. A combination of IL-2 (rIL-2), and interferons-α, -β, or -γ have been most widely used in clinical trials for treatment of cancer. Among various biological effects these cytokines, singly or in combination, have the consistent ability to increase spontaneous cytotoxicity in cancer patients as well as healthy individuals (Trinchieri et al., 1984). In vitro studies show that activated NK cells are responsible for the antitumor cytotoxicity of cultured human peripheral blood mononuclear cells when these cytokines are used in combination or individually (Herberman et al., 1987; Ortaldo et al., 1986). Activation induced by such combinations of cytokines occurs without a requirement for accessory cells or cofactors, and enhanced cytotoxicity can be measured within 4 hours post-lymphokine exposure, but is maximal at 18 hours (Trinchieri et al., 1984; Lanier et al., 1985).

Interferons (IFNs) were originally named to indicate their property of 'viral interference' meaning that IFNs induce an anti-viral state in target cells rather than interacting with the

virus itself. Since virus infection was known to induce IFN, it was hypothesized that the boosting of NK activity was due to the induction of IFN-γ following virus infection (Welsh, R. M., 1978). In fact, increased NK activity following virus infection or tumor response was found to be similar to that found following administration of IFN or IFN inducers in vivo. Trinchieri and co-workers (1977) first reported that human lymphocytes may be stimulated by some tumor cell lines to produce IFN, and although IFN can augment NK cell activity, this cytokine is not necessary for normal NK cell function in vitro (Trinchieri et al., 1978; Welsh et al., 1980; Fitzgerald et al., 1982; Minato et al., 1980; Casali et al., 1981; Copeland, et al., 1981). However, IFN made in response to a virus infection in vivo, does lead to an increased recruitment and activation of NK cells, thereby activating cells where they are needed most. Interferons are now known to inhibit the growth and proliferation of some tumors and normal cells, and to modulate immune responses by the control of cellular function. Interferons also inhibit replication via phosphorylation of a number of intracellular proteins required for protein synthesis, and activation of endogenous endonucleases that become actively engaged during an infection or tumor cell growth (Austyn and Wood, 1994).

Three different types of interferons are involved in the pleiotropy of antiviral and antitumor activities. Interferon-α and IFN-β, which are produced by macrophages and lymphocytes (including NK cells) have a more systemic effect participating in the first line defense before acquired immune mechanisms come into play (Thompson, 1991), IFN-γ induces an anti-viral state and is able to act directly on lymphocytes to promote differentiation and maturation of CTL (Abbas et al., 1993). Synthesis of IFN-γ is directly

initiated as a consequence of antigen activation, enhanced by IL-2, and produced by both by IL-2 secreting CD4+ helper T cells and nearly all CD8+ T cells (Abbas et al., 1993). Although NK cells are a minor source of interferon, NK responses to tumor cells and virus-infected targets is often accompanied by the production of some IFN (Austyn and Wood, 1994).

More specifically, IFK-γ may increase the number of NK cells binding to their targets and the rate of lysis by these cells, as well as the ability of NK cells to recycle and destroy more cells (Austyn and Wood, 1994). Moreover, IFN-gamma also enables non-cancer cells to become resistant to the increased lytic activity it grants NK cells, thereby protecting 'normal' cells (Austyn and Wood, 1994). T-cell secreted IFNγ is also a potent macrophage activating agent (Andrew et al., 1984; Kleinerman et al., 1984). Activated macrophages can release reactive oxygen intermediates that allow macrophages to kill phagocytosed microbes and tumor cells and produce other cytotoxic molecules such as tumor necrosis factor-alpha (Urban et al., 1986). Interferon-γ acts synergistically with other cytokines such as TNF enhancing the ability of macrophages to kill tumor cells (Abbas et al., 1993).

All IFN's have been shown to enhance NK activity and NK-like activity in PBL/PBMC and can partially (IFN-γ) or totally (IFN-α) induce LAK activity in purified NK cells (CD 56^+ $CD16^+$ respond better than $CD56^+$ $CD16^-$). Although LAK induction by IFN-γ can be totally independent of IL-2 (Trinchieri et al., 1984; Ellis et al., 1989), there is a synergistic effect between IL-2 and IFN-γ in NK cells and T cells resulting in maturation proliferation and/or activation (Cesano et al., 1993). Additionally, if cells are cultured in the presence

of IFN-α or IFN-γ are co-incubated with IL-2, enhancement of the IL-2 induced cytotoxicity is seen (Brunda et al., 1986; Tokuda et al., 1989).

Different cell types can vary greatly in their sensitivity to effects of IFNs, although tumor cells may generally be more sensitive than normal cells. The growth inhibitory activity of IFK-α and -β and the stimulation of lytic activity by IFK-γ suggests that IFNs may be potential tumor suppressors; currently studies are being performed using intraperitoneal administration of IFN-γ for treatment of ovarian cancer (Austyn and Wood, 1994).

Much research has focused on extracellular messengers or cytokines as activators of cytotoxic activity but some studies have shown that intracellular messengers may also be potent activators of spontaneous cytotoxicity. Phospholipid-dependent protein kinase C (PK-C) has been demonstrated to play a crucial role in post receptor-binding signal transduction in several cell systems (Nishizuka, 1986). Recently, it has been demonstrated that the synergistic action of $Ca2^{+}$ ionophores and TPA, an activator of PK-C, can induce proliferation and IL-2 production by human LGL which sugggests that protein kinase-C can play an important role in NK cell activation and function. Additionally, selective inhibition of PK-C strongly inhibits spontaneous cytotoxic activity of human LGL (Procopio et al., 1988).

In addition to PK-C, a receptor for C reactive protein (CRP) has been identified on the surface of human LGL. C reactive protein is an acute phase protein, activates complement, acts as an opsonin, and activates macrophages and neutrophils. C reactive protein is involved in NK cell function since antibody against CRP inhibits NK cell lysis of K562

(Baum et al., 1983) and MOLT-4 (Baum et al., 1987) human and mouse tumor cell lines, respectively. Anti-CRP does not block effector cell/target cell conjugate formation and, therefore, is not involved in effector-target cell recognition (Baum et al., 1987). However, anti-CRP does inhibit the calcium-dependent phase of signal transduction thereby blocking proximal events leading to cytoskeletal repolarization and the directed release of cytotoxic factors from activated NK cells and probably other cells that kill by degranulation (Hamoudi et al., 1991; and Khattri et al., 1994).

Lipopolysaccharides (LPS) have also been shown to rapidly enhance cytotoxicity of NK cells against tumor targets (Lindemann et al., 1988a; Lindemann 1989a; Tarkkanen 1986). Whereas IL-2 stimulation induces long term NK cytotoxicity and proliferation (Lindemann et al., 1988b), activation via bacterial products, especially LPS, is short-term with peak cytotoxicity occurring approximately 24 h after culture initiation (Lindemann 1989b). This cytotoxic induction appears to be dependent on release of interferon (IFN) by NK cells (Lindemann 1989b), however, significant concomitant release of IL-2 was not measured (Lindemann, 1988b).

Bitter Melon

Many compounds other than cytokines have been found to have immunostimulatory activity. The resurgence of herbal medicine has prompted examination of ancient folk remedies, such as the Chinese bitter melon, for their immunomodulatory properties.

Momordica charantia, the bitter melon, is a member of the Cucurbitaceae family and has been used for over 500 years as a folk remedy in China. Recent research has shown that

an aqueous extract of the bitter melon has antiviral (Lee-Huang et al., 1990), antitumor/anti-leukemic (Cunnick et al., 1990; Takemoto et al., 1982; Jilka et al., 1983), anti-AIDS (Ng et al., 1992) and immune enhancing properties (Cunnick et al., 1990). The proteins responsible for the antiviral activity appear to be two 30 kDa glycoproteins, alpha- and beta-momorcharin, that belong to a family of ribosome inactivating proteins (RIPs) capable of inhibiting ribosome function in eukaryotic cells (Ng et al., 1992). Ribosome inactivating proteins posses both cytostatic and non-specific anti-viral activity through the inhibition of RNA and protein synthesis. Several other plants have been found to have proteins with such anti-viral activity, such as trichoxanthin from *Tricosanthes kirolowii* and the ricin A chain from the castor bean. Additionally, these proteins have been found to have some sequence homology with the RIP from *Momordica charantia* (Lee-Huang et al., 1990).

The anti-tumor properties of the bitter melon appears to be related to both RIP activity and immune enhancing properties. Pretreatment of tumor cells in vitro with crude bitter melon extract has been successful in reducing tumor incidence and extending the life span of DBA/2 mice injected with L1210 tumor cells (Cunnick et al., 1990). Additionally, mice which received tumor cells pretreated with bitter melon extract and intraperitoneal (ip) injections of the bitter melon proteins (BMP) survived the longest. Researchers concluded the anti-tumor component of the BMP was protein in nature because it is degraded by heat and proteinases and is not degraded by DNAase (Takemoto et al., 1982).

A portion of the anti-leukemic and anti-viral action of the bitter melon appears to be associated with an activation of murine lymphocytes. A partially purified protein extract

from the bitter melon caused an infiltration and activation of peritoneal exudate cells in C57BL/6J, C3H/HeJ, and C3H/Hen mice. Bi-weekly injections of 8 micrograms of protein, ip, for up to 4 weeks enhanced cytotoxicity in a long-term (18hr) and short-term (4hr) ^{51}Cr release assay against a variety of tumor targets (Cunnick et al., 1990). Antibody depletion studies using asialo-GM1, was able to reduce a major portion of the anti-tumor activity of peritoneal exudate cells in mice. It was suggested that at least part of the anti-leukemic activity of the bitter melon extract is due to the activation of NK cells (Cunnick et al., 1990). However, asialo-GM1 is now known to be found on both CTL and NK cells (Austyn and Wood, 1994).

