MECHANISM OF MACROPHAGE ACTIVATION BY LARGE GRANULAR LYMPHOCYTES (LGL): IDENTIFICATION OF A CYTOKINE(S) CONTAINED IN RAT AND HUMAN LGL CYTOPLASMIC GRANULES THAT REGULATES MACROPHAGE TUMORICIDAL FUNCTION

BY

EUGENE ROUSSEL

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirement

for the Degree of

PHILOSOPHIAE DOCTOR (Ph.D.)

Department of Immunology

University of Manitoba

Winnipeg, Manitoba

ISBN 0-315-76841-X

MECHANISM OF MACROPHAGE ACTIVATION BY LARGE GRANULAR LYMPHOCYTES (LGL): IDENTIFICATION OF A UNIQUE CYTOKINE(S) CONTAINED IN RAT AND HUMAN LGL CYTOPLASMIC GRANULES THAT REGULATES MACROPHAGE TUMORCIDAL FUNCTION

BY

EUGENE ROUSSEL

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

A ma grand-mère Joséphine,
ma très chère dame Marie-Claude,
mon père et ma mère,
et tous ceux et celles dont la rencontre
furent une source d'évolution.

A mon propre devenir pour qui la réalisation de ce doctorat dans une langue seconde, une contribution scientifique inédite et la formation d'une personnalité scientifique internationale représentent dépassements et stoïcisme dignes de mention.

" Il n'y a pas de plus belle émotion que celle que l'on éprouve en approchant du mystère de la nature quand un fragment de l'inconnu se dévoile à votre esprit, vous ressentez une sorte d'émerveillement une joie intense qui vous envahit "

Albert Einstein

ACKNOWLEDGMENTS

I sincerely thank a thousand times Dr Arnold Greenberg for the very high quality of teaching and the support that he provided to me during all these years.

I thank the Manitoba Institute of Cell Biology and the Department of Immunology, professors, staff and graduate students for their collaboration and help all along these years.

I am very grateful to the Canadian Society for Immunology for their acknowledgments of the quality of my scientific work through these differents awards granted in 1986, 1987 and 1990.

I am grateful to the European Federation of Immunological Societies for their attention to my scientific achievements through their invitation at the 9th European Immunology Meeting in Rome, 1988.

Finally, I would like to thank Dr Monte S. Meltzer, Dr Ronald Herberman, Dr Robert Evans and Dr Fred Valentine for the very prestigious "International Presidential Award" granted to me during the 11th International Meeting of the Reticuloendothelial Society in Kauai, Hawaii, 1987.

ABBREVIATIONS USED IN THIS THESIS

BM, bone marrow; BSA, bovine serum albumin; Con A, concanavalin A; CSF-1, macrophage colony stimulating-factor; CTL, cytotoxic T lymphocytes; FCS, fetal calf serum; GM-CSF, granulocyte macrophage-colony stimulating factor; HBSS, Hank's balanced salt solution; HI, heat-inactivated; h, hour; IFN, interferon; IL, interleukin; kD, kilos Daltons; LAK, lymphokine activated killer; LGL, large granular lymphocyte; LK, lymphokine; LPS, lipopolysaccharide; Mϕ, macrophage; MAF, Mϕ activating factor; MHC, major histocompatibility complex; M.W., molecular weight; NK, natural killer; PBL, peripheral blood lymphocytes PBS, phosphatebuffered saline; PE, phycoerythrin; PHA, phytohemagglutinin A; PMSF, phenyl-methylsulfonyl fluoride; rhIL, recombinant human interleukin; rmIFN, recombinant murine interferon; RT, room temperature; SBTI, soy-bean trypsin inhibitor; SRBC, sheep red blood cells; TBS, tris buffer saline; TLCK, N-alpha-p-tosyl-L-lysyl-chloromethyl ketone; TNF, tumor necrosis factor; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

ABSTRACT

It has been previously shown by Greenberg et al. that LGL rapidly release preformed factor(s) that stimulate monocyte oxidative metabolism and microbicidal activity. We have hypothesized that such factors could also activate Mϕ tumor lysis and might be stored in LGL cytoplasmic granules. Using rat LGL leukemia cell lines, granules were isolated from disrupted cell homogenate by Percoll gradient fractionation. CSF-1 differentiated murine bone marrow-derived Mϕ activated with these granules in co-presence of LPS killed the TNF-resistant P815 tumor target cells. Solubilization in 2 M NaCl was necessary to recover activity from granules, suggesting that the MAF was tightly bound to an internal granule matrix. The rat granule-MAF appeared to have biochemical and biological properties distinct from IFN-τ (the most studied MAF). To verify if this MAF was present in the granules of normal human LGL, we examined human LAK cell granules. Human $CD3^+$ LAK cells were generated by incubating PBL in a two step method for 10-12 days with rhIL-2 10 U/ml. This particular method produced a morphologically homogeneous population of LGL, phenotyping $CD3^+$,$CD16^-$ at 95%. Human LAK cell granules were isolated and solubilized as above and then tested for MAF activity. Human LGL granules fully activated Mϕ tumoricidal activity against P815 without need for LPS. The human granule-MAF

showed a synergistic effect with rhIL-1ß, rmTNF-α and rmIFN-τ in the Mϕ cytolytic assay. In addition, proteose-peptone elicited murine peritoneal Mϕ profoundly increased H_2O_2 production after activation with human LGL granule-MAF but unlike with IFN-τ, they did not increase Ia antigen expression. Moreover, granule-MAF suppressed Ia induction by IFN-τ. This work shows for the first time the presence of immunoregulatory molecule(s) in granules of rat and human LGL. The human granule-MAF biochemical characteristics and distinct regulation of Mϕ activation for tumoricidal activity, H_2O_2 production and Ia antigen expression suggest the presence of a unique cytokine in the granules. It also suggests that the LGL degranulation process may do more than elicit a cytolytic process, it may regulate Mϕ priority as cytocidal effectors rather than antigen presenting cells.

TABLE OF CONTENTS

Dedication..... I

Citation..... II

Acknowledgments.....III

Abbreviations..... IV

Abstract..... V

Table of contents.....VII

List of figures.....VIII

List of tables..... IX

Chapter I..... 1

Introduction

Chapter II..... 46

Identification of a macrophage activating factor (MAF) in granules of the RNK large granular lymphocyte leukemia.

Chapter III..... 83

Long-term cultures of human peripheral blood lymphocytes with rhInterleukin-2 generate a population of virtually pure $CD3^+$,$CD16^-$,$CD56^-$ large granular lymphocyte LAK cells.

Chapter IV.....114

Granules of human $CD3^+$ large granular lymphocytes contain a macrophage regulating factor(s) that induces macrophage H_2O_2 production and tumoricidal activity but decreases cell surface Ia antigen expression.

Chapter V.....150

General discussion.

LIST OF FIGURES

Figure

1 NK-MAF activity of independent RNK cell lines... 60

2 Distribution of NK-MAF, ß-glucuronidase, cytolysin, and protein in the Percoll gradient fractions of the RNK 16 cell homogenate..... 61

3 NK-MAF activity is dependent on LPS..... 63

4 Kinetics of activation by NK-MAF..... 66

5 Heat resistance of NK-MAF..... 68

6 Morphology of the evolving LAK cell population at day 0 (left), 6 (center), and 12 (right)..... 95

7 Fluorescent stained granules of LGL $CD3^+$ LAK cells at day 12..... 96

8 Tumoricidal activity of bone marrow-derived macrophages after activation with solubilized LAK granules.....130

9 Synergy of human LAK granule-MAF with rhIL-1ß, rmTNF-α, or rmIFN-τ.....131

10 PMA triggering of H_2O_2 production after activation of peritoneal macrophages by human LAK granules.....133

11 Percent peritoneal macrophages expressing Ia antigen after activation by LAK granule-MAF.....135

LIST OF TABLES

Table

I Regulation of macrophage function by activating cytokines..... 18

II Solubilized NK-MAF activity in RNK cell granules 59

III NK-MAF activation of macrophage tumor killing following coincubation but not sequential activation by LPS..... 65

IV NK-MAF Sensitivity to Proteases..... 70

V Description of the antibodies used in this study 91

VI CD3,CD16 phenotyping and LU/10^7 cells of the evolving LAK cell population..... 97

VII Phenotyping and LU/10^7 cells of the evolving LAK cell population in function of the IL-2 concentration..... 99

VIII Phenotyping as a function of time of the T cell populations.....101

IX LAK granule-MAF optimum recovery from solubilized granules.....129

X Comparison between the rat and human LGL granule-MAF activities.....162

CHAPTER I

INTRODUCTION

I THE MACROPHAGES

1. Description and origin

The cells of the body that respond to antigens are categorized as belonging to the immune system. The immune system is made of different organs (tonsils, lymph nodes, thymus, spleen, Peyer's patches, appendix, lymphatic vessels) and a tissue the hemopoietic system. The hemopoietic system is made of two parts: the bone marrow precursor cells and the circulating blood cells. The marrow tissue gives rise to different cell lineages, the erythroid (red cells), the megakaryocitic (platelets), the lymphoid (lymphocytes) and the myeloid (granulocytes and monocytes (1,2). All these arise from a primitive undifferentiated hemopoietic stem cell which differentiates into separate committed precursors in response to microenvironmental stimuli and several growth factors like erythropoietin, colony-stimulating factors (GM-CSF, G-CSF, M-CSF) and cytokines (interleukins 1-2-3-4-5-6-7, IFNs, TNFs and TGFs) to eventually become red cells, platelets or mature immunocytes and activated effectors (2,3,4,5).

The myeloid lineage precursors in response to IL-3, IL-6, GM-CSF and M-CSF will differentiate into promonocytes and then circulating monocytes (6). The monocytes represent 1-3% of the circulating leukocytes in the human blood.

Monocytes have a circulating half-life of only 8-10 h. The diameter of these cells is ranging between 10 and 20 μm, their nucleus is large and usually occupies about half of the space within the cell with an oval or kidney shape. The cytoplasm is abundant and has a fine granular texture as a result of its generous content of lysosomal granules. Monocytes that disappear from the blood migrate to the tissue and become macrophages. Tissue macrophages have a life span of many months or years. Their form slightly modifies with a diameter ranging from 20-80 μm and according to their location they have specific tissue names: alveolar Mϕ in lungs, Kupffer's cells in the liver, microglia of the central nervous system, peritoneal Mϕ of the abdomen, Langerhans cells of the skin, Lamina propria of the large and small intestines, spleen Mϕ and bone marrow Mϕ (7). The macrophage population therefore consists of the circulating blood monocytes and the motile and fixed macrophages of the tissues.

2. Roles of macrophages and mechanism of function

Macrophages exert many roles. They are the most active phagocytic cells of the body and they are important in the cooperation with B and T cells as antigen presenting cells for the elaboration of the T cell dependent antigen specific immune response. Macrophages are very effective killers

against parasites and tumor cells. They are also important secretory cells, producing and secreting components of the complement system, hydrolytic enzymes, toxic forms of oxygen and different monokines.

Chemotaxis

Circulating monocytes and motile macrophages have the ability to migrate specifically to the infection or inflammation site. This phenomenon is called chemotaxis of macrophages. Chemotaxis occurs through the directional migration of cells along chemical gradient when chemical agents are released from bacteria or other leukocytes at the infection site. Several substances have been identified as chemotaxins for monocytes. These include soluble bacterial factors, peptide fragment of the complement activation C5a, cationic peptides released from neutrophils (1) and TNF-α (8).

Actual mechanisms for chemotaxis propose that it is mainly mediated by the Mac-1 and the p 150,95 adhesion molecules. *In vivo* chemoattractants diffuse from sites of inflammation into the circulation inducing Mac-1 and p150,95 up regulation on macrophages. Cells undergoing chemotaxis orient in the gradient with lamellipodia at their anterior end and the uropod at their posterior end. Sensing of the chemoattractant stimulates orientation and motility and also the fusion of secretory vesicules or granules with the macrophage plasma membrane releasing more Mac-1 and p150,95

glycoproteins at a focal point on the cell surface (9). Once arrived on site, adhesion occurs against the target through these recent highly expressed Mac-1 and p150-95, then the macrophages exert their subsequent function like phagocytosis or cytocidal activity.

Phagocytosis

Phagocytosis is the process by which the macrophages engulf particles, bacteria or cells for their neutralization and destruction. The process consists in adherence of the phagocytes to the target victim. This step is not always successful as some intended victims are slippery. Factors that promote attachment of phagocytes are called opsonins. The most potent opsonins are immunoglobulins but some other substances like complement fragments and sugars like mannose and other as yet undefined receptors can help attachment for phagocytosis (10). Opsonization of victims occurs when immunoglobulins attach to the antigens by their reactive site exposing their Fc portion to the phagocytes which bind them through their Fc receptors. Ingestion of the whole complex can follow. The phagocyte cytoplasm flows completely around the target and fuses with itself. The victim is held inside this phagocytic vesicle which is displaced centrally. Intracellular killing pathways have been already triggered at this stage through the recognition and attachment receptors (10,11). A major antimicrobial mechanism of activated macrophages is the production and intracellular

release of reactive oxygen species such as O_2^-, H_2O_2 and $\cdot OH$ (10,12,13). Oxygen independent pathways, by accumulation of lactic acid and the lowering of the pH to about 4.5 within the phagosome, can also create bacteriostatic if not bactericidal pH. Within the cytoplasm the phagosome contacts the lysosome granules which contain many types of digestive enzymes. Lysosome membranes fuse with the phagosome to form a large structure called phagolysosome and release their enzymatic content. From exposure to the toxic forms of oxygen and to the oxygen independent pathways (1,10), the viable antigen die and the particles are digested into residual bodies which are subsequently discharged. Meanwhile the phagocytes regenerate their lysosomes in preparation for the next phagocytic event.

Antigen presenting cells (APC)

Macrophages exert a central role in the initiation of the T cell mediated immune response. They have been shown to act as antigen presenting cells to the T cells that recognize antigens in the context of class II MHC molecules (14), the commonly called T helpers. Recognition of antigen by T lymphocytes involves an interaction of the T cell antigen receptor with fragments of the processed antigen and either class I of class II MHC complex molecules on the surface of the APC. The T cell receptor usually recognizes a complex formed between the processed antigens and the MHC

molecules. Macrophages are APC expressing MHC class II molecules. Antigens restricted by class II MHC molecules (soluble antigens) in order to be effectively recognized by T cells must first be taken by the macrophages. This step occurs either by pinocytosis, a non antigen specific mechanism where binding at the APC surface is not required and the antigen is immediately internalized, or by a receptor-mediated endocytosis where the antigen is bound to the APC through specific receptors such as immunoglobulin and Fc receptors, or bound non specifically to cell surface molecules and then internalized. APC subsequently degrade the internalized exogenous antigen by a process similar to the digestion step occurring in phagocytosis and is called antigen processing (15). Antigen fragments then associate with MHC molecules of the APC to form an immunogenic molecular complex. Observations suggest that antigenic fragments come into contact with class II MHC molecules in intracellular compartment and bind them (16). Binding to class II MHC molecules may serve to protect antigenic fragments from complete degradation, and function to direct intracellular trafficking up to the APC cell surface. The antigen-MHC complexes are finally expressed on the surface of the cell where they are recognized by the T cell antigen receptor.

The inflammatory process initiated by a variety of stimuli, including tissue damage due from trauma of

bactericidal infection occurs through the production of various factors promoting the attraction of polymorphonuclears, lymphocytes and monocytes into the site (17). Antigens introduced at the time of trauma or produced subsequently are likely to be ingested by a variety of cell types like the macrophages with the capabilities to express class II MHC molecules and function as APCs. Several studies have demonstrated a direct relationship between the density of expression of class II MHC molecules and APC function. Upregulation of the class II MHC molecules by cytokines is a mechanism whereby APC function is regulated (18,19).

Cytocidal activity

Macrophages are very efficient mediators of killing and lysis against different types of pathogens including bacteria, parasites and tumor cells. Dependent of the target nature, the macrophages will proceed using phagocytosis and intracellular mechanisms of killing previously described, or extracellular killing mechanisms which are the topic of this subsection. Macrophages are activated for extracellular killing by some forms of parasites like trypomastigotes of Trypanosoma cruzi or the schistosomula of Schistosoma mansoni and by neoplastic cells (20,21). There are different mechanisms used by Mϕ to kill extracellular targets. They will vary according to the kind of targets, the type of Mϕ population and the type of activating signal given to Mϕ (22). Generally, a recognition of

the target by Mϕ occurs by selective binding through surface receptors. This interaction initiates a complex series of biochemical events leading to the generation of toxic effector molecules. Target cells are killed by secreted effector molecules or by contact between Mϕ and target cells. In some cases, contact has been reported to be required (23,24) with subsequent exocytosis of Mϕ lysosome granules followed by their endocytosis by the target cell (25). Some targets are directly sensitive to the action of cytotoxic molecules like the murine fibrosarcome WEHI-164 or the L929 tumor cells which are killed by TNF-α without the requirement for Mϕ presence (26) whereas some other targets like the P815 mastocytoma are TNF-insensitive cells and require the presence of activated macrophages and other cytotoxic molecules for being killed (27).

Several effector molecules secreted by Mϕ have been reported, the most important ones have been identified as TNF-α, IL-1, H_2O_2 and proteases (24). (Two forms of TNF have been described, TNF-α which is secreted by Mϕ and TNF-ß or lymphotoxin secreted by lymphocytes.) TNF-α is a protein made of two subunits of 16-18 kD of which the monomeric form is active. TNF has pleiotropic effects depending of the cell type and the presence of other cytokines (24). For example, rhTNF has been tested on a panel of 27 human tumor cell lines and it was cytotoxic to

27% of the cell lines, cytostatic to 55% of the cell lines and growth stimulatory to 18% of the cell lines (28). In vivo, TNF induces tumor necrosis in some tumors sensitive to its action (24,29). TNF as a soluble cytotoxic factor mediating Mϕ extracellular cytotoxicity has been described with different types of tumor where cytotoxicity could be blocked with partially purified anti-TNF antibodies (30,31). Mechanisms of TNF killing are not fully understood but the target cells endocytose TNF after it bound to their cell surface receptors. Then TNF inhibits DNA and protein synthesis in the targets until death occurs (24).

Tumoricidal activated Mϕ are good producers of IL-1 (32). Over the past several years, IL-1 has been shown to have numerous biological activities in both immune and nonimmune responses including modulating IL-2 receptor expression (33), inducing IL-2 synthesis by T cells (34) and causing fever (32). Two forms of human IL-1 has been cloned (35). IL-1α, which is now known to be identical to hemopoietin-1 (36) and IL-1ß which has been shown to be cytocidal for several tumors (37). The mechanism by which IL-1 mediates cytotoxicity is unknown but some studies indicate that IL-1 affects metabolic activities of normal cells. It influences arachidonic acid metabolism (38) and depresses the activity of several key gluconeogenic enzymes (39). However, it remains to be determined whether any of these metabolic changes are related to the cytolytic activity of IL-1.

Oxygen metabolism has been shown to be important in the tumoricidal activity of Mϕ, particularly hydrogen peroxide. Elicited murine Mϕ triggered with PMA were shown to produce H_2O_2 and lysis of tumor cells (40). Oxygen deprivation reduced the production of H_2O_2 to an undetectable level and also blocked cytolytic activity. However, superoxide dismutase and free radical scavengers had no effect on cytolysis. In vivo experiments, where latex beads coated with glucose oxidase and capable of making H_2O_2 in quantities quite similar to intact Mϕ were injected with an H_2O_2 sensitive tumor line P388, significantly prolonged the host survival (24). However, the growth of an H_2O_2 insensitive tumor line P815 was unaffected. These studies indicate that H_2O_2 can be a major mediator of Mϕ cytotoxic reactions for tumor cell targets susceptible to this mode of killing.

Mϕ contain many degradative enzymes including proteases. It has been demonstrated that supernatants of Mϕ activated either in vivo or in vitro lysed neoplastic but not normal cells (41). It was shown that cytolytic activity of Mϕ could be blocked by protease inhibitors like PMSF and TPCK (42). In some systems, it was shown that activated Mϕ released arginases and that addition of arginine to supernatants of these cultures blocked their cytolytic activity (43). A recent study suggested that TNF and L-arginine-dependent mechanisms act synergistically as the

major cytolytic mechanism of activated Mϕ (44). However, most of these proteolytic factors have not yet been fully characterized, therefore their relative role to other well defined mediators like H_2O_2, IL-1 or TNF is still unresolved. Collectively, these studies indicate that proteases play a role in macrophage-mediated cytotoxicity.

Secretory functions

The importance and complexity of the Mϕ roles can be appreciated through the different types of products that they secrete. Mϕ secrete prostaglandins which have been shown to regulate a wide variety of cellular functions and to be involved in inflammation reactions (45). They secrete leukotrienes which are involved in anaphylaxis, are chemoattractants for polymorphonuclears, cause these cells to degranulate and may modulate mononuclear phagocyte functions (45). Mϕ secrete platelet activating factor and fibronectin, a 440 kD glycoprotein involved in cell to cell interactions, in the control of cellular morphology and are abundant in extracellular matrices (45). Mononuclear phagocytes synthesize most of the components of the alternative and classic components of the complement cascade which are C1q, C1, C2, C3, C4, C5, Factor B, Factor D and properdin (46). Mϕ are known to secrete several types of enzymes like arginase, lysozyme, collagenase, elastase, cathepsins, esterases, acid hydrolases, angiotensin-converting enzymes and other neutral proteases (1,45).