In light of the immunostimulatory properties, some AIDS patients have used a crude extract of the bitter melon as a homeopathic or supplementary treatment. Preliminary results indicate that HIV infected individuals who have used extract of BMP have an increased life span but the study included few individuals and was not well controlled (Zang, 1992). Bitter melon treated AIDS patients demonstrated an increase in the CD4+ (i.e., T-helper cell) population (Zang, 1992). Additionally, a new inhibitor of HIV replication has been isolated from the seeds and fruits of the bitter melon. Momordica anti-HIV protein, (MAP 30), is also about 30 kDa, and exhibits dose-dependent inhibition of cell-free HIV-1 infection and subsequent replication. No cytotoxic or cytostatic effects were noted under experimental conditions indicating that MAP 30 may be a useful therapeutic agent in the treatment of HIV-

1 (Lee-Huang et al., 1990).

The use of natural immunomodulators, such as the bitter melon, to stimulate immune function is very appealing to patients in light of adverse side effects often caused by drug treatment or the systemic administration of IL-2 that is now common practice in cancer treatment. In fact, food derived immunomodulators could also be used as a prophylactic, to keep immune function high at times when it might be suppressed such as during stressful life events.

Stress and Immune Function

Several researchers have found that stressors are able to alter the immune system in humans as well as animals (Cunnick et al., 1991; Keller et al., 1988; Hillhouse et al, 1991) thereby increasing the susceptibility to disease in humans. The effects of stress on the severity or frequency of disease can be seen in studies involving rheumatoid arthritis, diabetes, cancer, and repeated viral infections (Fitzpatrick et al., 1988; Eaton et al., 1979; Jemmott, et al., 1984; Baker et al., 1981). Additionally, stress has been identified as a co-factor in the progression of HIV infection to full blown AIDS (Wallace & Watson, 1990). Both psychological and physical stress have been shown to significantly decrease immune competency (Lysle et al., 1988), specifically, studies indicate that stress induces a robust suppression of lymphocyte function. Such suppression of lymphocyte function can be demonstrated by decreased levels of circulating antibodies (Solomon, 1969), decreased reactivity of lymphocytes to mitogen (Keller et al, 1981) or specific antigenic stimulation (Jasod and McKenzie, 1976), and increased susceptibility to tumor challenge (Lewis et al.,

1984). This increased susceptibility to tumor formation has been linked to suppressed function in NK and NK-like cells due to stress. In support of such a connection, there have been several studies in animals indicating that stress can have a prominent immunosuppressive effect on NK cell function (Lysle et al., 1988; Lewis et al., 1984). Experimentally, several types of stressors including physical (shock or drugs such as morphine) (Cunnick et al., 1988; Fecho et al., 1991), psychological (conditioned fear) (Lysle et al., 1990) have been used to examine the physiological effects of stress. In Lewis rats, electric foot shock (unconditioned stimulus) and conditioned fear/anxiety decrease the proliferation of lymphocytes when stimulated by mitogens (Lysle et al., 1987), decreases natural killer cell activity (Lysle et al., 1988), and increases susceptibility to tumor challenge (Lewis et al., 1984). A contributing factor to these stress induced decreases in functional NK activity are the decreased production of both IFN-γ and IL-2, both of which serve as robust activators of cytolytic function (Abbas et al., 1993; Trinchieri et al., 1984).

Natural killer cell activity is essential to protect the body from tumor or virally transformed cells. Stress can decrease NK cell function and the production of activators of cytolytic activity resulting in viral infection or development of tumors. During a time of stress, enhancement of immune function could serve as a key factor influencing the likelihood of illness. Use of immunomodulators/immunostimulators during times of stress could greatly reduce illness or disease severity if such immunomodulators could abrogate the effects of the stressor, and thereby reinstate normal immune function.

The hypothesis of this study is that administration of a crude extract of the bitter melon can reduce stress induced suppression of immune function.

MATERIALS AND METHODS

Media and Reagents

The two types of culture medium used were RPMI-1640 culture media supplemented with L-glutamine (50 mM), gentamicin (50 μg/ml) and Hepes buffer (200 mM) (all from Gibco Lab, Grand Island, NY), henceforth called supplemented media. Supplemented media prepared with 10% Fetal Bovine Serum (FBS) (JHR Biosciences, Lenexa KS) is referred to as complete media. All BMP treatments were diluted in Hanks balanced salt solution supplemented with Hepes buffer (200 mM) and gentamicin (50 μg/ml) (all Gibco Lab, Grand Island, NY). For use in the mitogen stimulation assay, mitogens concanavlin A (Con A) and lipopolysaccharide (LPS) derived from *Escherichia coli* were purchased from Sigma Chemical (St. Louis, MO) and phytohemagglutinin (PHA) was purchased from Burroughs Wellcome (England). ^{3}H-thymidine (specific activity 6.7 Ci/mmol) and ^{51}Chromium (specific activity 419.99 MCi/mg^{51}Cr, Dupont-New England Nuclear, Wilmington, DE) were used as described in the methods portion. Heparin 100 units/ml (ELKINS SINN Inc., Cherry Hill, NJ) was diluted in 0.9%NaCl solution for use in collection of blood samples. Phosphate-buffered saline (PBS) was prepared with NaCl (137 mM), Na_2HPO_4 (6 mM), and KH_2PO_4 (1.5 mM), pH 7.2, and sterilized by filtration through a 0.22 μm pore filter.

Cell Lines

The tumor cell line, YAC-1, a murine lymphoma line was originally obtained from the American Type Culture Collection (ATCC) Rockville, MD. The cell line was propagated in complete medium. The cells were maintained in log phase at 37°C in a humidified CO_2 incubator (5%CO_2, 95% air).

Animals

For preliminary experiments, female Balb/c scrub mice were obtained from Iowa State University Laboratory Animal Resources. Mice were housed 2-4 per cage and they received both food and water *adlibitum*. For subsequent experiments, male inbred rats of the Lewis strain, 52-58 days of age and 200-240 grams, were obtained from Harlan Sprague Dawley Laboratories (Madison, WI). The rats were housed individually for 2-3 weeks at the ISU Animal Care Facility in a colony room on a 12-h reverse light-dark cycle maintained through artificial illumination. The rats received both food and water *adlibitum* throughout the experiment. Animals were handled every other day for two weeks prior to the injection day, during their dark cycle, to acclimate the animals to the handling procedures and the new environment. The handling procedures included a simulation of the intraperitoneal (ip) injection procedure.

Bitter Melon Extract Preparation

The crude bitter melon protein extract (BMP) was prepared as previously described (Takemoto, 1982). Briefly, 50 pounds of bitter melon fruit, obtained from California, were chopped then homogenized in 10 mM Tris-HCl buffer (pH 6.7) with 1 mM $MgCl_2$, filtered

through cheese cloth, then centrifuged at 9,000 x G (Sorvall GSA) for 20 min. The resulting supernatant was precipitated with 50% saturated ammonium sulfate (Fisher Chemical, Fair Lawn, NJ). The precipitated protein was collected via centrifugation and the pellet was resuspended in PBS and dialyzed (Fisher Chemical, spectra/por 3, 45mm wide) against PBS overnight to remove residual ammonium sulfate. All procedures for BMP preparation were conducted at 4°C. This crude aqueous extract was stored at -80°C in 1.5 ml aliquots and used as the source of crude bitter melon preparation. This extract is stable for up to 1.5 years at -70°C and was tested for activity in mice prior to, and periodically throughout, the rat study (data not shown).

The crude protein content was determined using the colormetric Bio-rad Protein Assay and was determined to be 9.437 mg/ml using IgG as a standard. Prior to injection treatment, the crude bitter melon protein extract was thawed and centrifuged at 3000 rpm for 5 minutes. The supernatant was filter-sterilized using a 0.2 micron filter, and diluted in supplemented HBSS to 50 μg/ml. The denatured control samples were heated at a full boil for 10 minutes then filtered and diluted like the non-denatured samples. A total volume of 2 ml was injected ip four days prior to sacrifice. Animals in the control group were given 2ml ip of the diluent (HBSS). Injections of bitter melon protein (BMP) ranging from 30-3000 μg/ml as indicated in the results, denatured protein, BMP or HBSS were given 4 days prior to the animal sacrifice.

Preparation and Enumeration of Effector Cells

Peritoneal exudate cells (PEC) were obtained from BMP-treated rats and control rats via peritoneal lavage with 50 ml of supplemented HBSS. The lavage fluid was centrifuged at 1500 rpm for 10 minutes, and the cell pellet was resuspended in 4 ml complete media and counted using a Celltrak 2 (NOVA Biomedical, Waltham, MA). The cells were diluted to 2.5×10^6 cells/ml.

Spleens were ground between the frosted ends of glass slides, in 8 ml complete media, in order to obtain a single cell suspension. The cell suspension was allowed to settle for 5 minutes and the supernatant (containing the single cell suspension) was removed. The cells were enumerated as above and diluted to 5.0×10^6 cells/ml.

Differential leukocyte counts were performed by a certified Medical Technologist (ASCP) on cytospin prepared slides fixed in methanol and stained with Wright-Geimsa stain (Fisher Chemical, Fair Lawn, NJ). Slides were prepared by adding 35 μl or 55 μl spleen or PEC single cell suspension (above) to 160 μl of 1%BSA in PBS in the cytocentrifuge sample carrier. Slides were centrifuged at 700 rpm for 5 minutes, then immediately fixed and stained and at least 100 cells were counted.

Assay for Tumor Cytotoxicity

Tumor cytotoxicity was measured using a ^{51}Cr-release assay (Tadayoshi, 1981), utilizing the NK sensitive tumor cell line, YAC-1 (murine T-cell lymphoma) as tumor target cells. Briefly, the effectors (spleen or PEC) were prepared as above and plated in triplicate at 10, 5, 2.5, and 1.25×10^5 cells/well to obtain effector to target ratios of 100:1, 50:1, 25:1, and

12.5:1 for the spleen, and plated at 5, 2.5, 1.25 x 10^5 and 0.75 cells/well to obtain effector to target ratios of 50:1, 25:1, 12.5:1 and 6.25:1 for the PEC samples. All samples were plated in a 96-well, flat-bottom, microtiter plate and incubated for 4.5 hours at 37°C in a humidified CO_2 incubator. Tumor target cells were harvested and washed once with 10 ml of media. Tumor cells (7.0 x 10^6cells) were resuspended in 200-300 μl complete media and incubated for 70 minutes with 200 μCi sodium [^{51}Cr]-chromate (Dupont-New England Nuclear, Boston, MA). The cells were washed in 30 ml complete media, and incubated for another 30 minutes in 5 ml complete media to reduce spontaneous leakage of ^{51}Cr during the assay. After the 30 minutes incubation, the cells were washed twice more in 14 ml media to remove remaining extracellular chromium, then counted for viability using trypan blue exclusion. The tumor cells (targets) were resuspended at 2 x 10^5 cells/ml of complete media, and 50 ul of this suspension was added to the wells after the samples had been plated.