They can also secrete high-molecular weight inhibitors of proteolytic enzymes called α-proteinase inhibitor and α_2-macroglobulin under certain conditions (45). Mϕ have also been shown to secrete different products of oxygen metabolism such as hydrogen peroxide, hydroxylradical and singlet oxygen used as toxic factors for intra and extracellular killing (47). Finally, Mϕ play a central role in hemopoiesis and immunoregulation as secretory cells of growth factors and interleukins. Studies have shown that they secrete IL-1, Il-6, TNF-α, IFN-α, IFN-ß, M-CSF, G-CSF and TGF-ß (5).

3. Physiological activators, mechanisms of macrophage activation and function regulation.

Mϕ become activated to exert their functions as antigen presenting cells or to kill intracellularly or extracellularly as well as to secrete monokines upon exposure to a variety of different agents. The physiological activators include the bacterial product LPS and several cytokines. These activators have specific properties and will regulate Mϕ activation to exert different functions. The activation of Mϕ to perform a particular function can be assessed by specific biological assays. For instance, Mϕ up regulation of their cell surface Ia antigen expression indicates their activation to function as antigen presenting cells (14).

Their stimulation to secrete H_2O_2 indicates activation for intra and extracellular killing (24). The ability of Mϕ to perform extracellular killing is also expressed by their tumoricidal activity which has two pathways of activation, the first one is mediated by TNF and kills only TNF sensitive tumors, the second is more complex and kills TNF resistant and sensitive targets (48) (more details can be found in the discussion of chapter IV). Finally, the secretion of cytokines can be interpreted as an indication of activation of Mϕ secretory functions. This section presents LPS, GM-CSF, M-CSF, TNF-α, IL-1ß, IL-2, IL-4, IFN-τ and TGF-ß known as physiological regulators or the presently understood major mechanism responsible for Mϕ activation and function regulation.

LPS. Lipopolysaccharides are bacterial products also called endotoxin (24) which are present in high molecular weight (100-1,000 kD) complexes in aqueous solution (49). It has been shown that LPS suppresses Mϕ Ia antigen expression (50), LPS suppresses H_2O_2 production by resting and lymphokine activated macrophages (51) but it activates Mϕ tumoricidal activity pathways against TNF sensitive (31) and TNF resistant tumors (52). LPS also activates Mϕ secretion of IL-1 (24), TNF-α (53), IFN-α and ß (24) and TGF-ß (54).

GM-CSF. Granulocyte macrophage-colony stimulating factor is a glycoprotein of 18-30 kD classified as an

hemopoietic growth factor. GM-CSF at progressively increasing concentrations becomes an effective proliferative stimulus first for Mϕ then for neutrophils and lastly for eosinophils and megakaryocytes (55). T cells, Mϕ and endothelial cells are the major producer of GM-CSF (55). GM-CSF has regulating activity on Mϕ by increasing their Ia antigen expression (56) and H_2O_2 secretion (57). GM-CSF activates only the first pathway (TNF-mediated) of Mϕ tumoricidal activity (31). It also activates Mϕ secretion of IL-1 (55) and TNF.

M-CSF. Macrophage-colony stimulating factor is a glycoprotein of 35-50 kD that stimulates proliferation and differentiation of myeloid cells into monocytes (55). Numerous cell types are capable of producing M-CSF, these include T and B cells, Mϕ, endothelial and stromal cells (55,58). M-CSF has been shown to down regulate Mϕ Ia antigen expression (56). It induces Mϕ H_2O_2 secretion (57) but does not activate any Mϕ tumoricidal activity (59). M-CSF stimulates Mϕ expression of IFN-τ (55,60) and IL-1 (58).

TNF-α. Tumor necrosis factor-α is a monocyte/macrophage-derived cytokine of 17 kD that was originally described as the factor responsible for endotoxin induced necrosis of tumors <u>in vivo</u> (53). TNF is also known as a major cytolytic factor in Mϕ mediated tumor killing (31). TNF has been reported to induce Ia antigen expression on its own in some murine and human Mϕ cell lines (61,62) but its

activity is more powerful when it synergizes with IFN-τ (62). TNF-α also induces Mϕ H_2O_2 production (63) and TNF-mediated Mϕ tumoricidal activity (31).

IL-1ß. Interleukin-1ß is the dominant form (pI7) of the expression of the two IL-1 genes (64). IL-1ß is a 17 kD glycoprotein which acts on many cell types and was first detected as a Mϕ product that induced expression of IL-2 receptors and production of IL-2 by T lymphocytes (55). IL-1ß is responsible for many other biological activities among which is the induction of fever accompanying the inflammation reaction (64). IL-1ß is secreted by nearly all antigen presenting cells including Mϕ, dendritic cells, Langherhans cells, endothelial cells, astrocytes, microglial cells and fibroblasts (64). IL-1ß does not enhance Ia antigen expression by Mϕ (14) although it supports this reaction through activation of T cells. IL-1ß does not stimulate Mϕ H_2O_2 production (57) but induces the TNF mediated Mϕ tumoricidal activity (31). IL-1ß activates Mϕ secretion of IL-6 (65), TNF-α (31), M-CSF (55) and GM-CSF (66).

IL-2. Interleukin-2 is glycoprotein of 15 kD very well known for its activation of T cells to proliferate and differentiate into mature effectors (67). IL-2 is secreted mainly by T cells and its action seems to be limited specifically to lymphocytes (67). However, it has been reported that human monocytes express receptors for IL-2

(68). Il-2 does not stimulate Mϕ H_2O_2 production (57) but it activates both pathways of macrophage tumoricidal activity (48,69).

IL-4. Interleukin-4 is glycoprotein of 16-20 kD previously designated B cell stimulatory factor-1 for its induction of B cell proliferation (70). Experiments with recombinant IL-4 have shown that this cytokine has a wide range of effects on both B and T cells and other cells of the hemopoietic lineage (70). IL-4 is secreted by T cells (70). Normal Mϕ express IL-4 receptors and it has been reported that IL-4 increases Mϕ Ia antigen expression and activates TNF mediated Mϕ tumoricidal activity (71).

IFN-τ. Interferon-τ is a glycoprotein of about 20 kD that is secreted principally by T cells during the development of an immune response against a variety of antigens. Interferon-τ is also known to activate anti-viral mechanisms in a variety of cells (72). IFN-τ has been identified as the principal Mϕ activating factor (73). It induces Mϕ Ia antigen expression and it increases Mϕ H_2O_2 production (72). It also activates both pathways of Mϕ tumoricidal activity in copresence of LPS (48). IFN-τ induces Mϕ secretion of TNF-α (31), IL-1 (31) and M-CSF (14).

TGF-ß. Transforming growth factor-ß is a 25 kD peptide originally defined by its ability to induce transformation of non neoplastic cells in culture. TGF-ß is now known to play an important role in inflammatory cell recruitment and

TABLE I

Regulation of macrophage function by physiological activators

Activators	M.W.	Ia	H_2O_2	Tumoricidal TNF-s.[1]	Tumoricidal TNF-r.[2]	Secreted cytokines
LPS	100-1000 kD	-	-	+	+	TGF-ß, IL-1 TNF, IFN-α,ß
GM-CSF	18-30 kD	+	+	+	-	IL-1, TNF
M-CSF	45-50 kD	-	+	-	U[3]	IFNs, IL-1
TNF-α	17 kD	-	+	+	-	IL-6, IL-1 TNF
IL-1ß	17 kD	-	-	+	-	IL-6, TNF, M-CSF, GM-CSF
IL-2	15 kD	U	-	+	+	U, but TNF
IL-4	16-20 kD	+	U	+	-	U, but TNF
IFN-τ	20 kD	+	+	+	+	TNF, IL-1 M-CSF
TGF-ß	25 kD	-	-	U	U	IL-1, TNF TGF-ß

1: Activate Mϕ to kill TNF-sensitive targets
2: Activate Mϕ to kill TNF-resistant targets
U: Unknown

promotes tissue repair by down regulation of the immune response and enhancing of extracellular matrix formation (74). TGF-ß is produced by a large variety of cells including immune cells and almost every type of cell including Mϕ possess TGF-ß receptors (74). TGF-ß is very chemotactic for monocytes which possess high-affinity receptor for this peptide but monocyte/Mϕ susceptibility to TGF-ß stimulation decreases as the cells differentiate (74). TGF-ß was reported to be a deactivating factor for Mϕ Ia antigen expression and H_2O_2 production (75). TGF-ß has autocrine function on monocytes and also regulates monocytes synthesis of TNF and IL-1.

This section is summarized in Table I.

II LARGE GRANULAR LYMPHOCYTES

1. Description and origin

The large granular lymphocytes are a lymphocyte subset population characterized by a high cytoplasm to nucleus ratio, a diameter ranging between 10-20 µm compared to 5-7 µm for the small lymphocytes and the presence of numerous cytoplasmic granules. The LGL population is constituted of two different classes of lymphocytes, NK cells,

characterized by the expression of the surface antigen CD16 or Fcγ receptor in the absence of any T cell receptor related surface antigen and T cells, expressing the CD3 T cell receptor complex antigen but not the CD16 marker (76). The precursors of NK cells originate from bone marrow and have been shown to be large agranular lymphocytes but NK cells have also been obtained from small high density lymphocytes $CD3^-$ $CD2^+$ $CD11b^+$ cultures in presence of IL-2 (77). $CD3^+$ LGL have been shown to originate from small T cells that maturate by enlarging and acquiring numerous cytoplasmic granules after a few days in presence of IL-2 (78,79). NK LGL have the particular property to be spontaneously cytotoxic for different type of cells mainly against virus infected and neoplastic cells without prior sensitization or need for recognition of MHC surface molecules on the cell targets (76). Unlike NK cells, CTL normally recognize target cell antigens in association with MHC cell surface molecules and are therefore defined as MHC restricted in their cytolytic function (80). However, after culture in presence of IL-2 NK and CTL display an apparently similar LGL morphology and they both acquire the ability to kill a wide variety of tumor cells in a non MHC restricted manner defined as lymphokine activated killer (LAK) activity (26,78,79).

2. Roles of LGL

The roles possibly exerted by LGL have been examined in different systems. The data come mainly from study of LGL NK cells. NK cell interactions with the nervous system have been studied and it was found that depressed NK activity correlated with stress (81). The mechanisms of these interactions and their physiological significance are still unclear. A possible role for NK cells in reproduction has been proposed. Studies suggest that NK cells suppress the immune response of the mother against the embryo in the decidua (81). Evidence has also been reported for a role in hemopoiesis regulation by NK cells. Stimulated NK cells have been shown to produce GM-CSF, M-CSF and IL-3 (81). LGL has been reported to have anti-microbial activity. NK LGL have very limited ability for phagocytosis but they were reported to directly lyse certain bacteria (82). LGL have also been reported to have immunoregulatory activity on B and T cells (81). The major role of NK in bacterial infection and as immunoregulatory cells is probably the production of lymphokines. It has been shown that NK cells can produce several lymphokines, stimulation of NK cells with anti-CD16 and IL-2 induced high levels of mRNA accumulation and release of IFN-τ, TNF, GM-CSF and M-CSF (81,83). It was reported that NK cell clones can also secrete a B cell differentiating factor inducing

immunoglobulins production (84). Finally, a central role for NK and $CD3^+$ LGL is their anti-viral and anti-tumoral activity exerted through the recognition and killing of infected or transformed target cells (80,81).

3. LGL mechanism of cytotoxicity

NK or T LGL mediated killing can be defined in four different stages : 1) The cell contact between LGL and target; 2) specific binding with the rapid formation of a strong adhesion between the two cell surfaces; 3) the delivery of the lethal hit programming for lysis stage, which occurs within 12 minutes of cell contact; 4) the slower stage of killer cell independent lysis during which LGL-target dissolution occurs without interrupting the target death programming and after which the LGL is capable of initiating a new lytic cycle (80).

The recognition structure on NK LGL which is MHC independent, has not yet been identified but several studies suggest that more than one single structure is involved and that different targets might be recognized through different structures. For instance, CD16 (Fcγ receptors), CD11b (C3bi receptor), CD45 (T 200 molecule), laminin, CD2 (sheep erythrocyte receptor), C-reactive protein (CRP) and a 80 kD molecule defined by an anti-idiotypic antibody against an antibody recognizing a glycoprotein on K562 cells have all

been shown to be involved in target recognition by NK cells in some defined systems (81). Following recognition of the target, the triggering of the effector occurs. From all the different molecules for which a role has been proposed in NK-target recognition and binding events during NK cell-mediated cytotoxicity, only CD16 and CD2 have been shown to act directly in signal transduction and activation of the cytolytic mechanisms (85,86). Once activated, the LGL strongly bind their target through the LFA-1 (CD11a), LFA-2 and the CD2 molecules which bind to their respective ligands on the target cell the ICAM (CD54) and LFA-3 (CD58) (87). On the other hand, several antigens present on the target cell membranes have been proposed as possible NK cell target structures but evidence for the role of a single molecule has not been confirmed in many different systems as yet.

The following stage in this LGL mechanism of cytotoxicity is the delivery of the lethal hit by LGL concomitant with exocytosis of their cytoplasmic granules which fuse with the effector membrane and release their content against the target cell membrane. This phenomenon has been visualized using high-resolution optic cinematography (88). The exact nature of the lethal hit delivery is not yet fully understood, but granules are known to contain and release different types of molecules including perforin, proteases and proteoglycans. The triggering of target cell death program causes violent changes in the target including

membrane pore formation caused by perforin, membrane blebbing and DNA disintegration which are thought to be the effect of a combined action of the lethal contact and the released molecules (80).

4. The LGL cytoplasmic granules

Structure

LGL cytoplasmic granules have been observed under electron microscopy and described with a size ranging from 50 to 800 nm in diameter, containing a dense core surrounded by a lipid bilayer (89). Because of their higher density, granules can be easily separated from other cellular organelles using self-generated Percoll density gradient obtained by centrifugtion (90). Cytoplasmic granules have been shown to contain a variety of molecules including lysosomal markers, perforin, proteases, proteoglycans and immunoregulatory factors that are presented below.

Roles

Considerable evidence exists suggesting a role for cytoplasmic granules in LGL-mediated cytotoxicity. Morphologically, it has been observed that following contact of LGL with the target cell, the nucleus immediately moves away from the region of contact and is replaced rapidly by granules. As early as 4 minutes after binding, the polarized granules are seen to fuse with the plasma membrane

of the LGL in the vicinity of the area of its contact with the target (85,80). Soon after, circular lesions with inner diameter of 5 to 16 nm on the target cell membrane have been described and are thought to represent the pores formed by perforin polymers (91). The accumulated evidence suggests that upon specific LGL-target interaction, the granules are mobilized towards the target and deposit their contents into the intercellular space created by the binding between the two cells through exocytosis. Lysis of the target similar to that mediated by complement follows. The validity of this model is supported by the fact that cytoplasmic granules have been purified by several groups with isolation and characterizaton of the perforin molecule (92,93,94).

The biochemical trigger for NK LGL degranulation has not yet been established, but for the CTL the antigen receptor must be cross-linked to elicit T cell degranulation (95). Further biochemical changes have been reported where activated CTL or NK stimulate their phosphoinositide turnover with production of the calcium mobilizing messengers inositol triphosphates (96,97). Activated CTL have also been shown to first release calcium from intracellular stores followed by uptake from the external medium (96). The presence of external calcium is obligatory for degranulation and for the perforin pathway since calcium chelators were shown to inhibit exocytosis of granules and perforin (95).

The most recent model for LGL granule exocytosis in the lethal hit delivery proposes that after triggering of the antigen receptor, the activated CTL move their granules to fuse with their cell membrane in the area of contact with the target and then the granules release their contents which would be many smaller vesicles with T cell receptor molecules on their surface, used to recognize and attach to the target membrane. The advantage of this model is that it explaines how perforin can recognize the target without damaging the LGL effector. However, the exact mechanism of action of the granule components with the target cell is yet unexplained in this model (98). An analogous model for NK LGL granule-target interaction does not exist yet.

Evidence also exists for the existence of a granule independent mechanism of cytolysis by LGL. The degranulation and perforin pathway are absolutely calcium dependent but several studies have now reported CTL lysis of target in the complete absence of extracellular calcium and without the need for degranulation (99,100). CTL lines with absence of perforin were also shown to remain cytolytic (101). These studies indicate that CTL can kill by other mechanisms than degranulation and the perforin pathway. Subcellular fractionation has shown that cytolytic activity remained in the cytosolic fraction of the Percoll fractionation (101). Another molecule present in the LGL granules have been reported to kill by causing DNA breakdown in a

mechanism independent of the perforin pathway (102). A candidate to mediate such killing is TNF. LGL have been reported to produce TNF (103) and another similar molecule called NKCF (104). Other candidates include also proteolytic enzymes (105,106). Collectively, these studies indicate that LGL possess other pathways of killing than granule exocytosis and one may also think that mediated cytotoxicity is not the only function associated with granule exocytosis.

Principal molecular components of granules

Several types of molecules have been reported to be present in the LGL granules.

Perforin. This protein of 60-70 kD has been reported to be the granule component responsible for creating pores in the membrane of the target cell by insertion of its lypophilic end in the lipid layers of the target cell membrane followed by its polymerization to form channels causing ionic disequilibrium inside the cells (107).

TNF-related molecules. Two cytolytic peptides between 50-70 kD have been isolated from granules with the ability to induce DNA breakdown of the target. Both were shown to be antigenically related to TNF but different in a number of biochemical and functional properties (102,108).

Serines esterases. A family of seven serine esterases called granzymes A,B,C,D,E,F,G,H have been isolated from granules of CTL (109). The exact role of these proteases is

unknown in the mediated cytotoxicity. It has been proposed that perforin can be present in the granules in an inactive form and that proteolysis is required for activation (110).

Proteoglycans. Complex highly negatively charged proteoglycans of the chondroitin sulfate A type have been identified from NK and CTL granules (111,112). It is possible that this complex molecule functions as substratum to which other granule mediators bind. Their exact role is still unknown.

Immunoregulatory factors. Over the last 5 years, Greenberg et al. have reported two different granule factors acting on Mϕ and other leukocytes. A chemotactic factor in granules of rat LGL called NK-LCF has been found to act on polymorphonuclears and Mϕ (113). The second one is a Mϕ regulating factor contained in rat NK LGL and human $CD3^+$ LGL of which details are presented in this thesis.

III A MECHANISM OF MACROPHAGE ACTIVATION BY LGL; THE OBJECT OF THE THESIS

LGL have precedent as immunoregulatory cells through secretion of certain cytokines upon stimulation. These cells have also the particular ability to exert cytocidal activity by a rapid contact with the target followed by

cytoplasmic granule exocytosis. In this thesis, the principal hypothesis investigated was that LGL may use this granule exocytosis process to exert some immunoregulatory function. This hypothesis was based on observations that rapid release of preformed molecules able to activate Mϕ by LGL occurs after contact with a tumor target cell within the time limit of cell contact and lethal hit delivery (80,114). We tested this hypothesis by addressing the specific question: Is there any evidence for the presence of immuno-regulatory molecules in the LGL cytoplasmic granules. Chapter II of this thesis presents the first line of evidence in response to this question using a rat LGL tumor cell line as source of LGL granules. Chapter III presents an efficient method to generate human $CD3^+$ LGL beside proposing an explanation for the ambiguity about the nature of the LAK cells generated from human PBL. The chapter IV presents more elaborate evidence for the existence of an immunoregulatory molecule in the granules of normal human LGL.

REFERENCES

1. **J.T. Barrett.** 1983. Textbook of immunology. Chapter four. The C.V. Mosby Cie. St-Louis.

2. **Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and J.D. Watson.** 1983. Moecular biology of the cell. Chapter sixteen. Garland Publishing Inc. New York.

3. **Dexter, T.M. and E. Spooncer.** 1987. Growth and differentiation in the hemopoietic system. Am. Rev. Cell Biol. 3:423.

4. **Wahl, S.M., McCartney-Francis, N. and S.E. Mergenhagen.** 1989. Inflammatory and immunomodulatory roles of TGF-ß. Immunol. Today 10:258.

5. **Balkwill, F.R. and F. Burke.** 1989. The cytokine network. Immunol. Today 10:299.

6. **D. Metcalf.** 1989. The molecular control of cell division, differentiation commitment and maturation in hemopoietic cells. Nature 339:27.

7. **Tortora, G.J., Funka, B.R. and C.L. Case,** 3rd ed., 1989. Microbiology: an introduction. Chapter fifteen. The Benjamin/Cummings Publishing Cie Inc. Redwood City, CA.

8. **Ming, W.J., Bersani, L. and A. Mantovani.** 1987. TNF is chemotactic for monocytes and PMN. J. Immunol. 138:1469.

9. **Springer, T.A. and D.C. Anderson.** 1986. The importance of the Mac-1, LFA-1 glycoprotein family in monocyte and granulocyte adherence, chemotaxis and migration into

inflammatory sites: insights from an experiment of nature. In "Biochemistry of macrophages", Ciba Foundation Symposium 118. Pitman, London, p. 102.

10. **Adams, D.O. and T.A. Hamilton.** 1984. The cell biology of macrophage activation. Ann. Rev. Immunol. 2:283.

11. **R.B. Johnson.** 1981. Enhancement of phagocytosis-associated oxydase metabolism as manifestation of macrophage activation. Lymphokines 3:33.

12. **H.W. Murray.** 1984. Activation of macrophage to display enhanced oxidative and antiprotozoal activity. Contemp. Top. Immunobiol. 13:97.

13. **S.S. Klebanoff.** 1982. Oxygen-dependent cytotoxic mechanism of phagocytes. Ad. Host Defense Mechan. 1:111.