Control wells were plated using complete media and target cells to determine spontaneous release and maximum release. The maximum release was determined by lysing all of the targets with 10% trichloroacetic acid just prior to harvesting the samples. Next, the plates were centrifuged at 500 rpm for 5 minutes, 100 μl of the supernatant was removed, and counts per minute were determined on a gamma counter (Gamma Trac 1191: TMAnalytic, INC, IL). Cytotoxic activity is represented as lytic units. Lytic units are defined in terms of the number ($1x10^7$) of effector cells required to give 20% specific lyisis of 1 x 10^4 target cells [(LU) 20%/10^7 effectors]. Data from all E:T ratios are used to

calculate the LU.

Flow Cytometry

Flow cytometric analysis of cell surface proteins were performed on both the spleen and PEC cell suspensions using flourescently tagged monoclonal antibodies and an EPICS PROFILE-1 to perform the analysis. Anti-CD8alpha (OX-8) biotinylated for conjugation to strepavidin-cychrome, anti-CD4 antibody conjugated to fluorescein iso-thiocyanate (FITC) and anti-CD3 conjugated to phycoerythrin (PE) were used for three color analysis (all from PharMingen, San Diego, Ca). Additionally, mouse anti-rat NKR-P1 antibody, FITC conjugated (Harlan Laboratories, Madison,Wis) was used for one color cell staining. Isotypic controls used were FITC or Biotin conjugated mouse IgG1k or IgG2k antibodies (PharMingen, San Diego, Ca) to determine nonspecific binding. Briefly, $5x10^5$ cells/tube in 0.2 ml phosphate-buffered saline (PBS) containing 0.1% sodium azide were incubated with previously titered optimal concentrations of CD8 and CD3 or NKR1-P murine mAbs or isotypic control antibodies for 20 minutes at 4°C, in the dark. The cells were washed once with PBS/azide, the red blood cells were lysed using a 1x ammonium chloride lysing solution, and the cells were washed two more times. At this point the second-step flourochrome (i.e., strepavidin-cychrome) was added to the samples for 20 minutes at 4°C, in the dark. All samples were washed twice more, as above, and re-suspended in a 1% paraformaldehyde solution for analysis at the ISU Flow Cytometry facility. Analysis was performed by gating on the lymphocyte population as determined by forward light scatter vs side scatter. In order to determine phenotypic cell type, gates were set for each experiment,

based on isotypic controls, to optimize separation of the fluorescent signals and greater than 10,000 events were counted.

Mitogen Stimulation Assay

A mitogenesis assay was performed on splenic leukocytes (Lysle et al., 1988). In order to determine lymphocyte proliferation, con A (3.0 μg/ml) and PHA (10.0 μg/ml) were used as T lymphocyte mitogens and LPS (5.0 μg/ml) as a B lymphocyte mitogen. The whole spleen single cell suspensions were diluted in complete media to $5x10^6$ leukocytes/ml. The spleen cultures were pulsed with 1μCi ^{3}H-thymidine (specific activity 6.7 Ci/mmol; Dupont-New EnglandNuclear) in 50 μl of supplemented RPMI-1640 during the last 5 hours of a 48-hour incubation at 37°C in a CO_2 incubator. The cultures were harvested onto glass fiber filter paper using a Skatron Cell Harvester (Skatron Inc., AS, Norway) and the incorporation of ^{3}H-thymidine was measured with a liquid scintillation counter (Wallac Inc., Gaithersburg, MD). Data were expressed as counts per minute (cpm) $\pm$SEM.

Plasma Corticosterone Assay

A sample of heparinized blood obtained via heart puncture was centrifuged at 3000 rpm for 25 minutes, and 1 ml of plasma was removed and frozen at -80°C. Plasma corticosterone was determined using a competitive protein binding assay as previously described by Murphy (1967). Breifly, 400 μl of ethanol (100%) was added to 200 μl of each plasma sample in a microfuge tube, vortexed and centrifuged at 6000 rpm for 3 minutes. The supernate suspension, 100 or 200 μl, was transferred to polystyrene tubes, in duplicate and evaporated under air in a 45°C water bath. For samples from the stress group 100 μl of supernate was

used due to high levels of corticosterone. Additionally, a pooled high sample was run at the beginning, and end, of all samples to monitor assay variance. Corticosterone (Sigma), ranging from 0 to 32 μg/dl was prepared in 100% ethanol as a standard curve. The standards were pipeted directly into polystyrene tubes and evapoated to dryness under air in a 45° C water bath. After drying, 1 ml of a CBG isotope solution (0.08% ^{3}H corticosterone, 2% human salvage plasma, in 100% ethanol) is added to each tube of sample and standard. The tubes are vortexed vigorously and incubated in a 45° C water bath for 10 minutes. The tubes are subsequently cooled below 5°C in an ice-water bath for 20-50 minutes. Florisil (40 mg) is added to each tube, the tubes are shaken on a horizontal shaker for 2 minutes at a speed that just lifts the flourisil into suspension, and the tubes are placed in the ice-water bath for 10 minutes to allow the flourisil to settle. The sample or standard supernatant (0.5 ml) is transferred to a scintillation vial with 3 ml of scintillation fluid. To determine total counts 0.5 ml of CBG-isotope solution is added to 3 ml of scintillation fluid. Radioactivity is determined by counting for 2.5 minutes on a Beta-scintillation counter (LKB Rackbeta). Data reduction of the standard curve and interpolation of sample values using a Lotus program was performed in order to calculate corticosterone content of the samples. This assay is sensitive to 0.2 μg/dl.

Shock Appparatus

Eight identical rodent chambers (BRS/LVE Inc., Laurel, MD, small animal chamber, Model RTC-020), measuring 25 x 30 x 28 cm, served as the shock apparatuses. The chambers have clear Plexiglas side walls, sheet-metal top and end walls, and a grid floor

consisting of bars 0.24 cm in diameter, spaced 1.4 cm apart. The boxes are connected by timer circutry to the output of a shock generator and scrambler (BRS/LVE Models 903 and SC922) to provide a 5.0-second, 1.6-mA, foot-shock that was scrambled throughout the floor bars. The chambers were individually housed in identical sound-attenuating cubicles, 36 x 56 x 42 cm (BRS/LVE, Model SEC-002). A 100-W, 120-V bulb, recessed in the wall of the cubicle, was operated at 85-V, AC, to provide diffuse illumination of the chamber. An ambient sound level of 72dB was provided by operating the cubicle's ventilating fan at 57 V, AC.

Stress Paradigm

For experiments in which stress was a factor, half of each injection group was randomly assigned to receive shock or serve as home cage (control) animals. The stress subjects were presented with 16 shocks, randomly delivered throughout a single 64-min session. The session began approximately 1-3 hours into the dark phase of the day-night cycle. The shocks were delivered every 2-6 minutes, with the time between shocks averaging 4 minutes. There was no discreet stimulus accompanying the shock. Home cage control subjects were handled and transported in a manner identical to the stress subjects, but were not placed in the shock aparatus.

Twenty minutes after the last shock, the subject was rapidly sacrificed by cervical dislocation. The control subjects were transported from their home cages to the chamber room and sacrificed in the same mannner and at approximately the same time (one animal from each injection group in no-shock treatment then one animal from each injection group

in the shock treatment) as the experimental subjects. Blood was collected via heart puncture into 5-ml heparinized syringes, the peritoneum was washed twice with a total of 50 ml of supplemented HBSS and the liquid from each animal was removed and pooled in a 50 ml polystyrene centrifuge tube, the spleen was removed and placed in a 15-ml polypropylene centrifuge tube containing supplemented media. All samples were transported to the tissue culture laboratory for preparation.

Statistical Treatment of Data

A computerized program for analysis of variance (Statistix, NH Analytical Software) was used to determine differences among experimental and control groups. A one by six ANOVA compared differences among groups for the dose response curve study. A two x three ANOVA was used with two levels of stress (shock treatment or home cage) and three levels of drug (bittermelon-BMP, denatured bittermelon-DBMP or HBSS (saline) injection) for the experimental paradigm using stress.

The significance level for all analysis was set at a probability less than or equal to 0.05. All data are shown as mean $\pm$ SEM. All significant main effects and interactions were further examined using orthogonal contrasts and t-tests. Additionally the hypothesis led investigators to examine differences between stressed BMP group and non-stressed controls.

RESULTS

Preliminary Experiments

Stimulation of Tumor Cytolytic Activity

To initially determine whether the crude BMP contained the immunostimulatory proteins, the extract was tested in mice. Mice were injected ip with 0, 4, 8, 16 or 32 μg of protein, ip, from the BMP four days prior to sacrifice. Peritoneal exudate cells were harvested, enumerated and assayed for tumor cytotoxicity. It was determined that the administration of BMP produced in our laboratory increased tumor cytotoxicity of mouse PEC cells at a dose equivalent to previous reports (8 μg/ml) (data not shown).

Upon determination that the extract contained immunostimulatory properties in mice, investigators proceeded to determine if an increase in cytolytic activity could be demonstrated in rats, and what dose of protein would yield the highest lytic activity against NK sensitive targets. Intraperitoneal injections of 0-1000 μg of BMP were tested. Heat denatured protein (100-HD μg) and hanks balanced salt solution (HBSS-0 μg protein) injection were used to as control treatments. Both peritoneal and spleen leukocytes were assayed for anti-tumor activity and the results are shown in Figures 1 and 2, respectively.

In PEC and spleen cytotoxicity data there was a main effect of treatment (F's $(4,23)=6.44$ and $(4,24)=5.27$ respectively, p's $<.005$). While the PEC from all groups receiving either a bitter melon injection had significantly ($p<.05$) higher tumor cytotoxic activity than the HBSS control group. The PEC from the group receiving 100 μg of

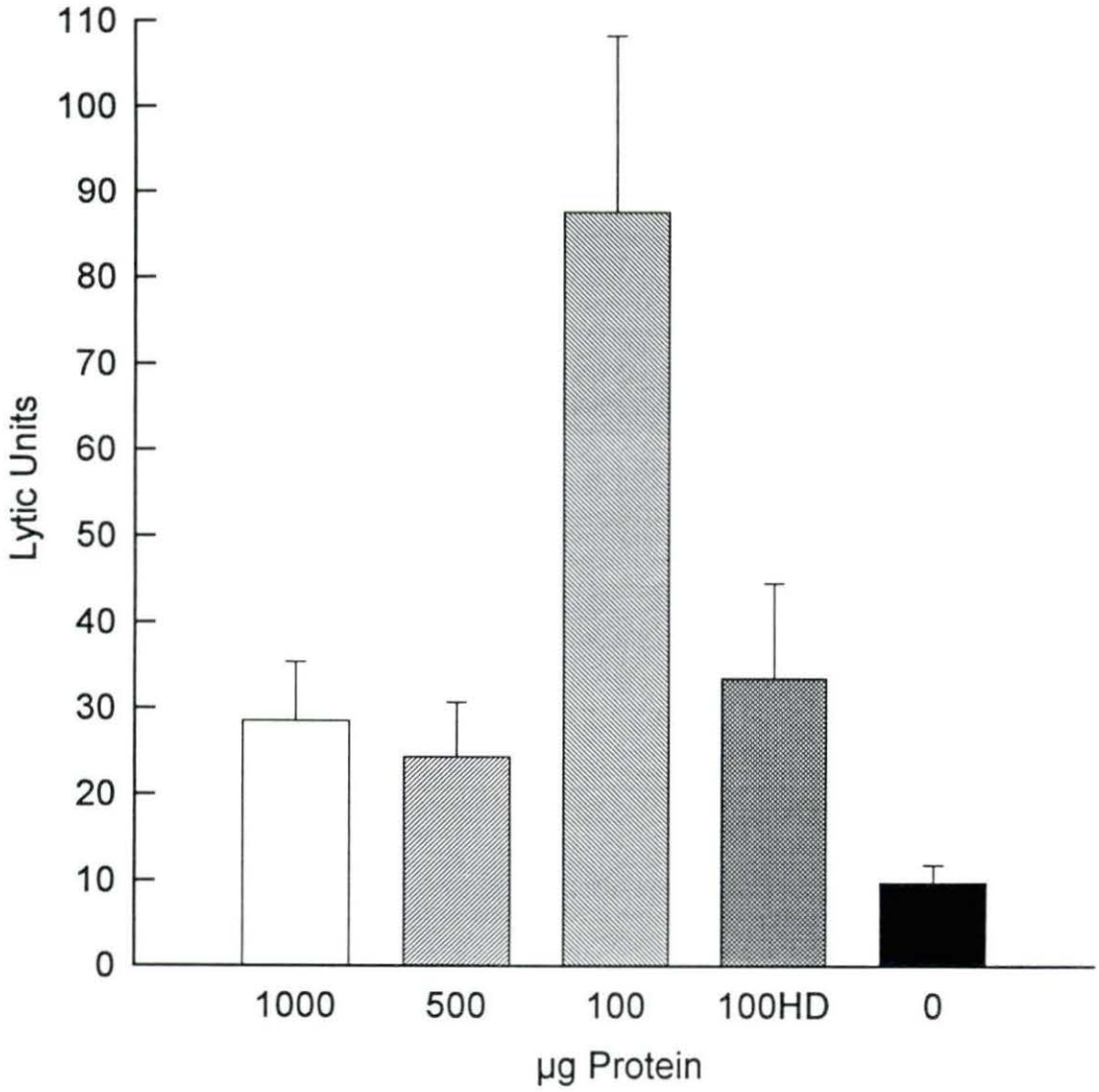

FIGURE 1. Tumor cytotoxicity of rat peritoneal exudate cells in response to various doses of bitter melon protein extract (BMP) or heat denatured bitter melon extract. The BMP was diluted in hanks balanced salt solution and all injections were given in 2 ml delivered i.p. The group which received 100 μg BMP had significantly higher levels of tumor cytolytic activity (lytic units) against YAC-1 tumor targets in comparison to both control groups: heat denatured protein (100 μg-HD) and 0 μg protein (HBSS) ($p < .05$). Results are represented the mean ($\pm$ SEM) for the designated group ($n=5$).

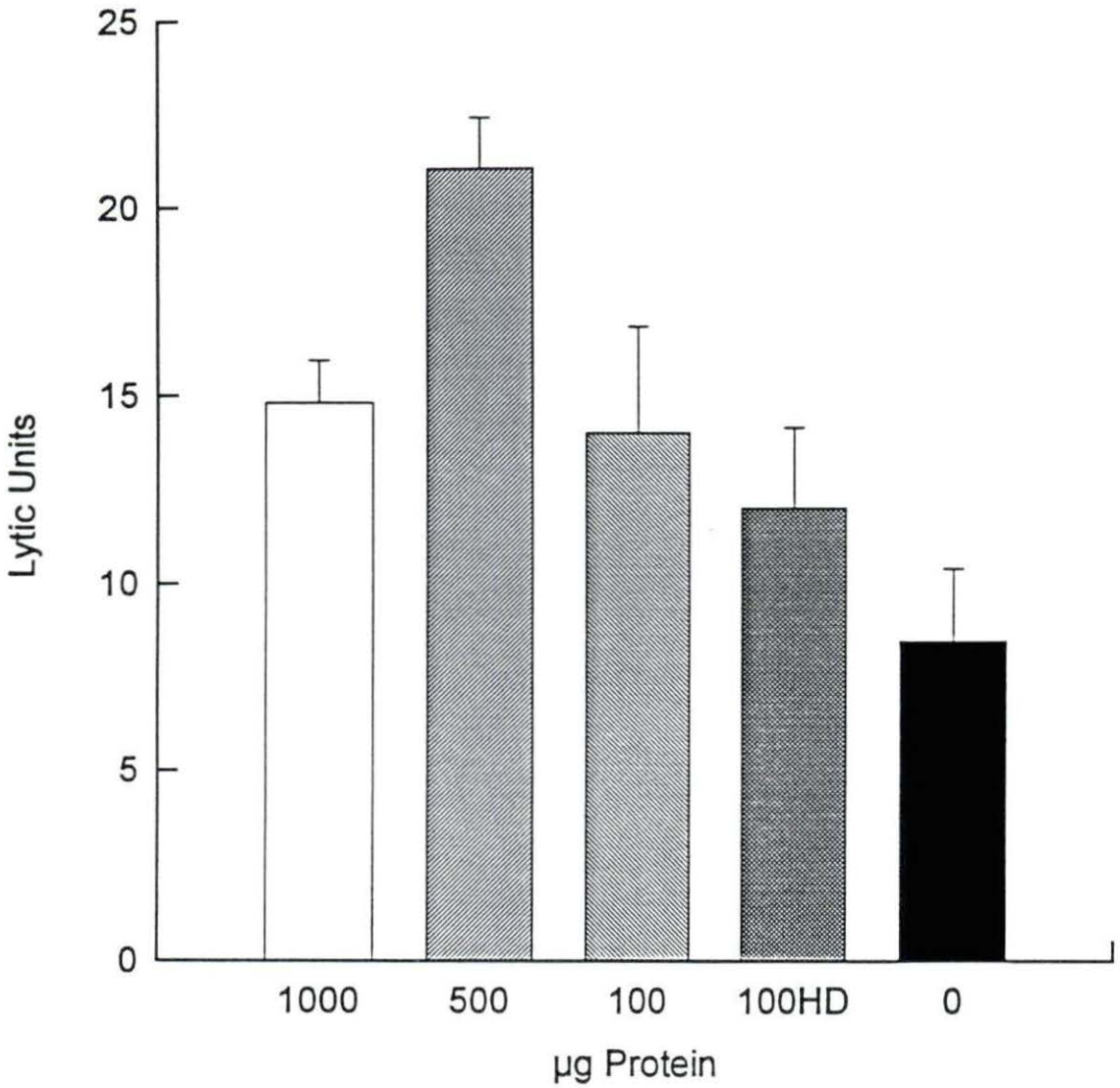

FIGURE 2. Tumor cytotoxicity of rat spleen leukocytes in response to various doses of bitter melon extract or heat denatured bitter melon protein extract. The BMP was diluted in hanks balanced salt solution and all injections were given in 2 ml delivered i.p. The group which received 500 μg bitter melon extract had significantly higher levels of tumor cytolytic activity against YAC-1 tumor targets in comparison to both control groups: heat denatured protein (HD) and 0 μg protein (HBSS) ($p < .05$). Each group represents the mean ($\pm$ SE) for the designated group ($n=5$).

protein had significantly ($p < .0005$) higher cytotoxic activity than all of the other groups that received a bitter melon injections. The 100 μg heat denatured control did have elevated cytolytic activity in comparison to the HBSS, but this was not significant.

In contrast, results of the tumor cytotoxicity of splenic leukocytes (Figure 2) show animals which received 500 μg protein had significantly ($p < .005$) higher anti-tumor activity than animals in the other bitter melon injection groups; while all animals receiving bitter melon injection were also shown the have significantly ($p < .05$) elevated cytotoxicity levels in comparison to the HBSS control group. There was no significant difference in cytotoxicity between the denatured and HBSS control groups.

Doses of BMP above 1000 μg protein and below 100 μg protein were also tested for stimulation of cytotoxicity (data not shown). The doses of BMP > 1000 μg induced weight loss in animals indicating illness or possible toxic effects. The data obtained from PEC of animals receiving such doses exhibited lytic units similar to the HBSS control groups confirming detrimental effects of such high doses. As our interest for this study was to examine modulation of peritoneal exudate cells, 100 μg bitter melon protein was chosen as our experimental dose for further studies.