14. **Geppert, T.D. and P.E. Lipsky.** 1989. Antigen presentation at the inflammatory site. Crit. Rev. Immunol. 9:315.

15. **E.R. Unanue.** 1983. Macrophages as regulators of lymphocyte function. In "The biology of immunologic disease" Dixon and Fisher eds. Sinauer Associate Inc. Sunderlands, MA. p. 87.

16. **Ellner J.J., Lipsky, P.E. and A.S. Rosenthal.** 1977. Antigen handling by guinea pig macrophages: further evidence for the sequestration of antigen relevant for activation of primes T lymphocytes. J. Immunol. 118:2053.

17. **Hugli, T.E. and H.J. Muller-Eberhard.** 1978. Anaphylatoxins: C3a and C5a. Adv. Immunol. 26:53.

18. **Sztein, M.B., Stoeg, P.S., Johnson, H.M. and J.J. Oppenheim.** 1984. Regulation of human peripheral blood monocyte DR antigen expression in vitro by lymphokines and recombinant interferons. J. Clin, Invest. 73:556.

19. **Zlotnik, A., Fisher, M., Roehm, N., and D. Zipori.** 1987. Evidence ofr effects of IL-4 an macrophages: ehhancement of antigen presenting ability of bone marrow-derived macrophages. J. Immunol. 138:4275.

20. **Nogueria, N. and Z.A. Cohn.** 1984. Activation of mononuclear phagocytes for the destruction of intracellular parasites: Studies with Trypanosoma cruzi. Cont. Top. Immunobiol. 13:117.

21. **Nacy, C.A., Oster, C.N., James, S.L. and M.S. Meltzer.** 1984. Activation of macrophages to kill Rickettsiae and Leishmania: Dissociation of intracellular microbicidal activities and extracellular destruction of neoplastic and helminth targets. Cont. Topics Immunobiol. 13:147.

22. **Nacy, C.A., Belosevic, M., Crawford, R.M., Healy, A.T., Schreiber, R.D. and M.S. Meltzer.** 1988. Lymphokine regulation of macrophage effector activities. Adv. Exp. Med. Biol. 239:1.

23. **Marino, P.A. and D.O. Adams.** 1980. Interaction of BCG-activated macrophages and neoplastic cells in vitro. I. Conditions of binding and its selectivity. Cell. Immunol. 54:11.

24. **Drysdale, B.-E., Agarwal, S. and H.S. Shin.** 1988.

Macrophage-mediated tumoricidal activity. Mechanisms of activation and cytotoxicity. Prog. Allergy 40:111.

25. **Fidler, I.J. and A. Raz.** 1981. The induction of tumoricidal capacities in mouse and rat macrophages by lymphokines. Lymphokines 3:345.

26. **Decker, T., Lohmann-Matthes, M.L. and G.E. Gifford.** 1987. Cell-associated TNF factor as a killing mechanism of activated cytolytic macrophages. J. Immunol. 138:957.

27. **Lu,C.Y., Lombardi, M.J., Shea, C.M. and L.B. Dustin.** 1988. High streght binding of P815 mastocytoma cells is not necessary for their lysis by macrophages which have been primed and triggered in vitro. J. Immunol. 141:1083.

28. **Sugarman, B.J., Aggarwal, B.B., Hass,P.E., Figari, I.S., Palladino, M.A. Jr and H.M. Shepard.** 1985. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells in vitro. Science 230: 943.

29. **Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A., Beutler, B., Cerami, A., Figari, I.S., Palladino, M.A. Jr and J.V. O'connor.** 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of IL-1. J. Exp. Med. 163:1433.

30. **Mannel, D.N., Falk, W. and M.S. Meltzer.** 1981. Inhibition of nonspecific tumoricidal activity by activated macrophages with antiserum against a soluble cytotoxic factor. Infect. Immunity 33:156.

31. **Philip, R., and L.B. Epstein.** 1986. Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon and interleukin-1. Nature 323:86.

32. **C.A. Dinarrello.** 1984. Interleukin-1.
Rev. Infect. Dis. 6:51.

33. **Kaye, J., Gillis, S., Mizel, S.B., Shevach, E.M., Halek, T.R., Dinarello, C.A., Lachman, L.B. and C.A. Janeway.** 1984. Growth of a cloned helper T cell line induced by a monoclonal antibody specific for the antigen receptor: IL-1 is required for the expression of the receptor for IL-2.
J. Immunol. 133:1339.

34. **Smith, K.S., Lachman, L.B., Oppenheim, J.J. and N.F. Favata.** 1980. The functional relationship of the interleukin. J. Exp. Med. 151:1551.

35. **Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Rich, A., Wolfe, S.M. and C.A. Dinarello.** 1984. Nucleotide sequence of human nonocyte IL-1 precursor cDNA.
P.N.A.S. USA 81:7907.

36. **Zsebo, K.M., Wypych, J., Yuschenkoff, V.N., Lu, H., Hunt, P., Dukes, P.P. and K.E. Langley.** 1988. Effects of hematopoietin-1 and Interleukin-1 activities on early hematopoietic cells of the bone marrow. Blood 71:962.

37. **Lachman, L.B., Dinarello, C.A., Lianso, N.D., and I.J. Fidler.** 1986. Natural and recombinat human interleukin-1ß is cytotoxic for human melanoma cells. J.Immunol. 136:3098.

38. **Beutler, B.A. and A. Cerami.** 1985. Recombinant IL-1 suppresses lipoprotein lipase activity in 3T3-L1 cells. J. Immunol. 135:3969.

39. **Hill, M.R., Stith, R.D. and R.E. McCallum.** 1986. Interleukin-1: a regulatory fole in glucocorticoid-regulated hepatic metabolism. J. Immunol. 137:858.

40. **Nathan, C.F., Silverstein S.C., Brukner, L.H. and Z.A. Cohn.** 1979. Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. J. Exp.Med. 149:100.

41. **Adams, D.O., Kao, K.J., Farb, R. and S.V. Pizzo.** 1980. Effector mechanisms of cytolytically activated macrophages. II. Secretion of a cytolytic factor by activated macrophages and its relationship to secreted neutral proteases. J. Immunol. 124:293.

42. **Piessens, W.F. and S.D. Sharma.** 1980. Tumor cell killing by macrophages activated in vitro with lymphocyte mediators. Cell Immunol. 56:286.

43. **G.A. Currie.** 1978. Activated macrophages kill tumor cells by releasing arginase. Nature 273:758.

44. **Higuchi, M., Higashi, N., Taki, H., and T.Osawa.** 1990. Cytolytic mechanisms of activated macrophage: Tumor necrosis factor and L-arginine-dependent mechanisms act synergistically as the major cytolytic mechanisms of activated macrophages. J. Immunol. 144:1425.

45. **Bonney, R.J. and P. Davies.** 1984. Possible auto-regulatory functions of the secretory products of mononuclear phagocytes. Cont. Topics Immunobiol. 13:199.

46. **K. Whaley.** 1980. Biosynthesis of the complement components and the regulatory proteins of the alternative complement pathway by human peripheral blood monocytes. J. Exp. Med. 151:501.

47. **Nathan, C.F., Murray, H.U. and Z.A. Cohn.** 1982. The macrophage as an effector cell. N. Engl. J. Med. 303:622.

48. **Philip, R.** 1988. Cytolysis of TNF-resistant tumor target. Differential cytotoxicity of monocytes activated by the interferon, IL-2, and TNF. J. Immunol. 140:1345.

49. **Wright, A., and S. Kanegasaki.** 1971. Molecular aspects of lipopolysaccharides. Physiol. Rev. 51:748

50. **Koerner, T.J., Hamilton, T.A. and D.O. Adams.** 1987. Suppressed expression of surface of Ia on macrophages by LPS: evidence for regulation at the level of accumulation of mRNA. J. Immunol. 139:239.

51. **Rellstab, P., and A. Schaffner.** 1989. Endotoxin suppresses the generation of O_2^- and H_2O_2 by resting and lymphokine-activated human blood-derived macrophages. J. Immunol. 142:2813.

52. **Roussel, E., and A.H. Greenberg.** 1989. Identification of a macrophage activating factor in granules of the RNK large granular lymphocyte leukemia. J. Immunol. 142:543.

53. **Ruff, M.R. and G.E. Gifford.** 1981. Tumor necrosis factor. Lymphokines 2:235.

54. **Assoian, R.K., Fleurdelys, B.E., Stevenson H.C., Miller, P.J., Madtes, D.K., Raines, E.W., Ross, R. and M.B. Sporn.** 1987. Expression and secretion of type ß transforming growth factor by activated human macrophages. P.N.A.S. 84:6020.

55. **D. Metcalf.** 1989. Haemopoietic growth factors 1. Lancet 8642:825.

56. **Willman, C.L., Stewart, C.C., Miller, V., Yi, T.L. and T.B. Tomasi.** 1989. Regulation of MHC class II gene expression in macrophages by hematopoietic colony-stimulating factors. Induction by granulocyte/macrophage CSF and inhibition by CSF-1. J. Exp. Med. 170:1559.

57. **Nathan, C., Campanelli, D., Ding, A., Fuortes, M., Gabay, J., Kwon, N.S., Srimal, S., and D. Stuehr.** 1990. Three antimicrobial systems in phagocytes: Regulation by cytokines. J. Cell. Bioch. (Suppl.) 14B:16.

58. **Moore, R.N., Hoffeld, J.T., Farrar, J.J., Mergenhagen, S.E., Oppenheim, J.J. and R.C. Shadduck.** 1981. Role of colony-stimulating factors as primary regulators of macrophage functions. Lymphokines 3:119.

59. **Ralph, P., Nacy, C.A., Meltzer, M.S., Williams, N., Nakoinz, I. and E.J. Leonard.** 1983. Colony-stimulating factors and regulation of macrophage tumoricidal and microbicidal activities. Cell. Immunol. 76:10.

60. **Kruze, A., Kirchner, H., Zawatzky, R. and I. Domke-Opitz.** 1989. In vitro development of bone marrow-derived macrophages. Influence of mouse genotype on response to colony-stimulating factors and autocrine interferon induction. Scand. J. Immunol. 30:731.

61. **Chang, R.J. and S.H. Lee.** 1986. Effects of interferon-γ and tumor necrosis factor-α on the expression of an Ia antigen on a murine macrophage cell line.
J. Immunol. 137:2853.

62. **Virelizier, J.L., Arenzana-Seisdedos, F. and S. Mogensen.** 1988. Respective role of lymphokines and monokines in macrophage activation.
Dev. Biol. Stand. 69:115.

63. **Hoffman, M. and J.B. Weinberg.** 1987. Tumor necrosis factor-α induces increased hydrogen peroxide production and Fc receptor expression but not increased Ia antigen expression by peritoneal macrophages.
J. of Leuk. Biol. 42:704.

64. **Oppenheim, J.J., Kovacks, E.J., Matsushima, K. and S.K. Durum.** 1986. There is more than one interleukin-1.
Immunol. Today 7:45.

65. **Kohase, M., Henriksen-Destefano, D., May, L.T., Vilcek, J. and P.B. Sehgal.** 1986. Induction of beta2-interferon by tumor necrosis factor: a homeostatic mechanism in the control of cell proliferation. Cell 45:659.

66. **Fibbe, W.E., van Damme, J., Biliau, A., Voogt, P.J., Duinkerken, N., Kluck, P.M.C. and J.H.F. Falkenburg.** 1986. Interleukin-1 (22-K factor) induces release of granulocyte-macrophage colony-stimulating activity from human mononuclear phagocytes. Blood 68:1316.

67. **R.J. Roob.** 1984. IL-2: The molecule and its function. Immunol. Today 5:203.

68. **Herrmann, F., Cannistra, S.A., Levine, H. and J.D. Griffin.** 1985. J. Exp. Med. 162:1111.

69. **Malkovsky, M., Loveland, B., North, M., Asherson, G.L., Gao, L., Ward, P. and W. Fiers.** 1987. Recombinant interleukin-2 directly augments the cytotoxicity of human monocytes. Nature 325:262.

70. **Paul, W.E. and J. Ohara.** 1987. B cell stimulatory factor-1/interleukin 4. Ann. Rev. Immunol. 5:429.

71. **Crawford, R.M., Finbloom, D.S., Ohara, J., Paul, W.E., and M.S. Meltzer.** 1987. B cell stimulatory factor-1 (IL-4) activates macrophages for increased tumoricidal activity and expression of Ia antigens. J. Immunol. 139:135.

72. **Greenfield, R.S., P.A. Kiener, D.P. Chin, R.C. Curry, and G.L. Spitalny.** 1986. Biochemical analysis and biological activities of immunoaffinity purified natural murine gamma interferon (MuIFN-γ). J. of Leuk. Biol. 40:665.

73. **D. Schreiber.** 1984. Identification of gamma-interferon as a murine macrophage-activating factor for tumor

cytotoxicity. Contemp. Top. Immunobiol. 13:171.

74. **Wahl, S.M., McCartney-Francis, N. and S.E. Mergenhagen.** 1989. Inflammatory and immunomodulatory roles of TGF-ß. Immunol. Today 10:258.

75. **Tsunawaki, S., Sporn, M., Ding, A. and C. Nathan.** 1988. Deactivation of macrophages by transforming growth factor-ß. Nature 334:260.

76. **Fitzgerald-Bocarsly, P., Herberman, R., Hercend, T., Hiserodt, J., Kumar, V., Lanier, L., Ortaldo, J., Pross, H., Reynolds, C., Welsh, R., and H. Wigzell.** 1988. A definition of natural killer cells. Immunol. Today 9:292.

77. **H.S. Warren.** 1984. Differentiation of NK-like cells from $OKT3^-$, $OKT11^+$, and $OKM1^+$ small resting lymphocytes by culture with autologous T cell blasts and lymphokine. J. Immunol. 131:2888.

78. **Roussel, E., Gerrard, J.M., and A.H. Greenberg.** 1990. Long-term cultures of human peripheral blood lymphocytes with rhIL-2 generate a population of virtually pure $CD3^+$,$CD16^-$,$CD56^-$ large granular lymphocyte LAK cells. Clin. Exp. Immunol. 82:416.

79. **Patel, S.S., Thiele, D.L.. and P.E. Lipsky.** 1987. Major histocompatibility complex-unrestricted cytolytic activity of human T cells. Analysis of precursor frequency and effector phenotype. J. Immunol. 139:3886.

80. **Young, J. D.-E.** 1989. Killing of target cells by lymphocytes: A mechanistic view. Physiol. Rev. 69:250.

81. **G. Trinchieri.** 1989. Biology of natural killer cells. Adv. Immunol. 47:187.

82. **Morgan, D.R., Dupont, H.L., Gonik, B. and S. Kohl.** 1984. Cytotoxicity of human peripheral blood and colostral leukocytes against Shigella species. Infect. Immunol. 46:25.

83. **Anegon, I., Cuturi, M.C. Trinchieri, G. and B. Perussia.** 1988. Interaction of Fc receptor (CD16) with ligands induces transcription of IL-2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. J. Exp. Med. 167:452.

84. **Vyakarnam, A., Brenner, M.K., Reittie, J.E., Houlker, C.H. and P.J. Lachmann.** 1985. Human clones with natural killer function can activate B cells and secrete B cell differentiation factors. Eur. J. Immunol. 15:606.

85. **Schmidt, R.E., Caufield, J.P., Michon, J. Hein, A., Kamada, M.M., Macdermott, R.P., Stevens, R.L. and J.Ritz.** 1988. T11/CD2 activation of cloned human natural killer cells results in increase conjugate formation and exocytosis of cytolytic granules. J. Immunol. 140:991.

86. **Cassatella, M.A., Anegon, I., Cuturi, M.C., Griskey, P., Trinchieri, G. and B. Perussia.** 1989. FcR (CD16) interaction with ligand induces Ca^{2+} mobilization and phosphoinositide turnover in human natural killer cells. Differential role of Ca^{2+} in FcR (CD16) and IL-2-induced transcription and expression of lymphokine genes.

J. Exp. Med. 169:549.

87. **Springer, T.A., Dustin, M.L., Kishimoto, T.K. and S.D. Marlin.** 1987. Annu. Rev. Immunol. 5:223.

88. **Yannelli, J.R., Sullivan, G.L., Mandell, G.L. and V.H. Engelhard.** 1986. Reorientation and fusion of cytotoxic T lymphocyte granules after interaction with target cells as determined by high resolution cinematography.

J. Immunol. 136:377.

89. **Grossi, C.E., Cadoni, A., Zicca, A., Leprini, A., and M, Ferrarini.** 1982. Large granular lymphocytes in human peripheral blood: ultrastructural and cytochemical characterization of the granules. Blood 59:277.

90. **Henkart, P.A., Millard, P.J., Reynolds, C.J. and M.P. Henkart.** 1984. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors.

J. Exp. Med. 160:75.

91. **Dennert, G., and E.R. Poddack.** 1983.

J. Exp. Med. 157:1483.

92. **P.A. Henkart.** 1985. Mechanism of lymphocyte-mediated cytotoxicity. Annu. Rev. Immunol. 3:31.

93. **E.R. Podack.** 1985. Molecular mechanism of lymphocyte-mediated tumor cell lysis. Immunol. Today 6:21.

94. **Tschopp, J. and M. Nabholz.** 1987. The role of cytoplasmic granules components in cytolytic lymphocyte-mediated cytolysis. Ann. Immunol. Paris 138:290.

95. **Takayama, H. and M.V. Sitkovsky.** 1987. Antigen

receptor regulated exocytosis in cytotoxic T lymphocytes. J. Exp. Med. 166:725.

96. **Treves, S.F., Di Virgilo, V., Cerulondo, P., Zanovello, D. and T. Pozzan.** 1987. Calcium and inositolphosphates in the activation of T cell mediated cytotoxicity. J. Exp. Med. 166:33.

97. **Steele, T.A. and Z. Brahmi.** 1988. Phosphatidylinositol metabolism accompanies early activation events in tumor target cell-stimulated human natural killer cells. Cell Immunol. 112:402.

98. **Peters, P.J., Geuze, H.J., van der Donk, H.A. and J. Borst.** 1990. A new model for lethal hit delivery by cytotoxic T lymphocytes. Immunol. Today 11:28.

99. **Trenn, G., Takayama, H. and M. V. Sitkovsky.** 1987. Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes. Nature 330:72.

100. **Pasternack, M.S. and H.N. Eisen.** 1985. A novel serine esterase expressed by T lymphocytes. Nature 314:743.

101. **Young, J. D.-E., Clark, W.R., Liu, C.-C. and Z.A. Cohn.** 1987. A calcium and perforin independent pathway of killing mediated by murine cytolytic lymphocytes. J.Exp. Med. 166:1894.

102. **Liu, C.-C., Steffen, M., King, F. and J.D.-E Young.** 1987. Cell 51:393.

103. **Peters, P.M., Ortaldo, J.R., Shalaby, M.R., Svedersky, L.P., Nedwin, G.E., Bringman, T.S., Hass, P.E., Aggarwal, B.B., Herberman, R.B., Goeddel, D.V. and M.A. Palladino.** 1986. Natural killer-sensitive targets stimulate production of TNF-α but not TNF-ß (lymphotoxin) by highly purified human peripheral blood large granular lymphocytes.
J. Immunol. 137:2592.

104. **Wright, S.C. and B. Bonavida.** 1982. Studies on the mechanism of natural killer cell-mediated cytotoxicity. I. Release of cytotoxic factors specific for NK-sensitive target cells (NKCF) during coculture of NK effectors cells and target cells. J. Immunol. 129:433.

105. **Quan, P.C., Ishzaka, T. and B.R. Bloom.** 1982. Studies on the mechanism of NK cell lysis. J. Immunol. 128:1786.

106. **Redelman, D. and D. Hudig.** 1980. The mechanism of cell mediated cytotoxicity. I. Killing by murine cytotoxic T lymphocytes requires cell surface thiols and activated proteses. J. Immunol. 124:870.

107. **Podack, E.R., Young, J.D.-E. and Z.A. Cohn.** 1985. Isolation, biochemical and functional characterization of perforin 1 from cytolytic T cell granules.
P.N.A.S. (U.S.A.) 82:8629.

108. **Konnigsberg, P.J. and E.R. Poddack.** 1987. A Cytolytic protein isolated from murine CTL granules (Abstract) Federation Proc. 46:1225.

109. **Masson, D.M. and J. Tschoop.** 1987. A family of serine esterases in lytic granules of cytolytic T lymphocytes. Cell 49:679.

110. **Krammer, M.D. and M.M. Simon.** 1987. Are proteases functional molecules of T lymphocytes. Immunol. Today 8:140.

111. **Kamada, M.M., Michon, J., Ritz, J., Holldack, J., Serafin, W.E., Austen, K., MacDermott, R.P. and R.L. Stevens.** 1989. Identification of carboxypeptidase and tryptic esterase activities that are complexed to proteoglycans in the secretory granules of human cloned natural killer cells. J. Immunol. 142:609.

112. **Young, J. D.-E., Damiano, A., Dinome, M.A., Leong, L.G. and Z.A. Cohn.** 1987. Dissociation of membrane binding and lytic activities of the lymphocyte pore-forming protein (perforin). J. Exp. Med. 165:1371.

113. **Greenberg, A.H., Khalil, N., Pohajdak, B., Talgoy, M., Henkart, P., and F.W. Orr.** 1986. NK-leukocyte chemotactic factor (NK-LCF): a large granular lymphocyte (LGL) granule-associated chemotactic factor. J. Immunol. 137:3224.

114. **Pohajdak, B., J.L. Gomez, J.A. Wilkins, and A.H. Greenberg.** 1984. Tumor-activated NK cells trigger monocyte oxidative metabolism. J. Immunol. 134:243.