Combined Effects of Stress and Bitter Melon

An experiment was designed to examine the effects of stress on the tumor cytolytic activity of PEC as well as the ability of BMP administration to block this modulation of tumor cytolytic activity. In this experiment, bitter melon was administered as three levels of drug (100 μg protein (BMP), 100 μg denatured protein (HD), or 0 μg

protein (HBSS)). On the experiment day, half of the animals from each injection group were stressed by presentations of mild electric foot-shock. Plasma, spleen and peritoneal exudate cells were collected and assayed in order to determine if the animals experienced stress and determine modulation of plasma corticosterone levels, splenic mitogen response, leukocyte cytolytic function, cell numbers, and phenotypic markers as a result of the drug and/or stress treatments.

Corticosterone

As an indicator of stress, plasma corticosterone was measured and the results are shown in Figure 3. ANOVA indicates that plasma corticosterone levels in the shock group are significantly ($p < .001$) higher than the levels in the no-shock (home cage) animals, indicating that the animals were stressed. There was no significant main effect of drug dose on corticosterone level or interactions of stress and drug. These results suggest that BMP treatment does not alter the animals response to stress. There was a main effect of replication and a main effect of replication and stress in corticosterone levels found in the plasma, due to the second replication having proportionally lower levels of corticosterone than the first replication. However, the directions of change due to stress treatment were similar in both replications.

Lymphocyte Proliferation

Spleen lymphocyte proliferation responses to mitogens were performed to determine immune suppression of this parameter which has previously been reported to be decreased due to stress. There was no significant effect of stress, drug or an interaction of stress and drug on the proliferation of lymphocytes in response to T cell

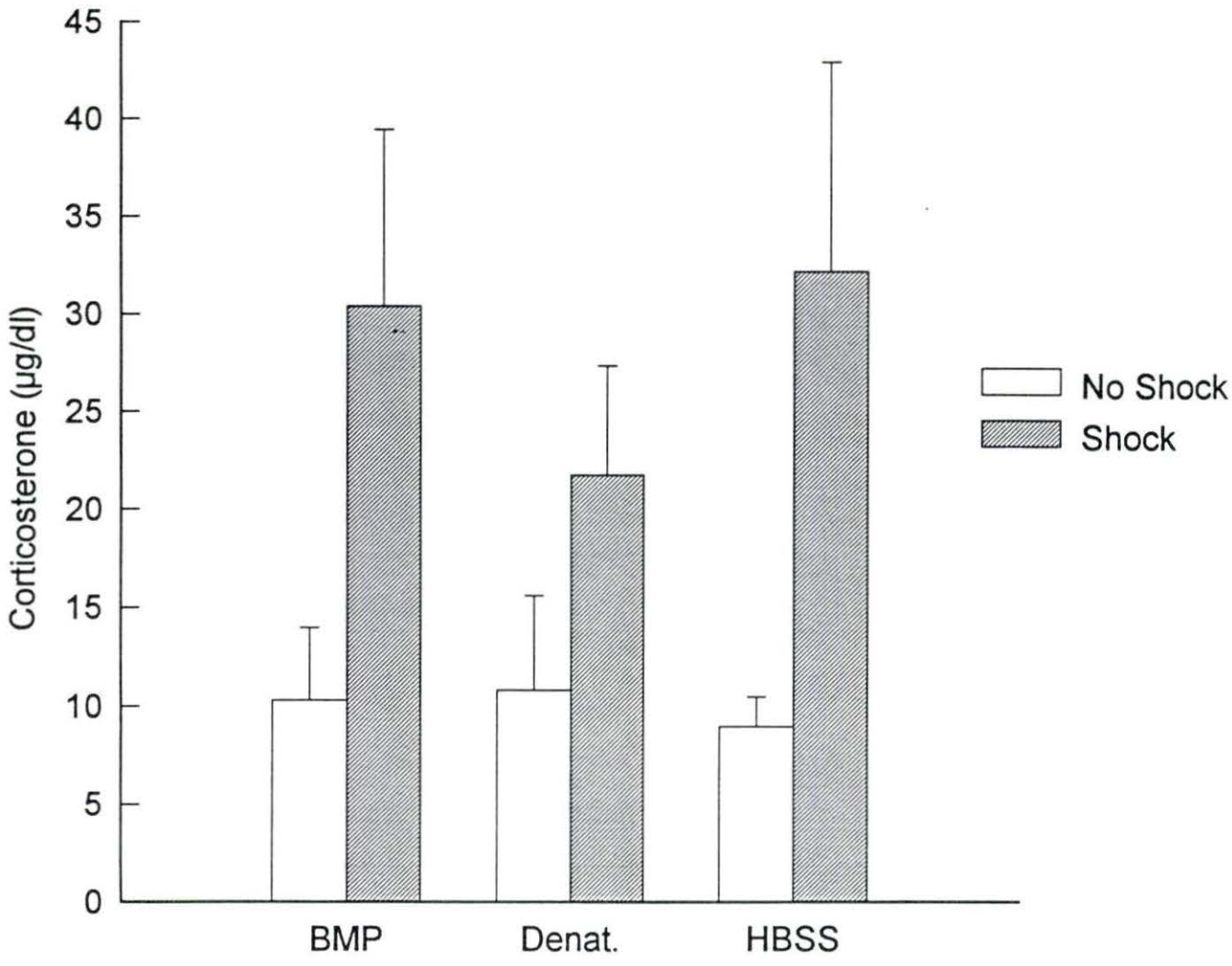

FIGURE 3. Concentration of corticosterone in plasma from stressed (electric foot shock) and non-stressed (home cage control) subjects. Stressor consisted of 16 random electric foot shocks, 5 second duration, 1.6 mAmps,in a 64 minute shock session. Corticosterone levels were significantly higher in the stress groups in comparison to the home cage controls ($p < .001$).There was not a significant difference in corticosterone levels between the drug - bitter melon ($n=6$), denatured bitter melon ($n=6$), or hanks balanced salt solution ($n=6$) treatment groups. Each bar representes the mean ($\pm$SE) for the designated group.

mitogens Con A and PHA or the B cell mitogen LPS (data not shown). The mean CPM in response to con A, PHA, or LPS was 2.92×10^5, 1.78×10^4 and 1.83×10^4, respectively.

Assay for Tumor Cytotoxicity

Cytotoxicity to YAC-1 cells by PEC and splenocytes was evaluated in rats receiving an injection of BMP and/or stress and the results are shown in Figure 4. In agreement with preliminary experiments, there was a significant main effect of drug, $F(2,20)=122.6$, $p<.0001$, which was due to a significantly higher killing of tumor cells by PEC in the BMP group compared to the two control groups ($p<.0001$). Additionally, stress induced a significant suppression of lytic activity against tumor target cells in the PECs, $F(1,20)=20.97$, $p<0.005$. Although no significant interaction was found between the bitter melon administration and stress treatment, planned contrasts indicated that the LU were significantly higher in the BMP-stress group than either the HD/No-shock or HBSS/No-shock control groups ($p's<.0001$).

Cytotoxicity to YAC-1 cells by splenocytes was evaluated and the results are shown in Figure 5. The administration of bitter melon protein did not show a significant increase in tumor cytotoxicity in comparison to the control groups. This is not surprising because 100 μg protein did not affect splenic tumor cytolytic activity in preliminary experiments (see Figure 2). However, there was a significant effect of stress in the splenocytes, $F(2,22)=6.4$, $p<.05$, that resulted in suppression of tumor cytotoxicity in comparison to the home cage control animals for all doses.

There was a significant main effect of replication in both the PEC and the spleen

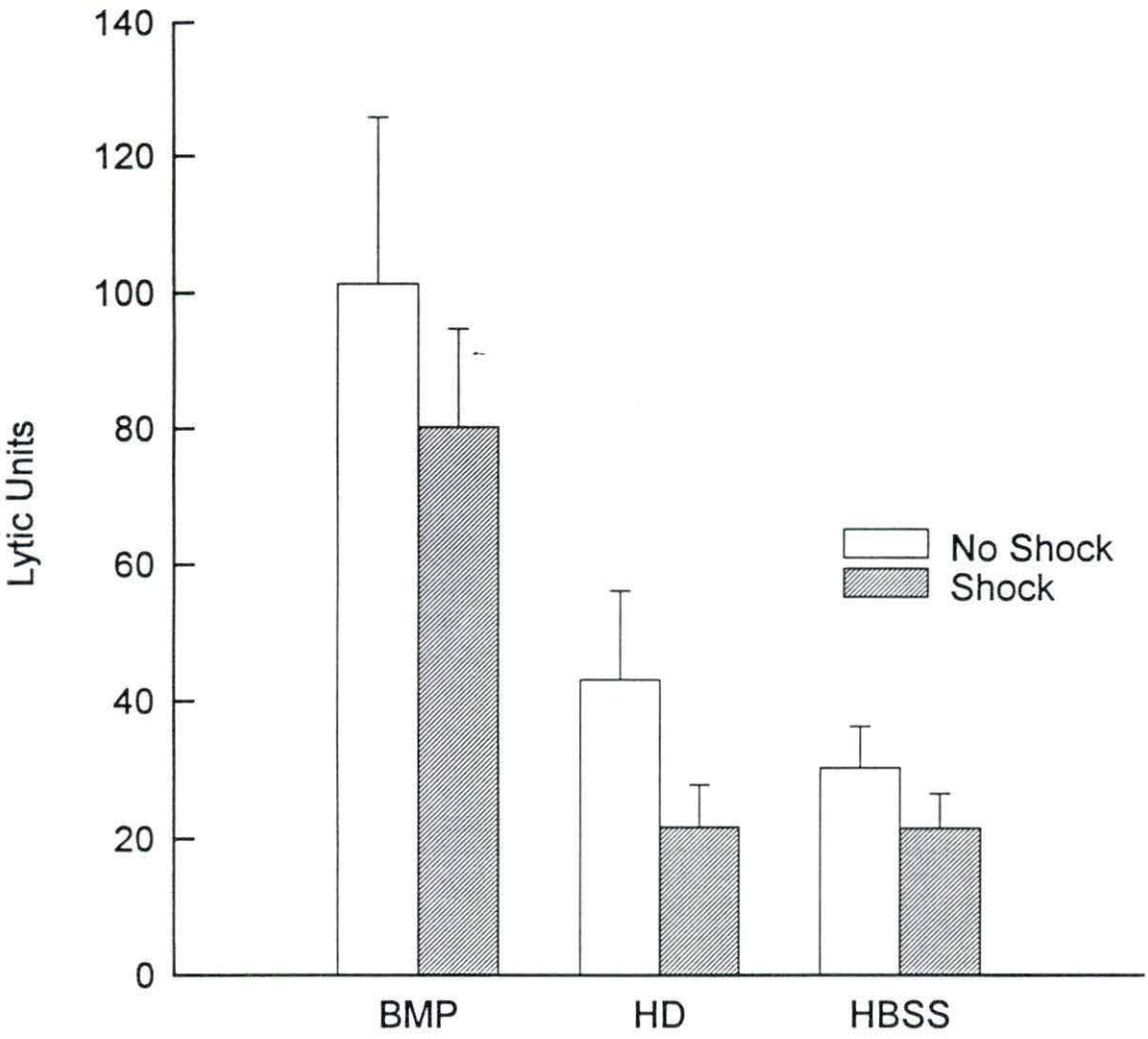

FIGURE 4. Tumor cytotoxicity of peritoneal exudate cells from stressed (electric foot shock) and non-stressed (home cage) subjects four days after an i.p. injection of 100 μg bitter melon protein extract (BMP; n=6), 100 μg heat denatured bitter melon (HD; n=6), or 0 μg protein (HBSS; n=6). Tumor cytotoxicity was significantly ($p < .0005$) suppressed by stress. The groups injected with bitter melon protein had significantly greater tumor cytotoxicity than the heat denatured or HBSS groups ($p < .0001$). Each bar represents mean ($\pm$SE) for the designated group.

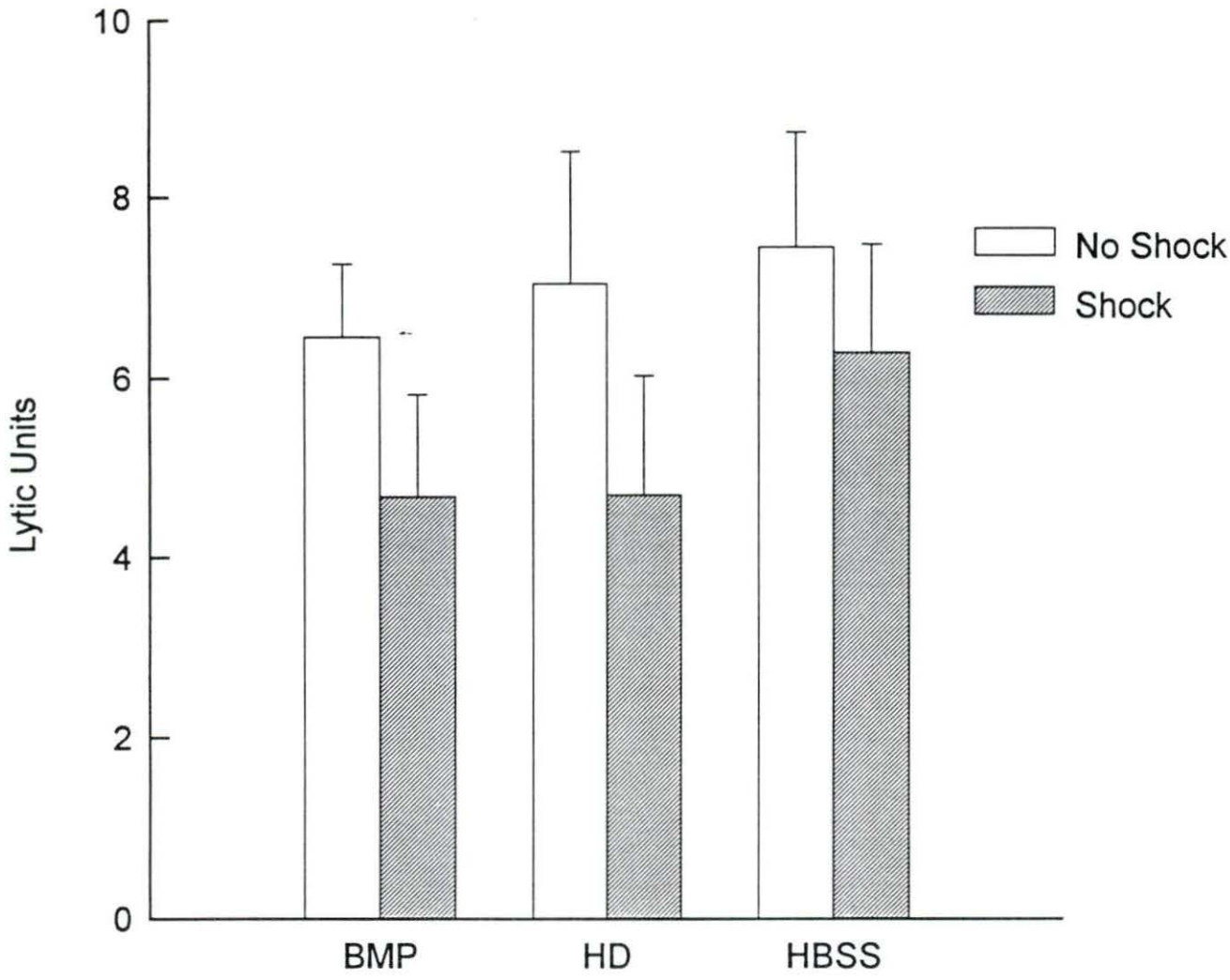

FIGURE 5. Mean (±SE) tumor cytotoxicity by splenocytes in stressed (electric foot shock) and non-stressed (home cage) subjects four days after an i.p. injection of 100 μg bitter melon protein (BMP; n=4), 100 μg heat denatured bitter melon protein (HD; n=6), or 0 μg protein (HBSS; n=6). Tumor cytotoxicity was significantly suppressed due to stress ($p < .05$). The groups injected with BMP did not have significantly higher tumor cytotoxicity in comparison to the HD and HBSS control groups.

that interacted with dose and stress. This was due to the second replication having proportionally lower lytic units than the first replication. However, the direction of change in both replications was similar.

Leukocyte Enumeration and Identification

In order to better understand the mechanisms of this immunostimulatory agent, the cells were enumerated and identified by Wright-Giemsa staining. As shown in Figure 6, the PECs displayed a significant increase in the number of cells recovered in the peritoneal wash as a result of administration of the BMP when compared to either the heat denatured or HBSS control, $F(2,21)=67.87$, $p<.0001$. Recovery of PEC in the BMP treated group, regardless of stress treatment, was in excess of 8.0×10^7 cells, but both the denatured and HBSS groups cell numbers were below 3.0×10^7 cells. There was no main effect of stress on number of leukocytes recovered. The spleen leukocyte counts did not vary significantly as a result of either the drug treatment or the stress treatment (data not shown).

To identify the cell types resident in the peritoneum and determine if the treatments favored an increase of one particular type of cell, leukocyte differential cell counts were performed (see Table 1). The major type of cell found in the peritoneum regardless of shock or drug treatment was macrophages. The majority of cells found in the spleen was lymphocytes, and there was no main effect of drug or shock treatment on cell type.

Flow Cytometry

In order to determine if the increased lytic activity was due to an increase in a particular cell linage or merely a global increase in activity, cell surface staining and