CHAPTER II

IDENTIFICATION OF A MACROPHAGE ACTIVATING FACTOR (MAF) IN GRANULES OF THE RNK LARGE GRANULAR LYMPHOCYTE LEUKEMIA

This chapter is the integral version of an article published by Eugene Roussel and Arnold H. Greenberg in **Journal of Immunology (1989) 142:543.**

ABSTRACT

Recent work from our laboratory has shown that NK cells rapidly release preformed factor(s) that stimulate monocyte oxidative metabolism and microbicidal activity. We have hypothesized that such factors could also activate Mϕ tumor lysis and might be stored in the cytoplasmic granules. Granules were isolated from the RNK LGL leukemias by nitrogen cavitation and percoll fractionation of the cell homo-genate. Utilizing CSF-1 differentiated murine bone marrow-derived Mϕ and P815 tumor target cells, a Mϕ activating factor (MAF) was found. The distribution curve of MAF activity showed two peaks, the first was coincident with dense granule enzymes and was 60 times more concentrated per mg protein than a second peak in the cytosol fractions. Solubilization in 2 M NaCl was necessary to recover activity from both peaks. Granule NK-MAF required the simultaneous presence of LPS in order to induce tumoricidal activity. Kinetics of NK-MAF activation peaked after 12 hours of exposure. The NK-MAF was short lived in the solubilized granules, however, its heat resistance allowed us to prepare enriched and stable preparations. Treatment of NK-MAF with pepsin but not trypsin completely abrogated its activity. The NK-MAF passed through an ultrafiltration membrane with a nominal cut-off of 10 kD. This work indicates that NK cell granules contain a small heat stable peptide capable of activating Mϕ tumoricidal activity.

INTRODUCTION

In addition to their role in resistance to malignant disease (1), NK cells may have important immunoregulatory functions which can be mediated through the release of specific cytokines. Some of these have been identified and include IL-1, IL-2, IFN, CSF and TNF (2,3,4). For NK cells to execute their cytolytic functions, it has been suggested that, following contact with the target cells, they exocytose cytoplasmic granules and release cytolysin, a molecule that is capable of damaging the target cell membrane (5). Chondroitin sulfate proteoglycans (6,7) and serine esterases (8,9) have also been shown to be released in this process, suggesting that the granules may contain and release a complex mixture of molecules. In earlier work we had suggested that immunoregulatory cytokines may be present in the cytoplasmic granules of the NK cells (10,11,12). This idea is based upon the observation that, following contact with a NK-sensitive tumor cells, there was the rapid release of a soluble factor which stimulated monocyte oxidative metabolism (12), and the intracellular lysis of facultative microorganisms by alveolar macrophages (10). Moreover, this macrophage activating factor was preformed and its release required an intact secretory apparatus in the LGL (10). The granules of the RNK LGL leukemias also contain a chemoattractant for leukocyte (NK-LCF) that appears to be distinct from the NK-MAF (11).

Collectively these results suggested that NK-MAF might be stored in the LGL granules. The present studies were undertaken to determine if the NK cell granules contain a MAF capable of activating Mϕ for tumor lysis.

MATERIALS AND METHODS

Preparation of RNK LGL granules

Isolation of cytoplasmic granules. The RNK tumor lines, used as a source of granules, were described previously (13,14). The RNK-8, RNK-10 and RNK-16 lines used in this studies were a gift from Dr C. Reynolds (NCI, Frederick, MD). They were grown in the peritoneal cavity of Fisher rats by inoculating 10^7 cells 5 days after the injection of 1 ml of pristane. Cells were harvested under sterile conditions, usually 15-18 days later, by washing the cavity with phenol red-free HBSS containing 100 U/ml of heparin (Sigma Chemical Co., St-Louis, MO).

The procedure for purification of cytoplasmic granules of rat LGL tumors has been described in detail (15), and was carried out under sterile conditions. Materials were gas sterilized in ethylene oxide and solutions were sterilized by filtration through 0.22 μm filters (Nalgene Co., Rochester, New York, USA). In brief, cells were washed in HBSS and resuspended at 1 x 10^8/ml in disruption buffer (0.25M sucrose, 0.01 M Hepes, 4mM EGTA, 100 U/ml heparin (Sigma Chemical Co., St Louis, MO), pH 7.4). They were lysed at 0°C by decompression after equilibrating at 450 psi nitrogen for 20 min.. After the addition of $MgCl_2$ to 5 mM, the homogenate was digested with DNAse I (from bovine

pancreas, type IV, Sigma) at 800 U/ml, 22°C, 25 min.. Nuclei were removed by filtration through Nucleopore filters (Nucleopore Corp., Pleasanton, CA) of 5 and 3 μm and the resulting homogenate was cooled to 0°C. Five ml aliquots were layered on 20 ml of 48% Percoll (in disruption buffer without heparin) and centrifuged in a 70 Ti rotor at 20,000 rpm for 10 min., deceleration without braking using a Beckman L8-M ultracentrifuge (Beckman Instruments, Fullerton, CA). The resulting gradient was fractionated from the bottom by careful insertion of a stainless steel tube from the top and the removal of 1.0 ml fractions via attached polyethylene tubing. Individual 1.0 ml fractions of these gradients were assayed for protein content, ß-glucuronidase, cytolysin, and MAF activities (see below). Dense granules have been shown to be present in the high density Percoll fractions on the basis of granule markers such as ß-glucuronidase, cytolysin, lysosomal enzymes, and confirmed by electron microscopy (15). In routine granule preparations fractions 1 to 4 were pooled and frozen at -20°C until used. For the Mφ tumoricidal assay, granules were quickly thawed in a 37°C water bath and then solubilized by dissolving 116 mg/ml of NaCl using low speed mechanical agitation. This 2 M NaCl solution was diluted in sterile water to normal osmolarity before use in the assay.

In some experiments granule proteins were heat denaturated after solubilizing in 2 M NaCl by diluting 1/5 with

water, then incubating in boiling water for 5 min.. The preparation was then centrifuged at 15,000 rpm in a 70.1 rotor (Beckman L8-M ultracentrifuge) for 15 min.. The supernatant was collected and assayed, or further treated with specific proteases. In these latter experiments heat extracted MAF was treated with 55000 U/ml of trypsin (from bovine pancreas type III, Sigma) for 15 min. or 2540 U/ml of pepsin (Millipore Corporation, Freehold N.J.) for 30 min. at 37°C. The mixture was then incubated 5 min. in boiling water to inactivate the enzymes and assayed. Controls substituted heat extracted MAF with PBS, and were then incubated either with non-activated macrophages or with IFN-τ plus LPS-treated macrophages. This was done to ensure that non-specific activation or inhibition of cytotoxicity was not produced by the heat denatured enzymes.

Protein content. Protein was measured by absorbency at 280 nm. Fractions of the Percoll gradient were solubilized in 2 M NaCl then centrifuged in a 70.1 Ti rotor 45 min at 34,000 rpm to remove the Percoll. Supernatants were removed and their absorbency at 280 nm was measured on a DU-8 spectrophotometer (Beckman). The protein concentration was calculated according to a standard curve obtained with albumin diluted in 2 M NaCl.

ß-glucuronidase assay. The method used has been also described elsewhere (16). Ten μl aliquots of Percoll gradient fractions were diluted 1/100 in water, 100 μl

of the dilution to be assayed for enzymatic activity was added in a tube followed by 50 µl of citrate-phosphate buffer 1.0 M pH 4.5 and 50 µl of substrate 4-MU-ß-D-Glucuro-nide (Sigma) 10 mM dissolved in 0.5 M NaCl. Tubes were incubated for 2 hours at 37°C then the reaction was stopped by adding 2 ml of 0.25 M glycine-NaOH buffer pH 10.3. The relative % of fluorescence was read on an Aminco-Bowman spectrophotofluorometer (American Instrument) previously calibrated with a standard curve of 4-Methylumbelliferone (Sigma) extended from 0.156 to 5.0 nMoles/2.2 ml. ß-glucuronidase activity was expressed in nMole of 4-MU-ß-D-glucuronide released/500nMole of substrate/ml/hr.

Cytolysin assay. This assay has been previously described in detail (15). Briefly, SRBC were labeled by incubation of 10^7 cells with 17 MBq of $Na_2{}^{51}Cr_2O_7$ (DuPont NEN Research Products) in 1 ml of HBSS containing 0.01 M Hepes and 10% FCS for 60 min. at 37°C. Three µl aliquots of Percoll gradient fractions were diluted into 147 µl of Ca^{++} and Mg^{++} free PBS in V-bottomed wells microtiter plates then 75 µl were serially diluted by half. Seventy-five µl of HBSS containing 0.01 M Hepes, 2 mg/ml BSA, pH 7.4, and $5x10^4$ ^{51}Cr-labelled SRBC were added to each well. Plates were incubated at 37°C for 30 min. then centrifuged and supernatants were harvested and counted in a LKB compugamma (LKB Wallac, Turku, Finland).

Macrophage tumoricidal assay

Cultivation of bone marrow macrophages. For each experiment bone marrow cells were obtained from 3 C3H/HeN mice by flushing the marrow from the femur and tibia with a 26 1/2 gauge needle and RPMI 1640. Cells were washed and resuspended in NH_4Cl 0.83% pH 7.3 for 3 min. to lyse the erythrocytes. They were washed twice again and resuspended in 5 ml of culture media. Cells were counted and adjusted to 3.2×10^4 large nucleated cells/ml then plated at 8×10^5 cells (25 ml) per 20-100 mm plastic Petri-dishes treated for tissue culture (NUNC, Kamstrup, Denmark) in RPMI 1640 containing sodium bicarbonate (0.2%), penicillin/streptomycin (50 IU/ml and 50 μg/ml respectively) and supplemented with 2 mM Glutamine, 10% v/v FCS, 10% v/v horse serum (GIBCO) and 10% v/v of pre-titred L-929 conditioned medium as a source of CSF-1. Petri plates were incubated for 5 to 6 days at 37°C, 7.5% CO_2 and 95% humidity. This method of bone marrow culture was previously shown to yield 98% pure macrophages after 5 days (17).

Activation of bone marrow-derived macrophages. At day 5 or 6, the cells were harvested by incubation for 15 min. at 22°C in PBS containing 5.0 mM disodium EDTA pH 7.4 and then gentle pipetting to help detach the macrophages. The cells were washed and resuspended in RPMI 10% FCS culture media at 1×10^6 big "foamy" cells/ml, as those cells are

thought to be the fully differentiated macrophages (18). Aliquots of 10^5 macrophages per well were plated in 96 microwells flat bottom plate (NUNC, Kamstrup, Denmark) and incubated for 4-6 hours. Then media and non-adherent cells were removed and activating agents were added. In most experiments the macrophages were activated for 9 hours. One hundred μl of purified mouse IFN-γ 20 U/ml (provided by Dr. G. Spitalny, Bristol Meyers Corp.) in culture media containing 50 ng/ml of LPS (from E. coli type B:4 or B:8 phenol extracted, Sigma), and Con A activated spleen cells supernatants (LK) at a concentration of 10% with 50 ng/ml LPS were used as positive controls. LK was prepared by stimulating Fisher rat spleen cells with 5 μg/ml of Con A (Miles Scientific) for 72 hours then absorbing Con A from the culture supernatant with 10 mg/ml of Sephadex G-25 at 4°C for one hour followed by filtration with 0.22 μm filter (Nalgene). RNK granule preparation containing the MAF were solubilised in 2 M NaCl then diluted with water to restore osmolarity to a physiological level and used in sequential dilution.

After the activation period the wells were washed with culture medium and 200 μl of 131-IUdR labelled P815 target cells at 5 x 10^4 cells/ml were added to the wells and incubated for 18-20 hours. The P815 target cells were labelled by incubating 2 x 10^5 cells/ml with 3 MBq/ml of 131-IUdR (Edmonton Radiopharmaceutical Centre, Canada) four

hours then washed four times. After the lytic period the plates were centrifuged at 600 g for 8 min. and 100 μl of the supernatant were harvested and counted in an LKB compugamma.

Experiments were conducted in triplicates and percent of specific 131-IUdR release was calculated by the formula:

$$\frac{\text{Experimental (\%) - mean of control (\%)}}{\text{100 - mean of control (\%)}} \times 100$$

Control values are % of 131-IUdR released from target cells incubated with non activated macrophages, referring to the total cpm contained in 200 μl of 131-IUdR labelled P815 target cells. Control levels varied from 8-22 %. Values presented are means $\pm$ SEM.

RESULTS

Identification of MAF activity in RNK cell granules

In order to obtain an adequate quantity of granule material for the study we used the RNK LGL tumor cell lines as a source of granules. These cells were derived from spontaneous leukemias of aged Fisher rats and were shown to resemble LGL morphologically, have high cytolytic activity without T cell receptor gene rearrangement (19), share certain surface markers with the peripheral blood LGL cells, and contained the characteristic cytoplasmic granules (13,14).

It has been shown on the basis of lysosomal enzymes as granule markers, cytolysin and with confirmation by electron microscopy that the dense granules of the LGL cytosol purified in the dense fractions of the LGL lysate Percoll gradient (15,5). With this system we isolated the granules of three independent RNK cell lines and tested for activity in a macrophage cytotoxicity assay using CSF-1 differentiated murine bone marrow derived macrophages with P815 murine mastocytomas as target cells in a 10:1 effector:target ratio. As a previous study had shown that better recovery of cytolysin from granules was achieved by solubilization of the granules in high salt buffers, we first examined several different methods of granule extraction in

order to increase the chances of detecting any MAF activity. Solubilization of the granules in sucrose or PBS including freezing and thawing, and sonication did not liberate the MAF, but when higher salt concentration buffers were used significant activity was detected. The optimum results were obtained with the 2 M NaCl salt extraction (Table II). MAF activity was identified in the solubilized granules of three independent RNK cell lines, with activity decreasing proportionally to the granule protein concentration, although some preparations showed inhibition at high concentration. The results shown in Figure 1 are characteristic of all the preparations tested over many experiments. The whole Percoll gradients of RNK cell homogenates were then analyzed to verify if MAF activity could be identified in other cell compartments. As shown in Figure 2, the cytolysin showed a major peak of activity in the first four fractions corresponding to the dense granules and another peak was observed in tubes 16-17. ß-glucuronidase activity peaked in the dense granules fractions and a second area of activity was noted in the last eight fractions, corresponding to the cytosol, with a peak in tubes 17-19. The distribution of BLT-esterase activity (20) superimposed that of ß-glucuronidase (data not shown). The MAF had a profile of activity similar to the ß-glucuronidase and the BLT-esterase with a first peak of activity in the dense granules and a second peak in the cytosolic fractions. On the basis

TABLE II

Solubilized NK-MAF Activity in RNK Cell Granules

Solubilizing agent[b]	Granule protein concentration (μg/ml)	LPS (ng/ml)	Specific lysis ± SEM[a] (%)	
			Expt 1	Expt 2
0.25 M Sucrose	12	50	0	0
	4	50	0	2 ± 1
PBS	12	50	0	5 ± 5
	4	50	0	0
1 M NaCl	12	50	4 ± 1	6 ± 3
	4	50	0	2 ± 1
2 M NaCl	12	50	23 ± 3	19 ± 5
	4	50	0	4 ± 2
---	0	50	0	0

a. In the experiment 1 the granule preparation was diluted from its original buffer of purification containing 0.25M sucrose and 48% of Percoll. In the experiment 2 the fraction containing the granules was centrifuged a second time at 85 000 g for 3 hrs to remove the Percoll.

b. Granules were solubilized in the agent at concentration indicated above, then assayed after readjustment to normal osmolarity, in the presence of 50 ng/ml of LPS.

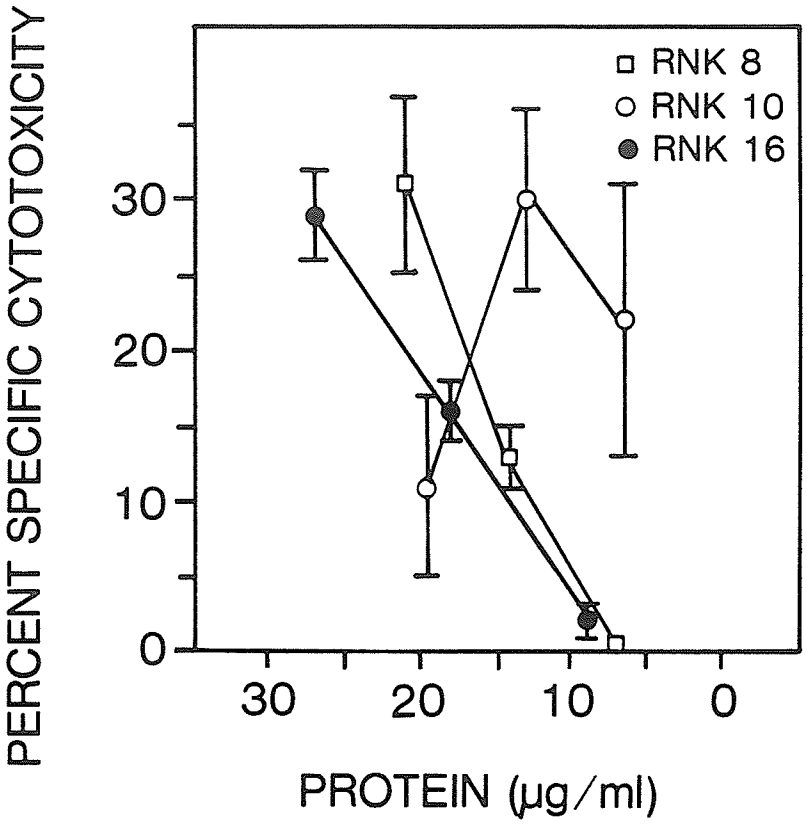

Figure 1. NK-MAF activity of independent RNK cell lines. In this assay, the granules from each cell line (□) RNK 8, (o) RNK 10, and (●) RNK 16 were purified and solubilized in 2 M NaCl then adjusted to normal osmolarity and assayed for activity in the presence of 50 ng/ml of LPS.

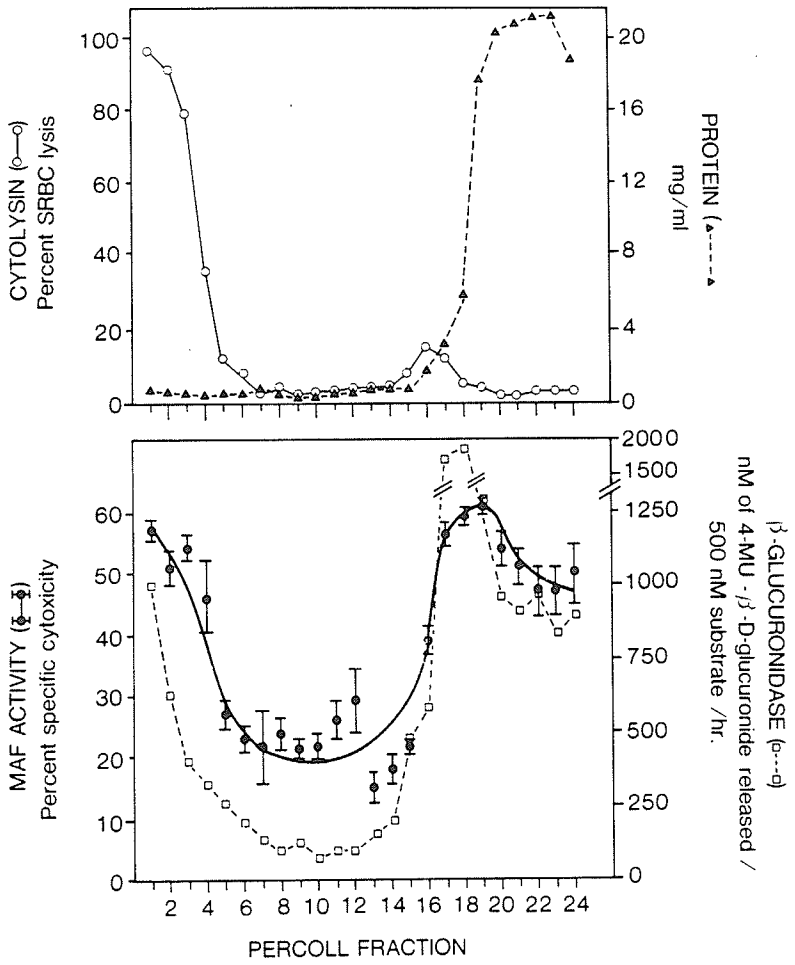

Figure 2. Distribution of NK-MAF, β-glucuronidase, cytolysin , and protein in the Percoll gradient fractions of the RNK 16 cell homogenate. The centrifugation of the cell homogenate on Percoll formed a self-generating density gradient which was fractionated starting at its highest density in 24 fractions. Aliquots were analyzed as described in the Materials and Methods section. In this figure, the cytolytic activity is presented at a dilution of 1/800 and the NK-MAF activity at a dilution of 1/30.

of protein concentration the MAF was sixty times more concentrated in the dense granule than in the cytosol fractions. The fractionation of MAF activity in the whole Percoll gradient was repeated five times and 4 different RNK cell lines were used with similar results.