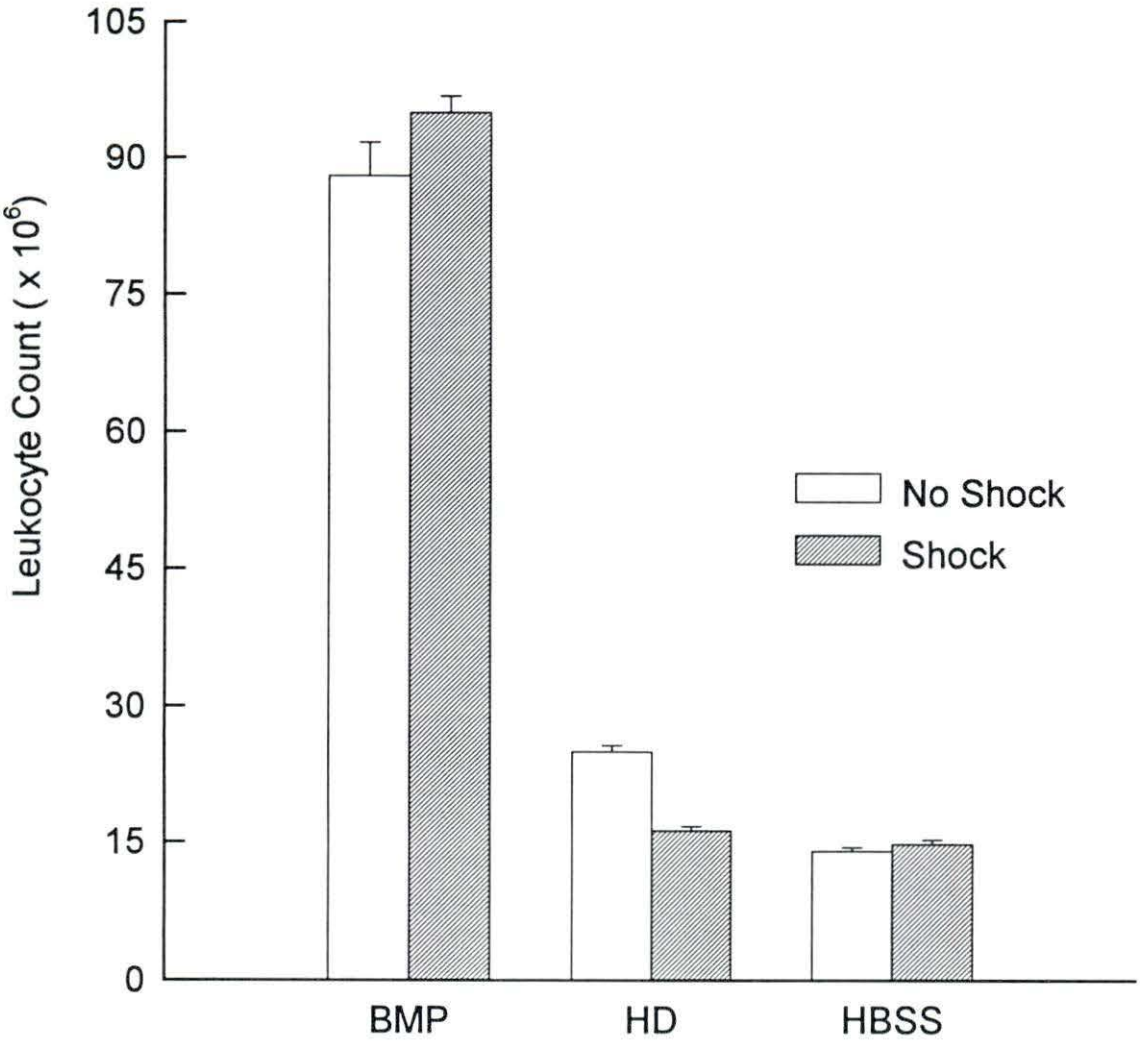

FIGURE 6. Mean ($\pm$SE) of number of leukocytes recovered from peritoneal lavage 4 days after ip injection of 100 μg bitter melon protein (BMP; n=4), 100μg heat denatured bitter melon protein (HD; n=6) or 0 μg protein (HBSS; n=6). All injections were 2 ml, ip. The groups which received BMP had significantly ($p < .0001$) higher leukocyte numbers than the HD or HBSS control groups. There was no significant difference due to stress.

Table 1. Lymphocyte differential cell counts of spleen and peritoneal exudate cells from stressed (electric foot shock) and non- stressed (home cage) four days after ip injection of bitter melon protein (BMP), denatured bitter melon protein (HD)or hanks balanced salt solution (HBSS). There was no significant difference in cell counts due to stress or drug. Greater than 100 cells were counted (n=6).

	basophil	neutrophil	monocyte	eosinophil	lymphocyte	macrophage
Spleen						
No shock				0%		
BMP	1%	0%	1%	0%	98%	0%
Denat.	0%	0%	2%	0%	98%	0%
HBSS	<1%	0%	2%		97%	0%
Shock						
BMP	0%	0%	1%	0%	99%	0%
Denat.	0%	0%	1%	0%	99%	0%
HBSS	0%	0%	1%	0%	99%	0%
PEC						
No shock						
BMP	0%	<1%	72%	7%	20%	0%
Denat.	0%	0%	73%	12%	16%	<1%
HBSS	0%	1%	75%	12%	10%	<2%

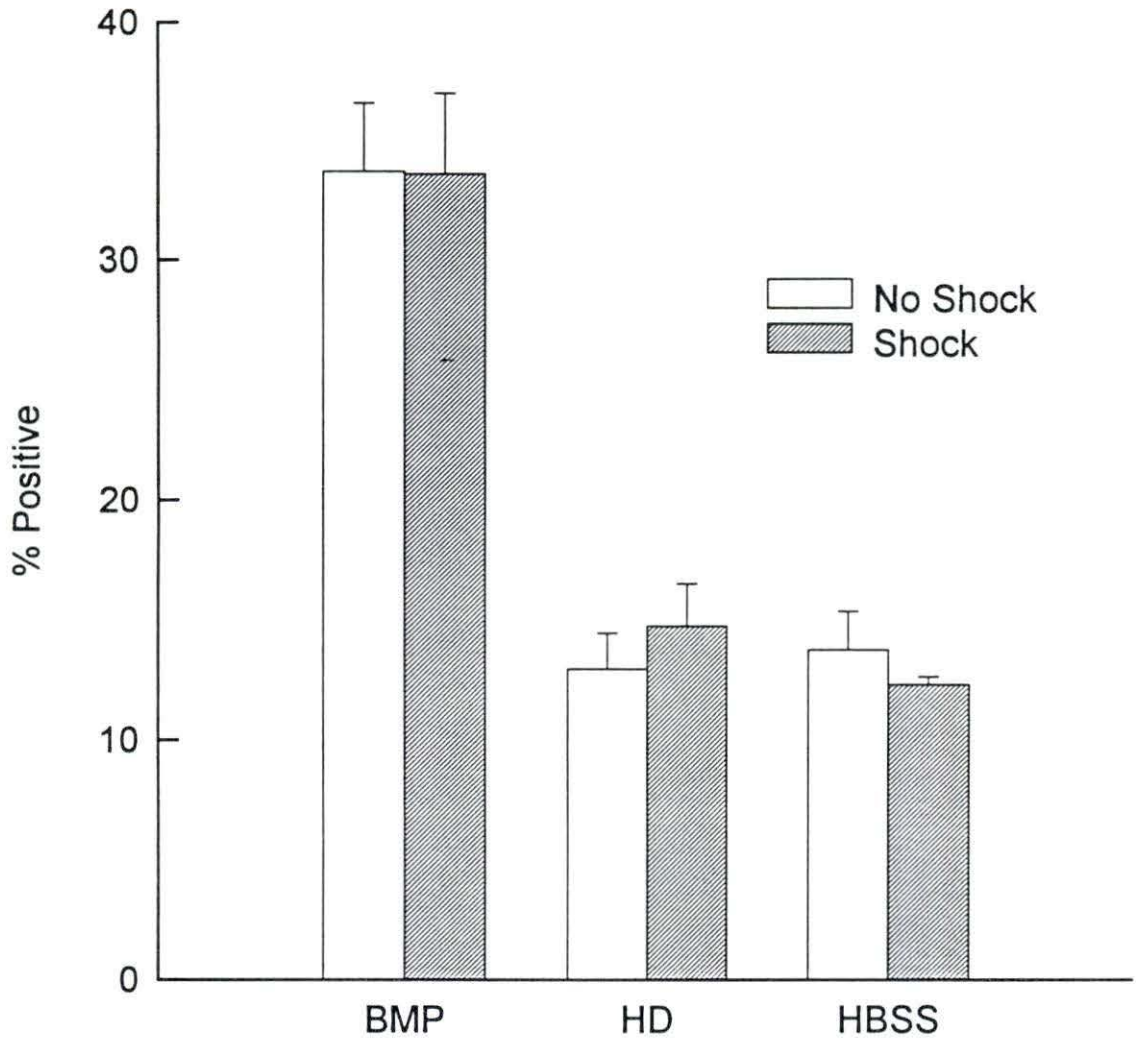

FIGURE 7. Percent CD3+ and CD8α+ leukocytes recovered from peritoneal lavage of rats 4 days after i.p. injection with 100 μg bitter melon protein (BMP; n=4), 100μg heat denatured bitter melon protein (HD; n=6) or 0 μg protein (HBSS; n=6). Animals that received bitter melon injections had a significantly ($p < .0001$) higher percentage of double positive cells than either the HD or HBSS control groups.There was no significant difference due to stress. Each bar represents mean ($\pm$SE) for the designated group.

flow cytometric analysis were performed. The cell phenotype was evaluated in both the spleen and peritoneal exudate leukocytes using antibodies to rat CD3 (T cell), CD8α (Tc or NK), or NKR-P1 (NK cell). Figure 7 shows the peritoneal exudate cells of the BMP treated animals contained a significantly higher proportion of $CD3^+CD8^+$ double positive lymphocytes than the other two treatment groups, $F(2,15)=50.23$, $p<.0001$. Figure 8 shows that the BMP administration also significantly increased the number of NK cells recovered from the peritoneum, $F(2,19)=16.25$, $p<.001$. There was no main effect or any interaction between drug and stress on number of NKR-P1 positive cells in the PEC. There was no main effect of stress or any interaction between drug and stress on the percentage of $CD3^+CD8^+$ or NKR-$P1^+$ cells in the PEC.

Splenocytes were also analyzed for any changes in phenotypic cell type, no significant changes in population occurred as a result of drug, stress or any interaction between stress and drug.