Characteristics of NK-MAF activation of BM-derived macrophages

It has been shown by many laboratories (21-23) that activation of mouse macrophages for tumor killing proceeds by two sequential steps, an initial priming signal followed by a second triggering step. Mouse IFN-γ can induce the priming step, and the second signal is usually fulfilled by LPS (24,25). This sequential presentation is not absolutely required for activation because the simultaneous presentation of both agents is as or more effective. Co-activation is now generally used instead of the sequential incubation (26,27). Similar to IFN-γ, LPS was required as a cofactor by NK-MAF (Figure 3A). When solubilized granules were incubated with macrophages no activity was seen whereas when LPS was added at 50 ng/ml, a dose which was inactive on its own, a proportional dose-response activity was obtained. By titrating the LPS in the presence of MAF we found that 50 ng/ml of LPS was the optimum dose, producing virtually no background activation with maximum expression of MAF

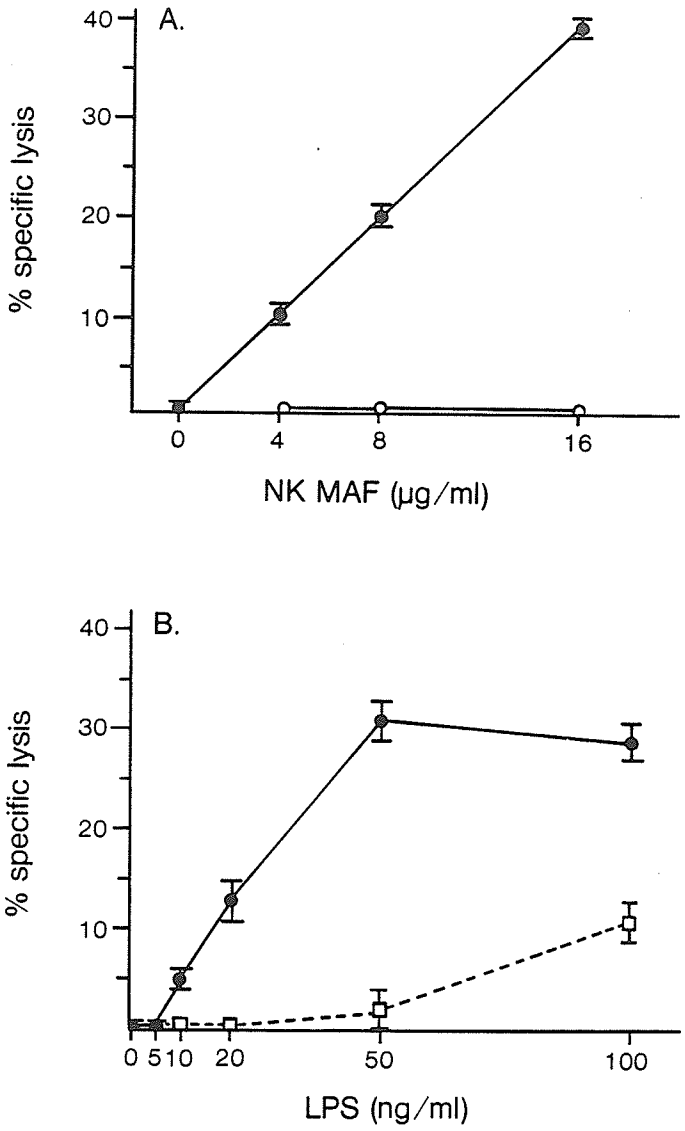

Figure 3. NK-MAF activity is dependent on LPS. A. Macrophages were incubated with solubilized granules in the absence (○) or presence (●) of LPS at 50 ng/ml then assayed for cytotoxicity. The results shown here are representative of many experiments. B. Macrophages were incubated with different concentration of LPS alone (□) as controls or in the presence of NK-MAF at 16 μg/ml (●) for 9 hours and then incubated with the P815 tumor target cells. Results were calculated by using the LPS alone as the control value in the equation described in the Material and Methods section to obtain the NK-MAF specific cytotoxicity.

activity (Figure 3B).

The NK-MAF appeared to differ from IFN-τ by its inability to activate in the sequential presentation of granule extracts and LPS. As shown in Table III, IFN-τ or LK incubated as a first signal followed by LPS as a second signal initiated substantial Mϕ tumoricidal activity whereas NK-MAF as a priming signal followed by LPS was completely inactive. Reversing the order of presentation using LPS as a first signal and NK-MAF, IFN-τ or LK as a second signal did not produce any NK-MAF activity, and it decreased IFN-τ and LK activation. To activate macrophages, the NK-MAF had to be simultaneously incubated with LPS as a cofactor. We next asked whether the NK-MAF, which appeared to act differently from IFN-τ, could replace LPS in IFN-τ activation, that is, whether IFN-τ and NK-MAF could activate in a simultaneous incubation. These macrophage preparations were not tumoricidal suggesting that NK-MAF was not LPS-like in its effect.

As a next phase in the characterization of NK-MAF activation, we tested and compared the kinetics of activation with IFN-τ. NK-MAF activity was detectable after 6 hours of activation with the optimum activity obtained after 12 hours, subsequently decreasing to zero at 24 hours (Figure 4). In the same experiment, IFN-τ showed optimum activity as early as 3 hours, then progressively decreased over the next 24 hours.

TABLE III

NK-MAF Activation of Macrophage Tumor Killing Following Coincubation but not Sequential Activation by LPS

Treatment of macrophages[a]		% Specific lysis ± SEM
First signal	Second signal	
NK-MAF(6 hrs)	LPS (1 hr)	1 ± 1
IFN-τ (6 hrs)	LPS (1 hr)	36 ± 4
LK (6 hrs)	LPS (1 hr)	44 ± 2
Medium(6 hrs)	LPS (1 hr)	10 ± 1
LPS (1 hr)	NK-MAF (6 hrs)	1 ± 0
LPS (1 hr)	IFN-τ (6 hrs)	4 ± 1
LPS (1 hr)	LK (6 hrs)	21 ± 1
LPS (1 hr)	Medium (6 hrs)	1 ± 1
Simultaneous incubation		
NK-MAF + LPS (6 hrs)		39 ± 2
IFN-τ + LPS (6 hrs)		65 ± 1
--- LPS (6 hrs)		2 ± 1
IFN-τ + NK-MAF (6 hrs)		0
IFN-τ --- (6 hrs)		0
NK-MAF --- (6 hrs)		0

a. In the sequential activation, the macrophages were incubated with the first agent for the specified time, then washed and incubated with the second agent and washed again before addition of the P815 tumor target cells. In the one step activation, the macrophages were incubated with the activator in the presence or absence of LPS for 6 hrs, washed and incubated with the P815. LK was prepared by stimulating rat spleen cells with 5 μg/ml of Con A for 72 hrs, followed by the absorbtion of Con A from the culture supernatant with Sephadex G-25 and then filtration with 0.22 μm filter. LK was used at 10%. NK-MAF was prepared by solubilization of the granules in 2 M NaCl which were then diluted to normal osmolarity with water and used at 30 μg/ml in both experiments. IFN-τ (20 U/ml) and LPS (50 ng/ml) were used throughout.

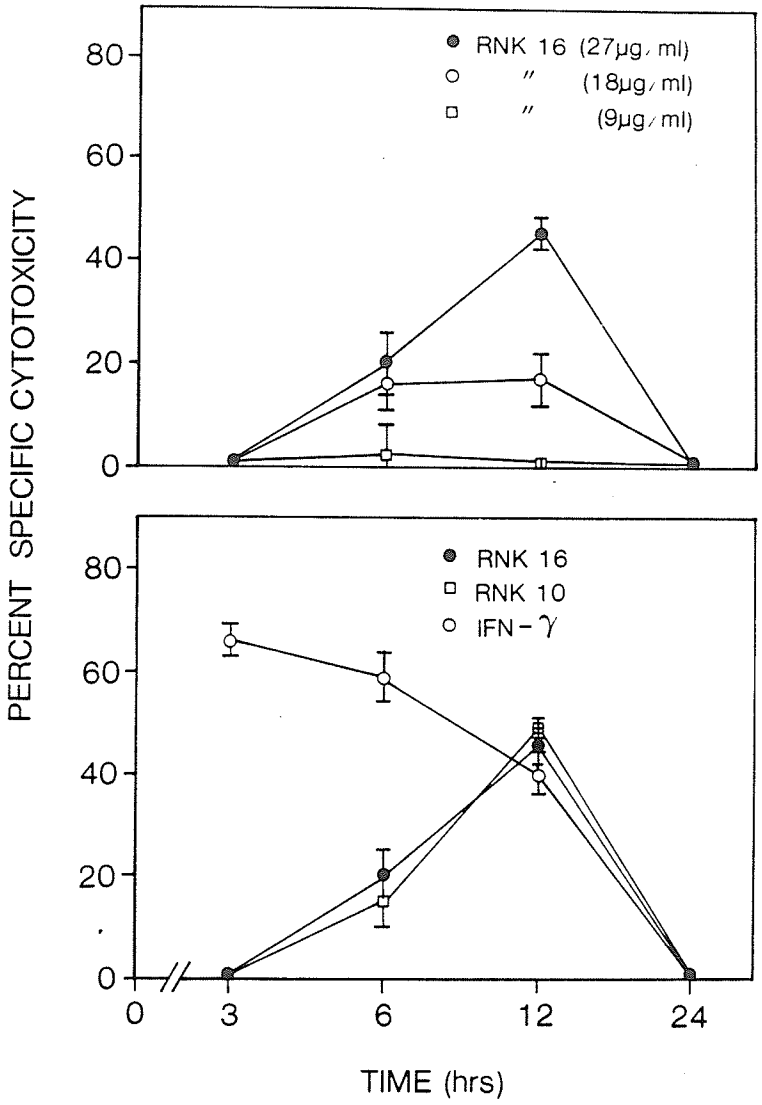

Figure 4. Kinetics of activation by NK-MAF. In the top panel, the macrophages were activated with different concentrations of NK-MAF in the presence of LPS (50 ng/ml). After each time, the activation was stopped by washing and then the macrophages were incubated with the P815 cells. The lower panel illustrates the kinetics of activation for two different RNK cell line granules and IFN-γ (20 U/ml) using the same procedure as described above.

Biochemical characteristics of NK-MAF

Neutral protease activity has been detected in LGL granules (15) as well as substantial serine esterase activity (20). In our initial attempts to biochemically characterize the MAF, we were confronted with its instability in solubilized granule preparations, more than 30% of the MAF activity was lost in solubilized granules stored overnight at 4°C. We therefore suspected that the NK-MAF degradation could be due to the effects of granule proteases, and subsequently isolated the LGL granules in the presence of different protease inhibitors (Benzamidine, PMSF, SBTI, TLCK, TPCK) but with little or no improvement. We next tried to produce stable MAF preparations by heat treatment of the solubilized granules, expecting to inhibit the proteases by heat denaturation. This approach was successful and no NK-MAF activity was lost after the heattreatment (Figure 5). Moreover, our preparations were usually 1.5 to 2 fold enriched on the basis of granule protein concentration. To determine whether the heat stable MAF was protein in nature, it was treated with pepsin or trypsin for 30 min. at 37°C then the mixture was incubated in boiling water for 5 min. to heat denature the protease. The supernatant was then assayed for MAF activity. In two experiments, the NK-MAF showed great sensitivity to the pepsin, however, similar treatment with trypsin was

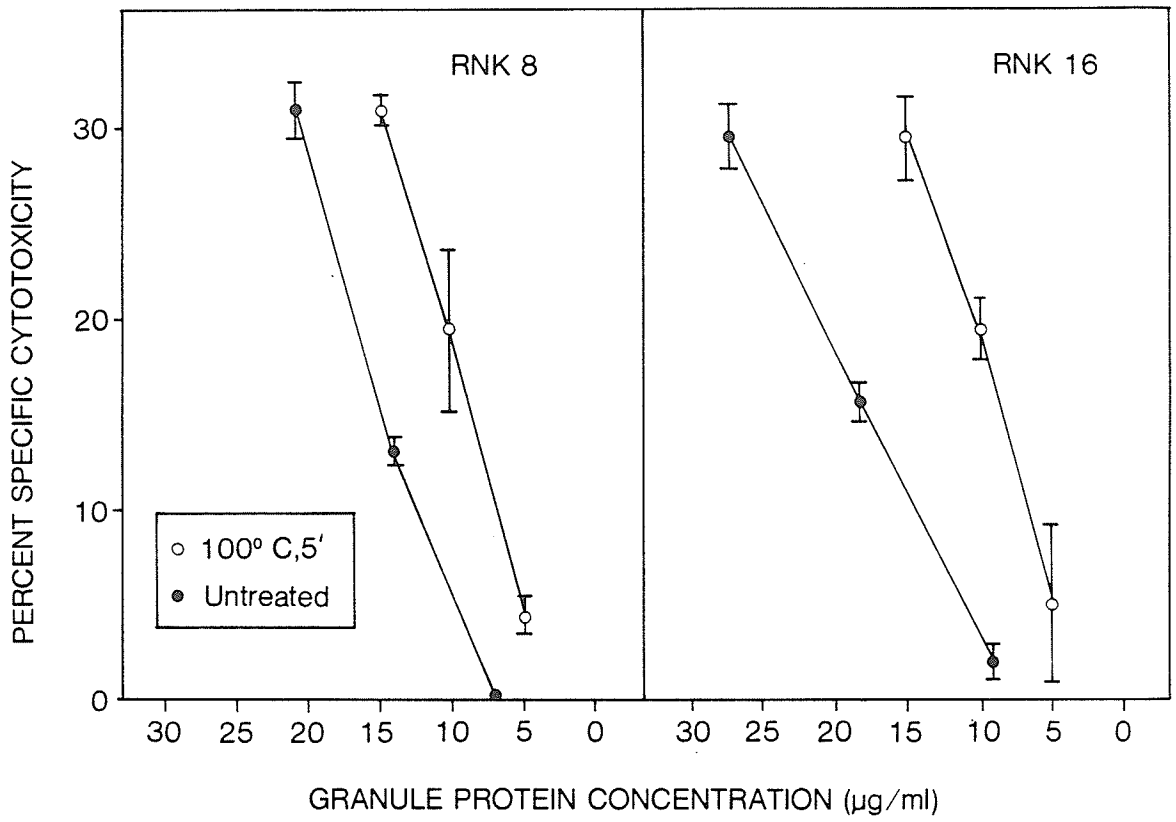

Figure 5. Heat resistance of NK-MAF. Granules were solubilized in 2 M NaCl then diluted 1/5 in water and incubated 5 min. in boiling water. They were centrifuged and the supernatant was assayed after returning to isoosmolarity. The untreated controls were solubilized granules diluted to normal osmolarity.

ineffective (Table IV) and even increased activity slightly. As our technique consisted of treating the NK-MAF with the enzyme and then heating the mixture to inactivate the protease, we controlled for the possibility that the denatured enzymes could block the activation of macrophages. Neither the heat inactivated pepsin nor trypsin affected the capability of IFN-τ and LPS to activate macrophage cytotoxicity.

Finally, we found that the heat extracted NK-MAF activity could pass through an Amicon ultrafiltration membrane with a nominal cut off of 10 kD. These results indicated that the NK-MAF is a small peptide.

TABLE IV

NK-MAF Sensitivity to Proteases

Treatment[a]	% Specific lysis ± SEM	
Pepsin	Expt 1	Expt 2
NK-MAF + LPS[1]	40 ± 5	21 ± 1
NK-MAF + pepsin + LPS	3 ± 2	8 ± 1
IFN-τ + LPS	62 ± 3	77 ± 4
Heat inactivated pepsin + IFN-τ + LPS[2]	47 ± 1	57 ± 3
Trypsin		
NK-MAF + LPS[1]	21 ± 1	32 ± 1
NK-MAF + trypsin + LPS	29 ± 1	41 ± 5
IFN-τ + LPS	77 ± 2	60 ± 7
Heat inactivated trypsin + IFN-τ + LPS[2]	63 ± 2	67 ± 1

a. Heat extracted NK-MAF was incubated in presence of pepsin (2,540 U/ml) or trypsin (55,000 U/ml) for 30 min. at 37°C and then incubated in boiling water for 5 min. to denature the protease. This mixture was then assayed with macrophages in presence of 50 ng/ml of LPS. Controls were NK-MAF incubated without enzyme at 37°C (1) or PBS incubated with either pepsin or trypsin then heat denatured as above and assayed in the presence of IFN-τ + LPS (2). This latter control tested any inhibitory effects that heat inactivated proteases may have on macrophage activation.

DISCUSSION

We have reported here that granules of RNK LGL leukemias contain a small heat stable peptide capable of activating Mϕ tumor killing. This is the first report of a macrophage activating factor contained in LGL granules. In addition to the MAF activity present in the granules of these RNK cell lines, a second peak of MAF activity was detected coincident with the cytosol fractions. It has been suggested that the cytosolic peak of esterases may be due to their presence in lighter granules, which would remain in the lighter fractions (20). Using finer fractionation, and lysosomal enzymes as granules markers, Henkart et al. have been able to obtain better dissociation of this second peak from the cytosolic fractions (20). The similar profile of activity for ß-glucuronidase, BLT-esterases, and the MAF, along with the requirement for 2 M NaCl solubilization of both peaks to detect MAF activity argues more in favor of the presence of lighter granules, than the release of MAF into cytosol from disrupted granules during nitrogen cavitation. However, we cannot exclude the possibility that MAF may be bound to granule fragments that fractionate in this cytosolic region.

The requirement for ionic solubilization of granules in 2 M NaCl to release the NK-MAF activity suggests that the NK-MAF must be tightly bound to an internal granule matrix.

This raises the question of what can be the physiological equivalent of this ionic solubilization. Although we cannot answer this question directly, we have previously shown that a chemotactic factor present in the granules, which also needed 2 M NaCl solubilization for best recovery, was released from intact cells following activation with a degranulating reagent (11). Exactly what mechanism would release the MAF from the matrix is not immediately apparent, however, granule proteases may act to digest an internal matrix to liberate some molecules stored in an inactive form. Since MAF appears to be a small peptide, it might be generated by the action of such proteases.

The identity of the MAF is a critical and unresolved issue. We have however, made several observations that allow us to argue against its identity with many known cytokines. The heat stability and size of the MAF excludes most of the known lymphokines. In addition, granules have been tested for the presence of TNF with an ELISA assay (E. Roussel, A.H. Greenberg and M. Palladino, unpublished) , IFN-τ on the basis of antiviral activity, IL-2 with the CTTL2 IL-2 dependent cell line, IL-1 by costimulation of thymocytes with PHA, and CSF-1 by incubation with murine bone marrow cells where 2 M NaCl solubilized granules were substituted for CSF-1-containing L-cell conditioned media. In each case no appreciable activity was detected. Of course, we cannot yet rule out the possibility that the NK-MAF is a fragment of one of these

molecules, but we think that it is rather unlikely if the parent molecule cannot be detected in the granules in appreciable quantity.

The study of the mechanism of NK-MAF macrophage activation indicated that there was some similarities with IFN-τ in its LPS requirement. NK-MAF required LPS as a cofactor for full Mϕ lytic activation, and accordingly it was always inactive using bone marrow-derived macrophages from LPS-resistant C3H/HeJ mice (E. Roussel, and A.H. Greenberg, data not shown). Differences with IFN-τ appeared at the level of the sequential activation protocol and time course for activation. However, it is possible that a suboptimal concentration of the NK-MAF in the granule preparations was responsible for these apparent dissimilarities. This will not be resolved until NK-MAF is further purified.

An important difference between NK-MAF and IFN-τ in our experiments came from the observation that some macrophage preparations that responded normally to IFN-τ, were not activated by NK-MAF. Subsequent study indicated that part of the variation was dependent on the conditions used for bone marrow cell differentiation. We noted that careful control of temperature and pH (6 days at pH 7.3, 37°C, 7.5% CO_2) yielded large number of macrophages called "big foamy cells" by some investigators (18, 29) and these macrophage subsets seemed to be the most responsive to NK-MAF, whereas IFN-τ did not exhibit this requirement. It is possible that

there are more macrophages susceptible to IFN-γ than to NK-MAF in a heterogeneous population of bone marrow-derived macrophages. The NK-MAF might target a more mature subset of macrophages present in sufficient number only under these certain conditions of differentiation. Further study in this direction with NK-MAF might help to define a subset of macrophages responsive to this particular factor.

Finally, the NK cells have been shown to be one of the first cells of the immune defense to infiltrate infected organs (30,31), and on contact with target cells, they would rapidly exercise their lytic function through a degranulation process which releases cytolytic molecules (32). This paper presents evidence for the existence of a granule-contained NK-MAF capable of activating Mϕ tumoricidal activity.

ACKNOWLEDGMENTS

We would like to thank Dr. Pierre A. Henkart for his continuing helpful advice. We also express our gratitude to Mike Talgoy for technical assistance.

REFERENCES

1. **Herberman, R.B.** 1981. Natural killer (NK) cells and their possible roles in resistance against disease. Clin. Immunol. Rev. 1:1.

2. **Scala, G., P. Allavena, J.D. Djeu, T. Kasahara, J.R. Ortaldo, R.B. Herberman, and J.J. Oppenheim.** 1984. Human large granular lymphocytes are potent producers of interleukin-1. Nature 309:56.

3. **Kasahara, T., J.D. Djeu, S.F. Dougherty, and J.J. Oppenheim.** 1983. Capacity of human large granular lymphocytes (LGL) to produce multiple lymphokines: interleukin-2, interferon and colony-stimulating factor. J. Immunol. 131:2379.

4. **Peters, P.M., J.R. Ortaldo, M.R. Shalaby, L.P. Svedersky, G.E. Nedwin, T.S. Bringman, P.E. Hass, B.B. Aggarwal, R.B. Herberman, D.V. Goeddel, and M.A. Palladino Jr.** 1986. Natural killer-sensitive targets stimulate production of TNF-α but not TNF-ß (lymphotoxin) by highly purified human peripheral blood large granular lymphocytes. J. Immunol. 137:2592.

5. **Henkart, P.A., P.J. Millard, C.W. Reynolds, and M.P. Henkart.** 1984. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. J. Exp. Med. 160:75.

6. **MacDermott, R.P., R.E. Schmidt, J.P. Caufield, A. Hein, G.T. Bartley, J. Ritz, S.F. Schlossman, K.F. Austen, and R.L. Stevens.** 1985. Proteoglycans in cell-mediated cytotoxicity. Identification, localization, and exocytosis of a chondroitin sulfate proteoglycan from human cloned natural killer cells during target cell lysis. J. Exp. Med. 162:1771.

7. **Schmidt, R.E., R.P. MacDermott, G. Bartley, M. Bertovich, D.A. Amato, K.F. Austen, S.F. Schlossman, R.L. Stevens, and J. Ritz.** 1985. Specific release of proteoglycans from human natural killer cells during target lysis. Nature 318:289.

8. **Pasternack, M.S., C.R. Verret, M.A. Liu, and H.N. Eisen.** 1986. Serine esterase in cytolytic T lymphocytes. Nature 322:740.

9. **Takayema, H., G. Trenn, W. Hemphrey, J.A. Bluestone, P.A. Henkart, and M.V. Sitkovsky.** 1987. Antigen receptor triggered secretion of a trypsin-type esterase from cytotoxic T lymphocytes. J. Immunol. 138:566.

10. **Gomez, J.L., B. Pohajdak, S. O'neill, J. Wilkins, and A.H. Greenberg.** 1985. Activation of rat and human alveolar macrophage intracellular microbicidal activity by a preformed LGL cytokine. J. Immunol. 135:1194.

11. **Greenberg, A.H., N. Khalil, B. Pohajdak, M. Talgoy, P. Henkart, and F. William Orr.** 1986. NK-leukocyte chemotactic factor (NK-LCF): a large granular lymphocyte (LGL) granule associated chemotactic factor.
J. Immunol. 137:3224.

12. **Pohajdak, B., J.L. Gomez, J.A. Wilkins, and A.H. Greenberg.** 1984. Tumor-activated NK cells trigger monocyte oxidative metabolism.
J. Immunol. 134:243.

13. **Ward, J.M., and C.W. Reynolds.** 1983. Large granular lymphocytic leukemia in F344 rats.
Am. J. Pathol. 111:1.

14. **Reynolds, C.W., E.W. Bere, and J.M. Ward.** 1984. Natural killer activity in the rat. III. Characterization of transplantable large granular lymphocyte (LGL) leukemias in the F344 rat. J. Immunol. 132:534.

15. **Millard, P.J., M.P. Henkart, C.W. Reynolds, and P.A. Henkart.** 1984. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors.
J. Immunol. 132:3197.

16. **Hoehn, S.K., and J.N. Kanfer.** 1978. L-Ascorbic acid and lysosomal acid hydrolase activities of guinea pig liver and brain. Can. J. Biochem. 56:352.

17. **Lee, K.-C., and M. Wong.** 1980. Functional heterogeneity of culture-grown bone marrow-derived macrophages. I. Antigen-presenting function.
J. Immunol. 125:86.

18. **Warren, M.K., and S.N. Vogel.** 1985. Bone marrow-derived macrophages: Development and regulation of differentiation markers by colony-stimulating factor and interferons.
J. Immunol. 134:982.

19. **Reynolds, C.W., M. Bonyhadi, R.B. Herberman, H.A. Oung, and S.M. Hendrick.** 1985. Lack of gene rearrangement and mRNA expression of the τ chain of the T cell receptor in spontaneous rat large granular lymphocyte leukemia lines.
J. Exp. Med. 161:1249.

20. **Henkart, P.A., G.A. Berrebi, H. Takayama, W.E. Munger, and M.V. Sitkovsky.** 1987. Biochemical and functional properties of serine esterases in acidic cytoplasmic granules of cytotoxic T lymphocytes. J. Immunol. 139:2398.

21. **Meltzer, M.S.** 1981. Macrophage activation for tumor cytotoxicity: Characterization of priming and trigger signals during lymphokine activation. J. Immunol. 127:179.

22. **Taramelli, D., H. T. Holden, and L. Varesio.** 1980. Endotoxin requirement for macrophage activation by lymphokines in a rapid microcytotoxicity assay. J. Immunol. Methods. 37:225.

23. **Pace, S.L., and S. W. Russell.** 1981. Activation of mouse macrophages for tumor cell killing. I. Quantitative analysis of interaction between lymphokine and lipopolysaccharide. J. Immunol. 126:1863.

24. **Schultz, R.M., and W. J. Kleinschmidt.** 1983. Functional identity between murine interferon and macrophage activating factor. Nature 305:239.

25. **Pace, J.L., S.W. Russel, B.A. Torres, H.M. Johnson, and P.W. Gray.** 1983. Recombinant mouse interferon induces the priming step in macrophage activation for tumor cell

killing. J. Immunol. 130:2011.

26. **Pace, J.L., S.W. Russel, P.A. LeBlanc, and D.M. Murasko.** 1985. Comparative effects of various classes of mouse interferons on macrophage activation for tumor cell killing. J.Immunol. 134:977.

27. **Krammer, P.H., C.F. Kubella, W Falk, and A. Ruppel.** 1985. Priming and triggering of tumoricidal and schistosomulicimal macrophages by two sequential lymphokine signals: interferon-gamma and macrophage cytotoxicity inducing factor 2. J. Immunol. 135:3258.

28. **Masson, D., M. Nabholz, C. Estrade, and J. Tschopp.** 1986. Granules of cytolytic T lymphocytes contain two serine esterases. EMBO J. 5:1595.

29. **Tushinski, R. J., I. T. Oliver, L. J. Guilbert, P. W. Tynan, J. R. Warner, and E. R. Stanley.** 1982. Survival of mononuclear phagocytes depends on a lineage-specific growth that the differentiated cells destroy. Cell 28:71.

30. **Marboe, C.C., D. M. Knowles, L. Chess, K. Reemtsma, and J.J. Fenoglia Jr.** 1983. The immunologic and ultrastructural characterization of the cellular infiltrate in acute cardiac allograft rejection: prevalence of cells

with the natural killer (NK) phenotype.
Clin. Immunol. Immunopathol. 27:141.

31. **Wiltrout, R. H., B. J. Mathieson, J. E. Talmagade, C. W. Reynolds, S. R. Zhang, R. B. Herberman, and J. R. Ortaldo.** 1984. Augmentation of organ associated natural killer by biological response modifiers. J. Exp. Med. 160:1431.

32. **Koren, H. S., and others.** 1987. Proposed classification of leukocyte-associated cytolytic molecules.
Immunol. Today 8:69.

CHAPTER III

LONG-TERM CULTURES OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES WITH rhINTERLEUKIN-2 GENERATE A POPULATION OF VIRTUALLY PURE $CD3^+, CD16^-, CD56^-$ LARGE GRANULAR LYMPHOCYTE LAK CELLS

This chapter is the integral version of an article published by Eugene Roussel, Jon M. Gerrard and Arnold H. Greenberg in **Clinical and Experimental Immunology (1990) 82:416.**

CHAPTER PRESENTATION

Following the identification of a rat granule-MAF in the RNK LGL leukemia cell lines and its partial purification, we were interested in looking for an homologous activity in normal human LGL granules. To purify enough granules we needed large amount of normal human LGLs. In order to easily obtain such a large amount we developed a method for generating a large pure population of $CD3^+$ LGL from human PBL cultured using a low dose of IL-2. This chapter describes and characterizes the details of the method and the nature of this LGL population which proliferated from human PBL cultures.

ABSTRACT

It has been reported that lymphocytes from peripheral blood cultured with IL-2 produce predominantly $CD16^+$ LAK cells. We developed a two step method to generate LAK cells from human PBL in long-term cultures (10-12 days) with rhIL-2 and characterized the evolving LAK cell population by testing its phenotype and cytotoxic activity as a function of time. The starting PBL displayed some NK cytotoxicity but no LAK activity. At day 6, the cells were a mixed population of about 80% $CD3^+$ and 6% $CD16^+$ cells. Little proliferation was evident but strong LAK activity was detected. After 10-12 days, major cell expansion had occurred and they were essentially a pure (>90%) $CD3^+$,$CD16^-$,$CD56^-$ cell population LGL by morphology that displayed strong non MHC-restricted killing activity (>200 LU). Over the same period of time, the $CD16^+$ cells had almost completely regressed in these cultures. This preferential induction of $CD3^+$ LAK cells was not an effect of IL-2 concentration as 10 U/ml was as effective as 500 U/ml. Further characterization revealed a major population of $CD4^+$ (60%) and $CD8^+$ (30%) with a smaller fraction (<9%) of $\tau\delta^+$ cells. These results indicate that a virtually pure $CD3^+$ LAK cells population was produced with long-term cultures of lymphocytes from peripheral blood in rhIL-2, in which active proliferation of the $CD3^+$ but not $CD16^+$ cells occurred.

INTRODUCTION

The in vitro culture of human peripheral blood lymphocytes in presence of rhIL-2 results in the generation of cytotoxic cells called LAK (lymphokine-activated killer) cells which are characterized by their ability to lyse tumor targets without MHC restriction (1-4). A considerable amount of work has been done over the past few years trying to define the lineage of the LAK effector cells. In an initial report, Grimm et al. (1) detected the generation of LAK cells which were $CD3^+$, but subsequently most investigators defined LAK activity generated from PBL as predominantly from large granular lymphocyte of the $CD3^-CD16^+$ phenotype which is typical of the natural killer (NK) cell (5,6,7,). However, the development of $CD3^+,CD16^-$ LAK cells was reported when PBL were cultured in the presence of IL-2 and the mitogenic OKT3 antibody in long-term cultures (8). These cultures were 14 days or more compared to most previous reports in which experiments were completed in short-term cultures of 2 to 4 days. We have developed a two step method of generating LAK cells from human PBL in long-term cultures (10-12 days) with IL-2 where a predominant proliferation of $CD3^+$ or $CD16^+$ cells can potentially be obtained. In this report, we have characterized as a function of time the evolving LAK cell population from the culture condition where preferential growth of $CD3^+$ cells

occured. We present evidence that the resulting LAK effector cells are a population of virtually pure $CD3^{+}$,$CD16^{-}$·$CD56^{-}$ large granular lymphocytes and we propose an explanation for the reported ambiguity about the lineage of the LAK cell effectors generated from PBL .

MATERIALS AND METHODS

PBL isolation and culture. Human buffy coat from normal donors were obtained through the Canadian Red Cross. The buffy coat cell population was diluted by half with phenol-red free HBSS (GIBCO) containing 100 U/ml of heparin (Sigma Chemical Co., St. Louis, MO), pH adjusted to 7.2. The diluted cells were layered on Ficoll-Paque (Pharmacia Fine Chemical, Piscataway) and centrifuged at 900g for 20 min.. The mononuclear band was removed and washed with HBSS containing 100 U/ml of heparin by centrifugation at 400g for 15 min.. The pellet was resuspended in HBSS without heparin and centrifuged at 100g for 8 min. to remove most of the remaining platelets. The resulting mononuclear cells were resuspended at $1x10^6$ cells/ml in RPMI 1640 containing sodium bicarbonate (0.2%), penicillin/streptomycin (50 IU/ml and 50 μg/ml, respectively), 10% v/v HI FCS (56°C, 30 min.), and newly supplemented with 2 mM glutamine. As a first step, the cells were incubated in 175 cm^2 tissue culture flasks (NUNC, Kamstrup, Denmark) at 100-150 x 10^6 cells per flask in presence of 10 U/ml of recombinant human interleukin-2 (AMGEN Biologicals, Thousand Oaks, CA) for 6 days at 37°C, 95% humidity, 5% CO_2. At day 6, the non adherent cells were centrifuged at 100g for 10 min. and resuspended at 0.25 x 10^6 cells/ml in fresh media containing 10 U/ml of IL-2. As a second step, the cells were incubated in new flasks at 150

ml of volume per flask for another 5-6 days until they had reached a concentration close to 1 x 10^6/ml.

Cell staining. Using cytospin centrifuged slides, cells were stained by the Diff-Quik stain technique (American Hospital Supply del Caribe Inc., Aguada, Puerto Rico). Diff-Quik is a modification of the Wright Stain technique. Cell smears were fixed by dipping the slides five times, one second each time, in fixative solution (1.8 mg/L Triarylmethane Dye in methylalcool) followed by five more dips in solution I (1 g/L Xanthene Dye buffered and sodium azide 0.01% as preservative) and five more dips in solution II (0.625 g/L Azure A and 0.625 g/L Methylene blue and buffer). The slides were then rinsed with deionized water and allow to dry.

Granule staining. Similar techniques have been previously described (9). Briefly, LAK cells were fixed with 2% paraformaldehyde in 0.1M cacodycate buffer for 1 h on ice. Cells were then washed 3 times with 0.1M TBS and permeabilized for 3 min. with 0.1% Triton X-100. Immediately after, the cells were washed 3 times with TBS/0.1% BSA and then incubated with the anti-granule mAb D519 (10) for 2 h at RT. After 3 washes with TBS/0.1% BSA, the cells were incubated with a goat anti-mouse antibody Biotin conjugated for 2 h at RT. Cells were washed as above and incubated with Fluorescein Avidin D for 2 h at RT. After 3 washes as above, cells were resuspended in glycerol and aliquots were

placed on slides and let sit for 30 min. before examination on a fluorescent microscope.

Phenotyping with monoclonal antibodies and flow cytometry. The cells were phenotyped on day 0, day 6, and on their last day in culture day 10, 11, or 12. The antibodies used in this study are described in Table V. The cells were treated with mAbs according to the following procedure: 1 x 10^6 PBL or LAK cells were labelled with 20 μl of mAb in a final volume of 200 μl of RPMI with 10% v/v HI FCS for 30 min. on ice. At the end of the incubation, the cells were washed once in medium and twice in PBS and finally resuspended and fixed in 1 ml of PBS containing 1% paraformaldehyde (Sigma). For the antibodies not conjugated with FITC or PE, the cells were washed 3 times with media after the first antibody and then incubated with 20μl of GAM-FITC in the same conditions as above until the final fixation. The proportion of fluorescent cells was determined using a Coulter EPICS V with an Argon ion laser of 500 mW output. Light scatter forward and orthogonal angles were used to select only viable cells. FITC single immunofluorescence was observed at 488nm whereas FITC double immunofluorescence was observed at 525nm with PE at 575nm. Each analysis was performed with a total count of 2 X 10^4 cells.

Cytotoxicity Assay. This assay was performed as previously described (11). Briefly, the NK-sensitive K562

TABLE V

Description of the Antibodies Used in this Study

CD	Name	Supplier	Major Reactivity
CD2	Leu-5b	B-D[1]	T cell
CD3	Leu4-FITC	B-D	T cell
CD4	OKT-4	ATCC (CRL8002)[2]	T cell subset
CD8	OKT-8	ATCC (CRL8014)	T cell subset
CD16	Leu11a-FITC	B-D	NK cells
CD56	Leu19-PE	B-D	NK cells, T cell subset
ND	TCR-τ1	DFCI[3]	$\tau\delta$-chains T cell receptor
--	Mouse IgG1-FITC	B-D	non specific control Ab
--	Mouse IgG1-PE	B-D	non specific control Ab
--	GAM(Fab^2)-FITC	TAGO[4]	mouse IgG
--	D519	--	LAK cell and platelet dense granules

1. Beckton-Dickinson, Mississauga, Ontario, Canada.

2. American Tissue Culture Collection.

3. Dana Farber Cancer Institute.

4. TAGO Immunologicals, Burlingame, CA.

and the NK-resistant Daudi targets were labelled with 100 μCi of ^{51}Cr for 45 min. at 37°C, washed three times, and 100 μl (1 x 10^4 cells) were incubated with 100 μl of PBL or LAK cells at 20:1, 10:1, 5:1, 2.5:1, 1.25:1, and 0.68:1 effector to target ratio in flat bottom microplates. After 5 h. at 37°C the plates were centrifuged and 100 μl of supernatant were removed and counted in a gamma counter. Three replicates were used for each experimental group, and the percent specific lysis was calculated as 100 X [(Exp.(%) - mean (%) of spont. rel.) / (100 - mean (%) of spont. rel.)]. Spontaneous ^{51}Cr release always ranged between 5-10% after 5 h of incubation. Our LU/10^7 cells was defined on the basis of the number of effectors required to cause 30% lysis of 1 x 10^4 target cells.

RESULTS

Many different methods to generate LGL LAK cells have been described starting with either whole peripheral blood lymphocytes or partially purified LGL, and using different doses of IL-2 (1-6,12), variable concentrations of FCS, and occasionally, mitogens such as PHA or OKT3 antibody to trigger better proliferation of the cells in culture (8,13,14). As there was evidence that LAK cells could develop in a low dose of IL-2 of around 5-10 U/ml (3,14), we cultured PBL at 1 x 10^6 cells/ml in RPMI 10% HI FCS and 10 U/ml of IL-2 for 6 days. At day 6, the cell concentration usually ranged from 0.9-1.1 x 10^6 cells/ml with a morphology still close to the small lymphocytes although the cells had usually developed a somewhat more abundant cytoplasm (Figure 6). Non adherent cells were removed, centrifuged then were plated at 0.25-0.3 x 10^6 cells/ml in fresh media. For the second period of culture, we tried 5, 10, and 15% of HI FCS. The cells did not proliferate in 5% FCS but did well in 10 and 15%, therefore we chose 10% FCS as a standard procedure. The presence of PHA made no difference to the IL-2 response of either a 10 or 50 U/ml dose, and both IL-2 doses on their own produced identical results in terms of cell recovery. We therefore standardized this second 4-6 days in culture using 10 U/ml of IL-2. Using this two step culture method and starting with 5 x 10^8 PBL at day 0, we were able to

obtain from 2-2.2 x 10^9 LGL after 10-12 days. The major expansion of the cells occurred in the second 4-6 days in culture. At harvesting, the cells appeared to be a homogeneous population of large granular lymphocytes (Figure 6). Subsequent study on these large cells revealed that at least 80% of them were specifically stained after permeabilization, with an anti-cytoplasmic granule MoAB (D519) confirming them as large granular lymphocytes (Figure 7).

As we were interested in fully characterizing the phenotype and the cytotoxic activity of the cells generated in these particular conditions, we proceeded to study their phenotype and cytotoxic activity at day 0, 6, and 10-12, depending on when they had reached 3 to 5 fold more than their starting concentration at day 6. Phenotyping as a function of time revealed a final population which was essentially $CD3^+, CD16^-$. Starting with 62-68% of $CD3^+$, we obtained an increase of 10-20% at day 6 eventually reaching up to 94% at the end of the experiment. Over the same period of time, the $CD16^+$ population showed continuous regression falling from 10-16% to as low as 0.9% at the end of the culture (Table VI and VII). Cytotoxic activity at day 0 was relatively low against the NK-sensitive K562 cells and no non MHC-restricted killing was detected against the NK-resistant Daudi cells (Table VI and VII). At day 6, full non MHC-restricted LAK cytotoxic activity against the Daudi cells as well as the K562 cells was seen and this LAK

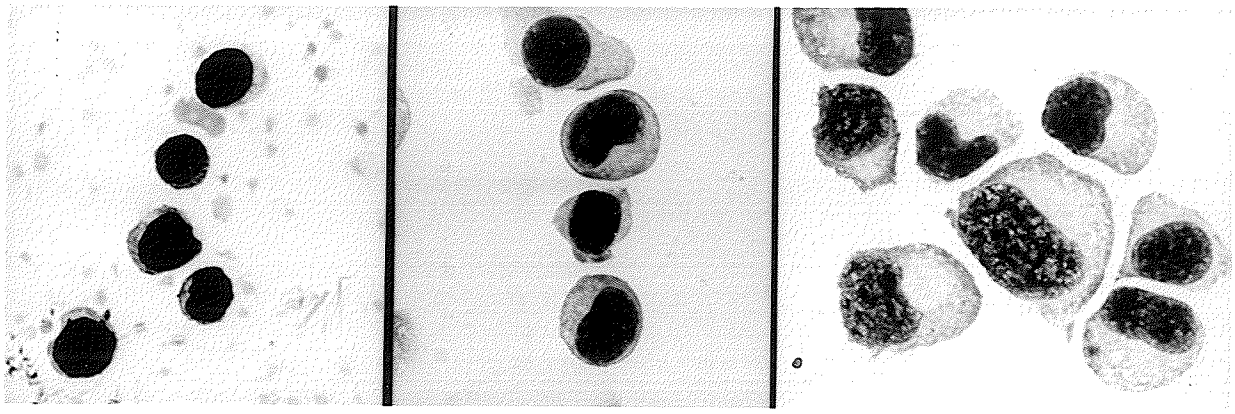

Figure 6. Morphology of the evolving LAK cell population at day 0 (left), 6 (center), and 12 (right). Cell smears were made and stained from PBL in culture at day 0, 6, and 12 as described in Material and Methods. Photographs were taken with 1000 X magnification.