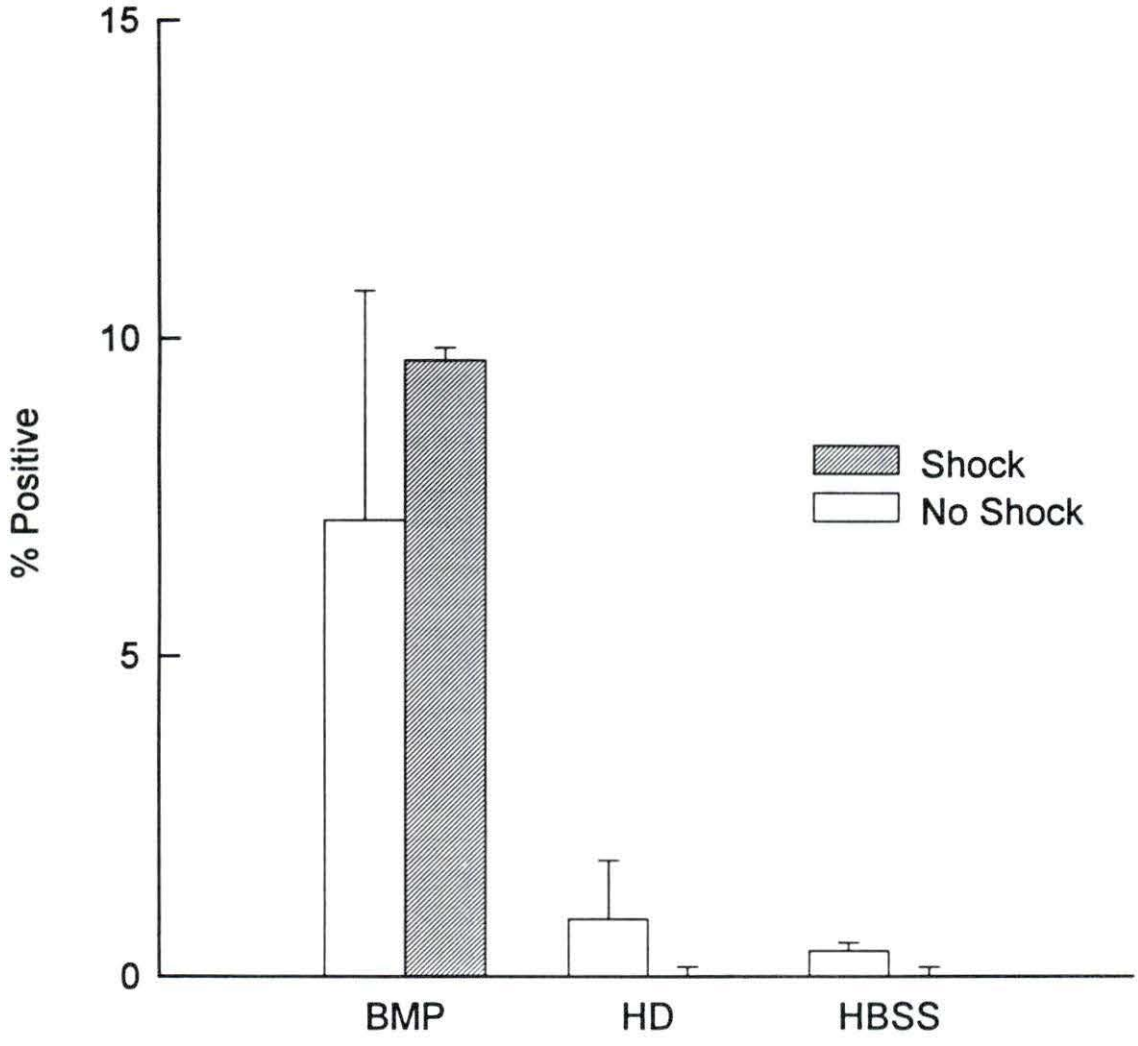

FIGURE 8. Percent positive NKR1-P leukocytes recovered from peritoneal lavage of rats 4 days after i.p. injection with 100 μg bitter melon protein (BMP), 100μg heat denatured bitter melon protein (HD) or 0 μg protein (HBSS). Animals receiving bitter melon injections had a significantly ($p < .05$) higher percentage of positive cells than either the HD or HBSS control groups. Our data indicates there are no positive cells in the stress-HD or stress-HBSS. There was no significant difference due to stress. Each bar represents mean ($\pm$SE) for the designated group ($n = 3$).

DISCUSSION

Cellular immunity is part of the bodys' primary defense against viral infection and tumor formation. A defect or suppression in this critical first line of defense would leave an individual more susceptible to disease. New cancer therapies consist of the administration of biologically active compounds, such as IL-2 or one of the IFNs, to stimulate anti-tumor activity in patients who are more susceptible to tumor formation. Previous studies have indicated that the Chinese bitter melon contains naturally occurring immunostimulatory agents (Cunnick, et al., 1990). The bitter melon has been shown to have both anti-viral effects (Lee-Huang et al., 1990) and anti-tumor effects (Cunnick, et al., 1990). Researchers have also indicated that stress induces a robust suppression of immune function resulting in decreased anti-tumor activity (Lysle et al., 1988). The administration of bitter melon agents may lessen stress induced suppression of tumor cytotoxic activity.

Preliminary experiments have demonstrated that ip injection of 100 μg of bitter melon protein extract stimulates the killing of tumor targets by rat peritoneal exudate cells in a short-term cytotoxicity assay. This same type of killing was demonstrated in spleen leukocytes, though a dose of 500 μg protein was required to detect a significant increase in tumor cell lysis (Figures 1 and 2). The difference in dose required to stimulate anti-tumor activity in the PEC and the spleen may reflect the fact that a higher dose causes activation sooner in the peritoneum, whereupon the cells then migrate to the spleen. Alternatively, the bitter melon protein, at higher doses, might circulate to the spleen

through the blood, and activate splenocytes directly. Although the heat denatured protein does increase the lytic activity of the effector cells, this increase was minimal when compared to the substantial increase in lytic activity seen in the groups that received the native BMP injections. Thus, the use of a HBSS (0 μg protein) and a heat denatured protein control demonstrates that only a minor part of immunostimulation by BMP could be attributed to a nonspecific stimulatory effect of injecting protein in a substantial volume into the peritoneum.

The second experiment used a common paradigm of unpredictable mild electric foot shock (Lysle and Lyte, 1987; Cunnick et al., 1988). To determine if administration of the BMP could reduce stress induced suppression of tumor cytotoxic native BMP, heat denatured bitter melon and HBSS were administered in combination with stress (electric foot shock) or no-stress (home cage) treatments. This paradigm was stressful to the animals as evidenced by the significant increase in corticosterone. Additionally, presentations of shock resulted in suppression of anti-tumor activity in both the spleen and peritoneal exudate cells. Administration of BMP induced a substantial increase in tumor cytolytic activity in the PEC but not the spleen (Figures 1 and 2). Although the tumor cytotoxic activity of PEC was suppressed by stress the level of cytotoxicity in animals receiving bitter melon and a stressor had significantly higher anti-tumor activity than did the no-stress animals in either of the control groups. Interestingly, suppression of lytic activity due to stress still occurs in the bitter melon group suggesting that administration of bitter melon does not affect the mechanism of stress induced

suppression, but rather is activating the immune system directly. It could be proposed that injection of a higher dose of BMP, as indicated in Figure 2, would result in similar activation of spontaneous splenic tumor cytotoxicity.

No enhancement was seen in splenic mitogen responsiveness due to injection of BMP. Additionally, there was not the expected suppression of splenic mitogen responsiveness due to stress as previously reported. The investigators theorize that the anomaly (lack of stress induced suppression) in the mitogen response is due to the change in the time of sacrifice after the shock session from 0-20 minutes to 20-50 minutes post-stress. Previous investigators have seen that stress induced suppression of mitogen responsiveness recovers in less than 24 hours (Lysle and Lyte, 1987), while the present study suggests a more rapid recovery. Indeed preliminary experiments demonstrated suppression of mitogen responsiveness when the rats were sacrificed 0-20 minutes post-stress (data not shown). However, stress induced suppression of NK activity was marginal at that time. The sacrifice times were shifted in order to optimize NK suppression, but suppression of mitogen responsiveness was lost, reflecting the possibility of different mechanisms mediating stress induced immune suppression. Interestingly, most reports of NK suppression examine animals at 24 hours post-stress (Shavit et al., 1984). Thus, a greater suppression of cytotoxicity may be seen if the sacrifice time was shifted beyond 50 minutes post-stress.

In the past, all spontaneous cytotoxic activity was attributed to natural killer cells because the standard assay used to detect tumor cytotoxicity (^{51}chromium release assay) is

a functional assay and can not distinguish spontaneous killing between cell types. Additionally, the cell lines used as tumor targets are known as NK sensitive targets (Deju, 1975), which fosters the erroneous notion that only NK cells can kill such tumor target cells. Recently, a number of different cell types have been shown to possess the ability to kill in a spontaneous, MHC-unrestricted manner (Cesano et al., 1993; Matthew et al., 1993; Okumura et al., 1995). It is reasonable to think that it is possible to stimulate such anti-tumor activity in a manner similar to that of NK cells. Activation of tumoricidal cells is clearly advantageous in any situation; however, during a time of stress when immune function is suppressed, augmentation of anti-tumor activity would be particularly significant. We have shown that the Chinese bitter melon possesses substantial immunostimulatory properties in both splenic and peritoneal lymphocytes and that suppression of PEC activity can be demonstrated as a result of stress. Plasma corticosterone levels in all groups indicate that the animals that received shock were stressed. For our purposes, corticosterone is measured as an indicator of stress, the data do not determine if the suppression is a direct effect of corticosterone or some other hormone acting on the cells.

Administration of bitter melon caused a large increase in cell numbers in the peritoneum, regardless of stress treatment. Cell differentials indicate that the number of lymphocytes remains relatively constant across all groups (Table 1). This finding leads us to question whether activation of lytic activity was a result of increased activational

state of all cell types or if certain key cell types were preferentially undergoing drug induced proliferation (Figures 6 ,7 and 8).

Through flow cytometric analysis it was demonstrated that treatment with bitter melon protein enhances the percentage of cells in at least two populations found in the peritoneum. Natural killer cells were seen to increase in numbers by approximately 10% (Figure 8), as did a CD3+CD8+ cell population (Figure 7), while the percentage of lymphocytes remained the same across all groups (Table 1). These findings suggest that it is a shift in lymphocyte cell type or maturational level and not an influx of lymphocytes that causes the increased anti-tumor activity. The results do not indicate if the increased anti-tumor activity was a direct result of the increased double positive population, increase in natural killer cell population, a combination of the two or some other factor. It is possible that the administration of the bitter melon causes a more systemic activation of immune function which is causing the release of cytokines that may stimulate tumor cell immunity. Many types of cells are capable of NK-like killing, particularly in response to cytokines. It is possible that administration of the bitter melon activates macrophages which secrete IFN-α, -β and/or TNF-α, known activators of NK-like killing in CD3+CD8+ cells.

In summary, the results indicate administration of bitter melon protein induces increased cell numbers and activation of tumor cytotoxic peritoneal exudate cells in rats, and that this enhancement persists in the face of stress. It has been shown that this activity may not be entirely due to natural killer cell activity as previously thought but

that it may be partially due to activation of CD3+CD8+ cells; a cell line only recently identified as having the capacity for MHC-unrestricted killing.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Joan Cunnick for teaching me so much more than experimental procedures and protocols. Her faith in my ability to perform sound experiments has given me a confidence as a scientist I could not have learned anywhere else. I would like to thank Shiu-huey Chou for being an endless source of answers, questions and patience. I would also like to thank my other committee members, Dr. Richard Hughes and Dr. Mike Wannemuehler for their useful suggestions and stimulating questions. I would especially like to thank my family for their faithful support - especially my parents, Jack and Bobbie Nordyke who unconditionally believed in me, even when I was unsure of myself. Last, I would like to thank my husband Mike, who challenges me to learn more every day - about science, about Immunology, and about myself.