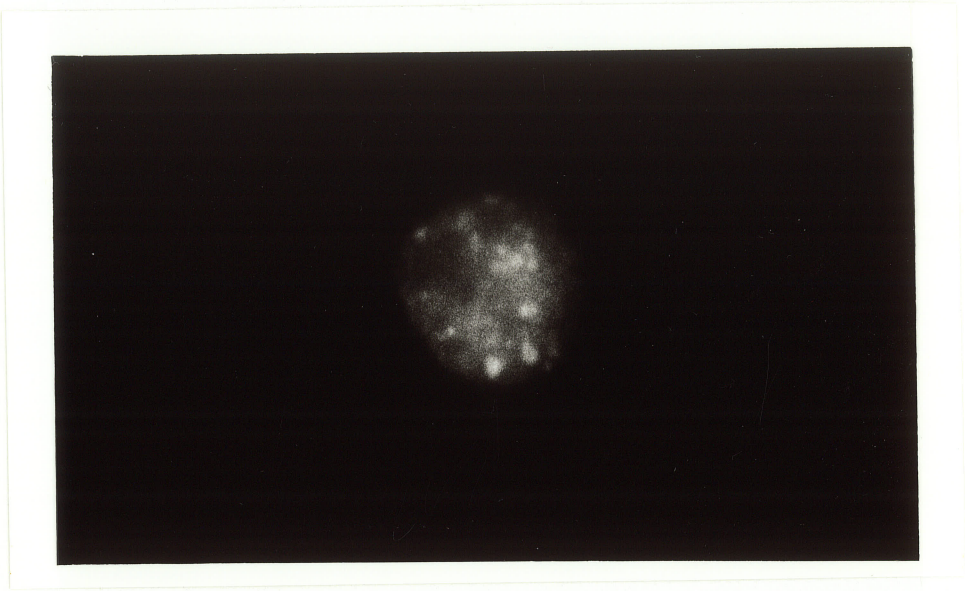

Figure 7. Fluorescent stained granules of LGL $CD3^+$ LAK cells at day 12. LAK cells were permeabilized then stained with mAb anti-granule D519 as described in Materials and Methods. The photograph was taken with 1000 X magnification.

TABLE VI

CD3,CD16 Phenotyping and LU/10^7 cells of the Evolving LAK Cell Population[a]

Day	0	6	10-12
Expt A:			
CD3	68.1	68.5	83.3
CD16	10.7	6.6	4.9
LU K562	ND	269	484
LU Daudi	ND	266	498
Expt B:			
CD3	62.1	81.2	93.4
CD16	11.4	5.5	2.7
LU K562	0.1	438	85
LU Daudi	0	283	15
Expt C:			
CD3	65.3	79.8	94.2
CD16	16.1	6.4	1.4
LU K562	4.0	441	249
LU Daudi	0	358	285

[a]Peripheral blood lymphocytes of three different donors were stained with the monoclonals anti-CD3 and anti-CD16 at day 0, 6, and at day 10-12 the last day of culture as described in Material and Methods. The results are percentage of positive cells after flow cytometry analysis. The cells were also assayed as effectors in a NK assay with K 562 or Daudi as target cells as described in Material and Methods. LU/10^7 cells were calculated by computer on the basis of the number of effectors required to cause 30% lysis of 1 X 10^4 target cells.

activity was still present at the end of the second period of culture where the final population was almost totally $CD3^+$. These studies of the cell phenotype and LAK activity as a function of time were repeated 4 times with 3 different donors and gave very similar results with a final population of LAK cells of 90 ± 5% $CD3^+$, displaying strong non MHC-restricted killing activity (Table VI and VII).

We subsequently asked the question if this $CD3^+$ LAK cell population with a LGL morphology was bearing the CD56 antigen. Two color fluorescence analysis of the evolving cell population with anti-CD3 and anti-CD56 revealed that our final LAK population was $CD3^+$,$CD56^-$ and suggest that its precursor was also $CD3^+$,$CD56^-$ as the other subsets $CD3^+$, $CD56^+$ and $CD3^-$ cells showed almost complete regression at the end of the cultures (Table VII).

As other studies on LAK cells had reported a $CD3^-$,$CD16^+$ phenotype and had used a high dose of IL-2 from 500-1000 U/ml (5,6), we repeated our study examining the effect of high versus low IL-2 doses with the 2 step culture method using either 500 U/ml or 10 U/ml. No difference was seen in the percentage of $CD3^+$ LAK cells recovered with either dose throughout the study (Table VII). However, higher cytotoxicity of LAK cells grown in presence of 500 U/ml of IL-2 was noticed suggesting that the higher dose might accelerate the development of the non MHC-restricted killing in those $CD3^+$ LAK cells.

TABLE VII

Phenotyping and LU/10^7 Cells of the Evolving LAK Cell Population as a Function of the IL-2 Concentration[a]

IL-2	--	10 U/ml		500 U/ml	
Day	0	6	11	6	11
$CD3^+$ $CD56^-$	62.1	72.0	93.0	73.5	94.2
$CD3^+$ $CD56^+$	4.1	5.3	1.5	5.2	2.4
$CD3^-$ $CD56^+$	12.0	7.3	3.3	5.5	1.9
$CD3^-$ $CD56^-$	21.8	12.0	2.3	9.4	1.5
$CD16^+$	16.1	6.4	1.4	2.4	0.9
LU K562	4.0	441	249	422	369
LU Daudi	0	358	285	512	445

[a] The PBL were cultured in a two step method as described in Material and Methods for 11 days with either 10 or 500 U/ml of IL-2. Phenotyping and NK assay were done at day 0, 6, and 11. The cell population was phenotyped using two color immunofluorescence and analyzed by flow cytometry. Results are percentage of positive cells and LU/10^7 LAK cells.

Finally, we investigated what type of T cell subsets constituted this LAK cell population. Using anti-CD4, anti-CD8, and a monoclonal anti-T cell receptor $\gamma\delta$ chain antibody, we found that this resulting LAK cell population was predominantly a mixture of $CD4^+$ (60%) and $CD8^+$ (30%) cells, with a small fraction of (<9%) $\gamma\delta^+$ cells (Table VIII).

TABLE VIII

Phenotyping as a function of Time of the T Cell Populations[a]

Day	0	6	10
$CD2^+$	ND[b]	ND	90.3
$CD3^+$	54.0	80.0	86.0
$CD4^+$	29.0	49.0	59.3
$CD8^+$	25.0	35.0	30.3
$\tau\delta^+$	2.5	10.0	8.8

[a] The PBL were cultured and phenotyped as described in Material and Methods. Numbers are percentage of positive cells.
[b] Not Done.

DISCUSSION

This study shows for the first time that human PBL long-term cultured with IL-2 generate a virtually pure $CD3^+$ LAK cell population. These $CD3^+$,$CD16^-$,$CD56^-$cells acquired strong non MHC-restricted killing activity (>200 LU/10^7 cells) as well as a LGL morphology and are therefore, within the definition of a LAK cell. This work relies on a time-function study of the phenotype and LAK activity which suggest that the peripheral blood $CD3^+$ T cells proliferated and developed into LAK cells. We found that the two step culture could generate predominantly $CD3^+$ or $CD16^+$ LAK cells depending upon whether the initial mitogenic signal was rhIL-2 or co-incubation with the previously irradiated EBV transformed RPMI 8866 B cell line. Cultures supplied only with IL-2 developed into an expanded population of almost exclusively $CD3^+$ cells, where the $CD16^+$ cells continuously regressed until their virtual disappearance. The two possible phenotypes responsable for LAK activity in these cultures were the $CD3^+$ and the $CD16^+$ cells. The $CD16^+$ cells were effectively removed from the $CD3^+$ cells by the culture conditions with an efficiency superior or similar to that obtained with antibody and complement or with a FACS sorting. Thus, we concluded that the LAK activity displayed at the end of these cultures was due to the $CD3^+$ cells. This finding of $CD3^+$,$CD16^-$ LAK activity from cultured PBL

essentially agrees with an early report by Grimm et al. (1) who also identified the LAK effector as a $CD3^+$ cell. However, this conflicts with later reports (5,6,7) that LAK effector cells from PBL were predominantly $CD16^+,CD3^-$ cells. The disagreement between these reports may be a result of the proliferation of the $CD3^+$ cells during the longer culture conditions. In the reports where $CD16^+,CD3^-$ cells were the LAK effectors, the cells were tested for LAK activity after short-term culture (usually 2-4 days) in presence of IL-2 (5,6,7) and no proliferative activity was noted. We also did not find evidence of proliferation before day 5 or 6 of experiments. In other work by Perussia et al. (14) where the proliferation of peripheral blood mononuclear cells was initiated by incubation in presence of irradiated B lymphoblastoid cell lines, the proliferation of NK cells started only at day 6. Therefore, in reports where short-term cultures were used, it appears that the NK $CD16^+$ cells rapidly developed strong LAK activity in presence of 500-1000 U/ml of IL-2 (even within one day), whereas the LAK activity generated from $CD3^+$ cells exposed to IL-2 for the same amount of time was very low. Additional evidence supporting the idea of rapid development of LAK activity by NK cells without proliferation comes from a recent report in which mouse spleen cells were shown to acquire LAK activity within 18 hours, independent of DNA synthesis (16). In contrast to $CD16^+$ LAK cells, proliferation of PBL in the

presence of IL-2 is required to generate $CD3^+$ LAK cells. This was suggested by both Grimm et al.(1) and by our experiments where the PBL expanded 3-5 fold in the second 5-6 days in culture. Ochoa et al. (8) found that $CD3^+$ LAK effector cells develop in long-term cultures with proliferation of PBL in presence of IL-2 and mitogenic OKT3 antibody. These workers also detected the expansion of $CD16^+$ cells, whereas almost complete regression of the $CD16^+$ cells occurred in our experiments. This difference may be related to the use of a mitogen with IL-2. Using the same culture conditions described for the generation of the $CD3^+$ LAK cells, we have been able to stimulate the proliferation of $CD16^+$ cells in long-term cultures by co-incubating the PBL in step one with previously irradiated EBV transformed cell line RPMI 8866 at a ratio of 30:1 and subsequently cultured the resulting non adherent cells for 3-4 days in presence of 10 U/ml of rhIL-2. These cultures yielded up to 75% $CD16^+$ cells and 25% $CD3^+$ cells (Roussel and Greenberg, unpublished data). Similar preferential induction of $CD16^+$ cells with PBL cultured in presence of B lymphoblastoid cell lines was obtained by Perussia et al. (15) who reported that IL-2 alone was not sufficient to induce substantial NK proliferation. This is further supported by previous work suggesting that IL-2 and a second stimulus was necessary to maintain NK cell to proliferate in culture (17,18).

Taken together, these studies on LAK cells from short

and long-term cultures suggest that NK $CD16^+$ cells acquire LAK activity in presence of a high dose of IL-2 in a short period of time (within 1 to 4 days) without proliferation. IL-2 alone is sufficient to induce proliferation of the $CD3^+$ LAK cell precursors from cultured PBL. However, LAK activity mediated by the $CD3^+$,$CD16^-$ cells requires a longer time ($\geq$ 10 days) in culture to develop, and such proliferation occurs as well with either a low or a high dose of IL-2. Accordingly, LAK activity at day 6 of our cultures is likely due to a mixed population of surviving $CD16^+$ LAK cells and some $CD3^+$ LAK cells that have already proliferated. The LAK activity at day 10-12 comes from the $CD3^+$ cells which constituted close to 95% of the total population while the $CD16^+$ cells were effectively eliminated by the culture conditions. The slight decrease in LU observed between day 6 and day 10-12 in 3 out of 4 of our experiments is explained by the regression of the $CD16^+$ LAK cells within the maturing $CD3^+$ LAK cell population. The observation that LAK LU appeared to increase with time as the LAK cells were harvested at day 10,11 or 12 also supports this idea, as the disappearing $CD16^+$ LAK are replaced with more mature $CD3^+$ LAK cells. The phenotyping of the resulting $CD3^+$ T cell population with anti-CD4, anti-CD8 and anti-$\tau\delta$ chains of the T cell receptor indicated that these cells were essentially a mixture of $CD3^+$,$CD4^+$ and $CD3^+$,$CD8^+$ cells with a small minority of $CD3^+$,$\tau\delta^+$ cells. Non MHC-restricted

LAK activity developed by T cell subset $CD3^+,CD4^+$ or $CD3^+,CD8^+$ cloned or isolated after long-term culture with IL-2 have also been observed by other groups (8,19,20,21). The contribution by the $CD3^+,\tau\delta^+$ cells to the LAK activity in our cultures is also very probable as these cells were shown to be able to mediate non MHC-restricted activity (22,23). It is necessary by definition that the proliferating T cells become LAK cells by developing a mechanism of target recognition other than the classical CD3 $\alpha\beta$ chains T cell receptor responsible for MHC restriction. Therefore, it has been suggested that the CD2 surface antigen which is involved in T cell target binding (24,25) could serve in conjunction with the Zeta-chain of the TCR as a non MHC-restricted recognition mechanism for $CD3^+$ T cells (26). Accordingly, we noticed in our experiments that over 90% of the final population also expressed the CD2 T cell determinant (Table VIII). Interestingly, a recent study on LAK cells induced by <u>in vivo</u> therapy of four repetitive weekly cycles of 4 days with low dose of IL-2 (concentration of 30 U/ml serum) showed that the predominant LAK effectors were $CD2^+,CD16^-$ cells (27).

In conclusion, there is growing evidence indicating that LAK cell effectors from long-term stimulated human PBL with IL-2 are predominantly a T cell population $CD3^+,CD2^+,CD16^-$, whereas early LAK cells that develop in PBL after short-term stimulation with IL-2 are $CD16^+$ NK cells.

ACKNOWLEDGMENTS

We would like to thank Dr. Edward Rector for his expertise in performing the flow cytometry analysis. We are grateful to Dr. John Wilkins and Lisa Selin for their help and advice in performing some of these experiments. We also thank Eileen McMillan for her help with granule staining.

REFERENCES

1. **Grimm, E.A., A. Mazumder, H.Z. Zhang, and S.A. Rosenberg.** 1982. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2 activated autologous human peripheral blood lymphocytes. J. Exp. Med. 155:1823.

2. **Grimm, E.A., K.M. Ramsey, A. Mazumder, D.J. Wilson, J.Y. Djeu, and S.A. Rosenberg.** 1983. Lymphokine-activated killer cell phenomenon. II. Precursor phenotype is serologically distinct from peripheral T lymphocytes, memory cytotoxic thymus-derived lymphocytes, and natural killer cells. J. Exp. Med. 157:884.

3. **Grimm, E.A., R.J. Robb, J.A. Roth, L.M. Neckers, L.B. Lachman, D.J. Wilson, and S.A. Rosenberg.** 1983. Lymphokine-activated killer cell phenomenon. III. Evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer cells. J. Exp. Med. 158:1356.

4. **Trinchieri, G., M. Matsumoto-Kobayashi, S.C. Clark, J. Seehra, L. London, and B. Perussia.** 1984. Response of resting human peripheral blood natural killer cells to interleukin 2. J. Exp. Med. 160:1147.

5. **Phillips, J.H., and L.L. Lanier.** 1986. Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. J. Exp. Med. 164:814.

6. **Ortaldo, J.R., A. Mason, and R. Overton.** 1986. Lymphokine-activated killer cells. Analysis of progenitors and effectors. J. Exp. Med. 164:1193.

7. **Herberman, R.B., Hiserodt, J., Vujanovic, N., Bach, C., Lotzova, E., Bolhuis, R., Golub, S., Lanier, L.L., Phillips, J.H., Riccardi, C., Ritz, J., Santoni, A., Schmidt, R.E., Uchida A.** 1987. Lymphokine-activated killer cell activity. Characteristics of effector cells and their progenitors in blood and spleen. Immunol. Today 8:178.

8. **Ochoa, A.C., D.E. Hasz, R. Rezonzew, P.M. Anderson, and F.H. Bach.** 1989. Lymphokine-activated killer activity in long-term cultures with anti-CD3 plus Interleukin 2: Identification and isolation of effector subsets. Cancer Res. 49:963.

9. **Wencel-Drake, J.D., Plow, E.F., Zimmerman, T.S., Painter, R.G., Ginsbey, M.H.** 1984. Immunofluorescent localization of adhesive glycoproteins in resting and thrombin-stimulated platelets. Amer. J. Pathol. 115:156.

10. **Gerrard, J.M., Lint, D., Sims, P.J., Wiedmer, T., Fugate, B., Sudhof,T., McMillan, E., and S. Israels.** Identification of a platelet dense granules membrane protein which is deficient in a patient with Hermansky-Pudlak syndrome. Blood: (In press).

11. **Pohajdak, B., Gomez, J.L., and A.H. Greenberg.** 1984. Tumor-activated NK cells trigger monocyte oxidative metabolism. J. Immunol. 133:2430.

12. **Lowrey, D.M., A. Hameed, M. Lichtenheld, and E.R. Podack.** 1988. Isolation and characterization of cytotoxic granules from human lymphokine (Interleukin 2) activated killer cells. Cancer Res. 48:4861.

13. **Zalman, L.S., M.A. Brothers, F. John Chiu, and Hans J. Müller-Eberhard.** 1986. Mechanism of cytotoxicity of human large granular lymphocytes: Relationship of the cytotoxic lymphocyte protein to the ninth component(C9) of human complement. Proc. Natl. Acad. Sci. USA 83:5262.

14. **Zalman, L.S., M.A. Brothers, and H. J. Müller-Eberhard.** 1988. Self-protection of cytotoxic lymphocytes: A Soluble form of homologous restriction factor in cytoplasmic granules. Proc. Natl. Acad. Sci. USA 85:4827.

15. **Perussia, B., Ramoni, C., Anegon, I., Cuturi, M.C., Faust, J., and G. Trinchieri.** 1987. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. Nat. Immun. Cell Growth Regul. 6:171.

16. **Punturieri, A., F. Velotti, M. Piccoli, R.B. Heberman, L. Frati, and A. Santoni.** 1989. Overnight incubation of mouse spleen cells in recombinant IL-2 generates cytotoxic cells with NK characteristics from precursors enriched with or devoid of LGL. Clin. exp. Immunol. 75:155.

17. **Olabuenaga, S.E., C.G. Brooks, S. Gillis, and C.S. Henney.** 1983. Interleukin 2 is not sufficient for the continuous growth of cloned NK-like cytotoxic cell lines. J. Immunol. 131:2386.

18. **Hercend, T., E.L. Reinherz, S.C. Meuer, S.F. Schlossman, and J. Ritz.** 1983. Phenotypic and functional heterogeneity of human cloned natural killer cell lines. Nature (Lond.), 301:158.

19. **Irle, C., Beatty, P.G., Mickelson, E.M., and J.A. Hansen.** 1984. Change in functional phenotype of clone human alloreactive cytolytic T cells. Human Immunol. 11:183.

20. **Pawelec, G., Kahle, P., and P. Wernet.** 1982. Specificity spectrum and cell surface markers of mono- and multi-functional mixed leukocyte culture-derived T cell clones in man. Eur. J. Immunol. 12:607.

21. **Patel, S.S., Thiele, D.L., and P.E. Lipsky.** 1987. Major histocompatibility complex-unrestricted cytolytic activity of human T cells. Analysis of precursor frequency and effector phenotype. J. Immunol. 139:3886.

22. **Biassoni, R., S. Ferrini, I. Prigione, A. Moretta and E.O. Long.** 1988. CD3-negative lymphokine-activated cytotoxic cells express the CD3ϵ gene.
J. Immunol. 140:1685.

23. **Biassoni, R., S. Ferrini, I. Prigione, R.P. Sekaly and E.O. Long.** 1989. CD3-negative lymphokine-activated cytotoxic cells express the CD3ϵ and TCRδ genes. In Natural Killer Cells and Host Defense. 5th Int Natural Killer Cell Workshop, Hilton Head SC, 1988. E.W. Ades, and C. Lopez, eds. Karger, Basel, Switzerland, pp. 9-11.

24. **Anderson, P., Caligiuri, M., Ritz, J., and S.F. Schlossman.** 1989. CD3-negative natural killer cells express Zeta TCR as part of a novel molecular complex. Nature 341:159.

25. **Shaw, S., and G.E. Ginther Luce.** 1987. The lymphocyte function-associated antigen (LFA)-1 and CD2/LFA-3 pathways of antigen-indenpendent human T cell adhesion.
J. Immunol. 139:1037.

26. **Scott., C.F.Jr., Lambert, J.M., Kalish, R.S., Morimoto, C. and S.F. Schlossman.** 1988. Human T cells can be directed to lyse tumor targets through the alternative activation/T11-E Rosette receptor pathway.
J. Immunol. 140:8.

27. **Weil-Hillman, G., P. Fisch, A.F. Prieve, A.J. Sosman, J.A. Hank and P.M. Sondel.** 1989. Lymphokine-activated killer activity induced by in vivo interleukin 2 therapy: Predominant role for lymphocytes with increased expression of CD2 and Leu19 antigens but negative expression of CD16 antigens. Cancer Res. 49:3680.

CHAPTER IV

GRANULES OF HUMAN $CD3^+$ LARGE GANULAR LYMPHOCYTES CONTAIN A MACROPHAGE REGULATING FACTOR(S) THAT INDUCES MACROPHAGE H_2O_2 PRODUCTION AND TUMORICIDAL ACTIVITY BUT DECREASES CELL SURFACE Ia ANTIGEN EXPRESSION

This chapter is the integral version of an article published by Eugene Roussel and Arnold H. Greenberg in **Cellular Immunology (1991): (In Press).**

CHAPTER PRESENTATION

In this chapter we tested normal human LGL granules for a macrophage activating factor. We purified normal human LGL cytoplasmic granules according to the method and principle described in the chapter II using the human LGL $CD3^+$ population of cells characterized in chapter III. Then, we performed three different lines of biological assays related to different macrophage functions. The results constitute significant evidence to suggest the presence of a macrophage regulating factor(s) in human LGL granules.

ABSTRACT

CTL and LGL exocytose cytoplasmic granules on activation after recognition of their target, releasing granule associated molecules. We have previously suggested that this process could release immunoregulatory molecules. In this study we investigated if normal human LGL granules contained a factor regulating different macrophage activity. Human $CD3^+$ LGL cells were generated by activating PBL for 10-12 days with rhIL-2 and granules were isolated from disrupted cell homogenate by Percoll gradient fractionation. Solubilized granules were tested for macrophage activating factor activity in three different macrophage assays. When M-CSF differentiated murine bone marrow-derived macrophages were incubated 9 h with human LGL granules, they were fully activated to lyse the TNF-resistant P815 tumor cells. The granule-MAF showed a synergistic effect with rhIL-1ß, rmTNF-α, and rmIFN-τ in the cytolytic assay. In addition, proteose-peptone elicited murine peritoneal macrophages profoundly increased H_2O_2 production after activation with human LGL granules. However, unlike IFN-τ, no increase in peritoneal macrophage Ia antigen expression was detected after incubation with granules. Moreover, granule-MAF suppressed Ia induction by IFN-τ. These results confirm that human $CD3^+$ LGL granules contain a molecule(s) capable of regulating macrophage function.

INTRODUCTION

Human peripheral blood T cells cultured in the presence of IL-2 for 10 days and more will differentiate into $CD3^+$ cytotoxic T lymphocytes with non MHC restricted killing and are one of the cell populations with LAK (lymphokine activated killer) activities (1,2,3). During their maturation, the T cells enlarge and acquire numerous cytoplasmic granules with the morphology of large granular lymphocytes similar to the natural killer (NK) cells (1,4,5). When these CTL or LGL execute their cytolytic functions, they exocytose cytoplasmic granules (6,7). It is generally accepted that granule exocytosis is an important mechanism of lethal hit delivery in some cytotoxic cells (7,8). In this degranulating process, many molecules such as cytolysin /perforin (9,10), chondroitin sulfate proteoglycans (11,12) and serine esterases (13,14) have been shown to be released indicating that the granules contain and release a complex mixture of molecules. In our laboratory, we have investigated whether immunoregulatory functions could also be exerted through this granule exocytosis process. This idea was based upon the observation that following contact with NK-sensitive tumor cells, LGL released a soluble factor which stimulated monocyte oxidative metabolism (15) and intracellular lysis of microorganisms by alveolar macrophages (16).

The rapidity of the release implied that the factor was preformed and we hypothesized that it might be stored in cytoplasmic granules. In following work, we identified a factor in granules of RNK rat LGL leukemia cell lines activating macrophage tumor lysis (17). In this study we present evidences that normal human $CD3^+$ LGL cytoplasmic granules contain a regulating factor able to control macrophage function.

MATERIALS AND METHODS

Generation of human LGL. Human buffy coat from normal donors were obtained through the Canadian Red Cross. The buffy coat cell population was diluted by half with phenol-red free HBSS (GIBCO) containing 100 U/ml of heparin (Sigma, St. Louis, MO), pH adjusted to 7.2. The diluted cells were layered on Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged at 900g for 20 min.. The mononuclear band was removed and washed with HBSS containing 100 U/ml of heparin by centrifugation at 400g for 15 min.. The pellet was resuspended in HBSS without heparin and centrifuged at 100g for 8 min. to remove most of the remaining platelets. The resulting mononuclear cells were resuspended at 1 x 10^6 cells/ml in RPMI 1640 containing sodium bicarbonate (0.2%), penicillin/streptomycin (50 IU/ml and 50 μg/ml, respectively), 10% v/v heat inactivated FCS (56°C, 30 min.), and newly supplemented with 2 mM glutamine. As a first step, the cells were incubated in 175 cm^2 tissue culture flasks (NUNC, Kamstrup, Denmark) at 100-150 x 10^6 cells per flask in presence of 10 U/ml of rhIL-2 (AMGEN Biologicals, Thousand Oaks, CA) for 6 days at 37°C, 95% humidity, 5% CO_2. At day 6, the cells were centrifuged at 100g for 10 min. and resuspended at 0.25 x 10^6 cells/ml in fresh media containing 10 U/ml of IL-2. As a second step, the cells were incubated in new 175 cm^2 flasks at 150 ml of volume per flask for

another 5-6 days until they had reached a concentration of 1-1.2 x 10^6 cells/ml.

Isolation of LGL cytoplasmic granules. The procedure for purification of cytoplasmic granules was described in an earlier publication (17), and was carried out under sterile conditions. Materials were gas sterilized in ethylene oxide and solutions were sterilized by filtration through 0.22 μm filters (Nalgene, Rochester, NY). In brief, 2 x 10^9 cells were washed in HBSS and resuspended at 1 x 10^8/ml in disruption buffer (0.25M sucrose, 0.01 M Hepes, 4mM EGTA, 100 U/ml heparin (Sigma) ph 7.4). They were lysed by decompression at 0°C after equilibrating at 450 psi nitrogen for 20 min.. After the addition of $MgCl_2$ to 5 mM, the homogenate was digested with DNAse I (from bovine pancreas, type IV, Sigma) at 800 U/ml, 22°C, 25 min.. Nuclei were removed by filtration through Nucleopore filters (Nucleopore, Pleasanton, CA) of 5 and 3 μm and the resulting homogenate was cooled to 0°C. Five ml aliquots were layered on 20 ml of 48% Percoll (in disruption buffer without heparin) and centrifuged in a 70 Ti rotor at 20,000 rpm for 10 min., deceleration without braking using a Beckman L8-M ultra-centrifuge (Beckman, Fullerton, CA). The resulting gradient was fractionated from the bottom by careful insertion of a stainless steel tube from the top and the first 5 ml high density Percoll fraction was removed via attached polyethylene tubing. Granules has been shown to be

present in the high density Percoll fractions on the basis of granule markers such as ß-glucuronidase and cytolysin and confirmed by electron microscopy (18). To eliminate the Percoll, the granule fractions were centrifuged at 35,000 rpm in 70.1 Ti rotor (Beckman L8-M ultracentrifuge) for 2 h and then the granules were removed and diluted to 1 x 10^9 cell equivalent/ml with sterile water, giving a concentration of 0.2 mg granule protein /ml with the Biorad protein assay, before being aliquoted and stored at -70°C. For the macrophage biological assays, the granules were quickly thawed at room temperature and then solubilized by dissolving 116 mg/ml of NaCl (99.999% pure, Aldrich, Milwaukee, WIS), for 2 h on ice. The 2 M NaCl solubilized granules were then partially diluted first in sterile water then with medium to restore isoosmolarity before use in the assays.

Macrophage tumoricidal assay.

Cultivation of bone marrow macrophages. For each experiment bone marrow cells were obtained from 3 C3H/HeN mice by flushing the marrow from the femur and tibia with a 26 1/2 gauge needle and RPMI 1640. Cells were washed and resuspended in NH_4Cl 0.83% pH 7.3 for 3 min. to lyse the erythrocytes. They were washed twice again and resuspended in 5 ml of culture media. Cells were counted and adjusted to 3.2 x 10^4 large nucleated cells/ml then plated at 8 x 10^5 cells (25 ml) per 20-100 mm plastic Petri-dishes not treated for tissue culture (Fisher), in RPMI 1640 containing sodium

bicarbonate (0.2%), penicillin/streptomycin (50 IU/ml and 50 μg/ml respectively) and newly supplemented with 2 mM Glutamine, 10% v/v FCS, 10% v/v horse serum (GIBCO) and 10% v/v of pre-titred L-929 conditioned medium as source of CSF-1. Petri plates were incubated for 6 days at 37°C, 7.5% CO_2 and 95% humidity. This method of bone marrow culture was previously shown to yield 98% pure macrophages after 5 days (19).

Activation of bone marrow-derived macrophages. At day 6, the differentiated marrow cells were harvested by incubation for 15 min. at room temperature in fresh PBS containing 0.5 mM disodium EDTA pH 7.4 and then gentle pipetting to help detach the macrophages. The cells were washed and resuspended in RPMI 10% FCS culture media at 1 x 10^6 big "foamy" cells/ml, as those cells are thought to be the fully differentiated macrophages (20). Aliquots of 10^5 macrophages per well were plated in 96 microwells flat bottom plate (NUNC, Kamstrup, Denmark) and incubated for 90 min.. Then media and non-adherent cells were removed and activating agents were added. In most experiments the macrophages were activated for 9 hours. One hundred μl of recombinant mouse IFN-τ 50 U/ml (Holland Biotechnology, Leiden, The Netherlands) in culture media containing 50 ng/ml of LPS (lipopolysaccharides from E. coli type B:4 or B:8, phenol extracted, Sigma) were used as positive controls. LGL granule preparations containing the MAF and

made isoosmolar as described above were used in sequential dilutions. In some experiments the solubilized LGL granules were diluted with media already containing the appropriate amounts of rhIL-1ß (a gift of Dr. D. Urdal, Immunex, Seattle), or rmTNF-α (a gift of Genentech, San Francisco), or rmIFN-τ (Holland Biotechnology).

After the activation period the wells were washed with culture medium and 200 μl of ^{131}IUdR-labelled P815 target cells at 5 x 10^4 cells/ml were added and incubated for 18-20 hours. The P815 target cells were labelled by incubating 2 x 10^5 cells/ml with 3 MBq/ml of ^{131}IUdR (Radiopharmaceutical Centre, Edmonton, Canada) for 4 h then washed 4 times. After the lytic period the plates were centrifuged at 600 g for 8 min. and 100 μl of the supernatant were harvested and counted in an LKB compugamma.

Experiments were conducted in triplicates and percent of specific ^{131}IUdR release was calculated by the formula:

$$\frac{\text{Experimental (\%) - mean of control (\%)}}{\text{100 - mean of control (\%)}} \times 100$$

Control values are % of ^{131}IUdR released from target cells incubated with non activated macrophages, referring to the total cpm contained in 200 μl of ^{131}IUdR labelled P815 target cells. Control levels varied from 8-22 %. Values presented are means $\pm$ SEM.

$\underline{H_2O_2 \text{ production.}}$ This assay measured H_2O_2 production by macrophages triggered with PMA, based on the loss of fluorescence by scopoletin when one mole of H_2O_2 oxidized one mole of scopoletin catalyzed with horseradish peroxidase. This method is described in detail elsewhere (21). Briefly, C3H/HeN mice were primed with one ml i.p. injection of 15% proteose-peptone (Fisher, Fair Lawn, NJ) dissolved in saline prepared immediately before use. After 4 days, mice were killed and the peritoneal macrophages were harvested using 4°C RPMI 1640 containing 2 mM EDTA. Cells were washed and resuspended at 1 x 10^6 macrophages/ml in RPMI 1640, 10% FCS. Macrophages were plated at 10^5 cells/well in 96 microwells flat bottom tissue culture plates (NUNC) and allowed to adhere for 90 min.. Then medium and non adherent cells were removed, the activating agents were added and the macrophages were incubated for 24 h at 37°C, 95% humidity, 5% CO_2. After this activation time, the adherent cells were washed twice with 37°C HBSS then incubated for 2 h at 37°C with 100 μl of a mixture containing 30 nanomoles/ml of scopoletin (Sigma), 2 μg/ml horseradish peroxidase (Sigma) and 30 ng/ml of PMA (Sigma). The plates were then read with a computer controlled fluorometer Fluoroskan II (Flow Laboratories, Mississauga, Ont.). Each sample performed in triplicates was read for 1 second and the value recorded in arbitrary units. Data were analyzed with Microsoft II (Flow Laboratories) to calculate the amount of H_2O_2 produced/well

according to a standard curve of H_2O_2 oxidized scopoletin ranging from 0-30 nanomoles of H_2O_2/ml. Results were expressed as mean $\pm$ SD nanomole of H_2O_2 produced/10^6 macrophages/2 h.

Ia antigen expression. Primed peritoneal macrophages were obtained from C3H/HeN mice exactly as described above in the H_2O_2 assay. The macrophages were resuspended in RPMI 1640, 10% FCS and plated at 1 x 10^6 cells/well in 24 wells tissue culture plates and adhered for 90 min.. Medium and non adherent cells were removed, the activating agents were added and the macrophages were incubated for 24 h at 37°C, 95% humidity, 5% CO_2. After the activation time, the cells were recovered by incubation for 10 min. at room temperature in PBS-EDTA 0.5 mM then gently detached using a sterile policeman. The macrophages were then washed in medium (RPMI 1640, 10% heat inactivated FCS) and ten μl of a mouse mAb anti-mouse Ia^k (Beckton-Dickinson, Mississauga, Ont.) was added to the pellet and the mixture incubated for 30 min. on ice. The cells were washed 3 times then 20 μl of a goat anti-mouse $F(ab)^2$ FITC-conjugated antibody (TAGO Immunologicals, Burlingame, CA) was added to the pellet. The mixture was incubated for 30 min. on ice then washed once with medium followed by 3 washes in PBS and finally resuspended and fixed in 1 ml of PBS containing 1% paraformaldehyde (Sigma). The proportion of fluorescent cells was determined using a Coulter EPICS V with an Argon ion laser of 500 mW output.

Light scatter forward and orthogonal angles were used to select only viable cells. FITC immunofluorescence was observed at 488nm. Each analysis was performed with a total count of 2 X 10^4 cells.

RESULTS

Isolation of human LGL cytoplasmic granules.

Large numbers of human LGL were required to obtain sufficient granule protein for analysis. These were generated (>2 x 10^9 cells) by incubating PBL in long-term culture (10-12 days) in a two step method with 10 U/ml of rhIL-2 (1). Characterization of the cells at the end of the culture revealed that they were a virtually pure population of $CD3^+$,$CD16^-$·$CD56^-$ cells, LGL by morphology, contained multiple cytoplasmic granules and displayed strong non MHC-restricted cytolytic or LAK activity (1). Cytoplasmic granules were isolated from these human LGL LAK cells as described in the Method section.

Regulation of macrophage functions by an LGL granule factor.

The presence of a macrophage regulating factor in human LAK cell granules was assayed by either induction of murine macrophage tumoricidal activity, secretion of H_2O_2, or increase in cell surface Ia antigen expression.

Macrophage tumoricidal activity. Using conditions similar to those developed to detect granule-MAF in the RNK LGL leukemias (17), a 9 h activation with human LAK granules was sufficient to induce optimum macrophage cytotoxicity. The non solubilized granules produced little activity and often none at all, whereas optimum activity was obtained when the granules were first solubilized in 2M NaCl or KCl

for 2 h on ice immediately before use (Table IX). The solubilized human LAK granules were able to fully activate macrophage tumoricidal activity to lyse the TNF-resistant P815 tumor cells without addition of LPS, unlike IFN-τ which requires the presence of LPS (22,23,24). LGL granule-MAF activity was detectable at concentration of 2.2 μg/ml of granule protein with a rapid increase as the concentration was raised to 4.4 and 6.7 μg/ml. These experiments were repeated 8 times with 3 different LAK granule preparations giving similar results to these shown in Figure 8. Since it has been shown that the cytokines rmTNF-α, rhIL-1β, and rmIFN-τ interact to promote murine macrophage activation (25,26), we tested the ability of these lymphokines to synergize with the human granule-MAF. Using a concentration of 4.4 μg granule protein/ml, which produced suboptimal levels of macrophage cytotoxicity, we observed a pronounced synergy with rhIL-1β, rmTNF-α, and rmIFN-τ at concentrations that were practically inactive on their own (Figure 9). These experiments were repeated 4 times, each time showing significant synergy between the granule-MAF and these particular lymphokines.

Macrophage H_2O_2 production. To determine whether the human LGL granule-MAF was able to promote other macrophage functions, we examined the ability of LAK granules to stimulate H_2O_2 secretion in mouse peritoneal macrophages primed <u>in vivo</u> with proteose-peptone. As previous studies

TABLE IX

LAK Granule-MAF Optimum Recovery from Solubilized Granules[a]

Granule protein concentration (μg/ml)	Percent specific lysis ± SEM				
	Expt 1			Expt 2	
	.15M NaCl	2M NaCl	2M KCl	.15M NaCl	2M NaCl
6.7	7 ± 2	27 ± 2	24 ± 3	0	37 ± 3
4.4	10 ± 2	17 ± 1	13 ± 1	0	19 ± 3
2.2	8 ± 2	10 ± 4	5 ± 1	0	3 ± 2

[a] Two different LAK granule preparations were assayed for induction of macrophage tumoricidal activity as described in Materials and Methods. Each experiment was performed comparing the non solubilized granules (.15M NaCl) with granules solubilized with 2M NaCl or KCl for 2 h on ice immediately before use.

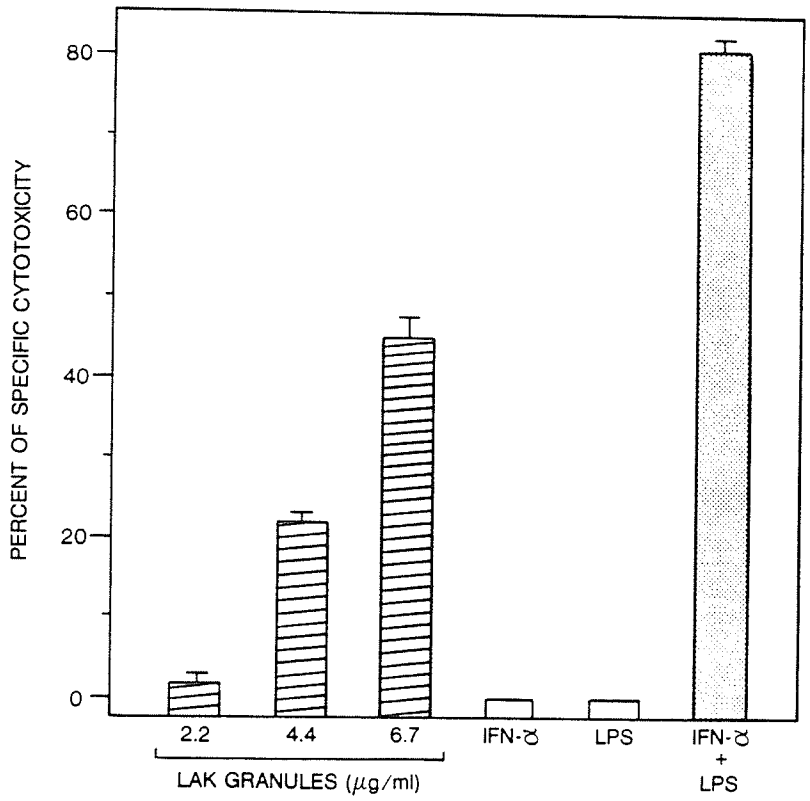

Figure 8. Tumoricidal activity of bone marrow-derived macrophages after activation with solubilized LAK granules. Bone marrow derived-macrophages were incubated for 9 h with the solubilized LAK granules, IFN-τ alone (50 U/ml), LPS alone (50 ng/ml), or IFN-τ + LPS as controls, then the macrophages were washed and assayed for lytic activity against ^{131}IUdR-labelled P815 tumor cells for 18h. This experiment was repeated 8 times with three different granule preparations.

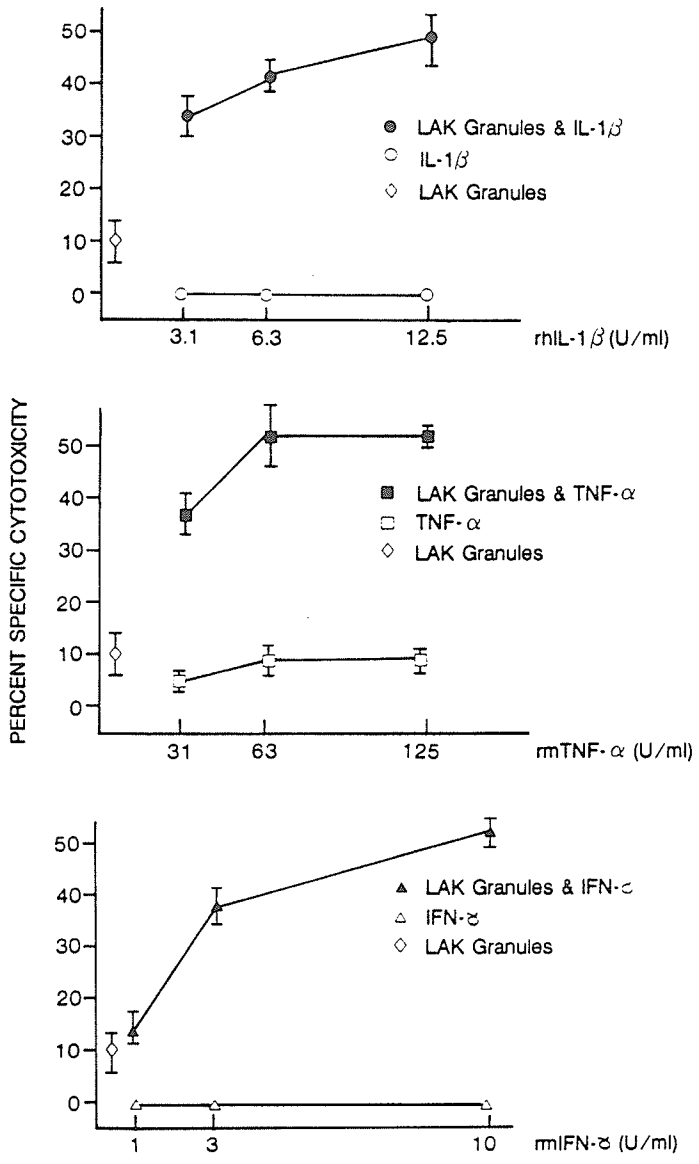

Figure 9. Synergy of human LAK granule-MAF with rhIL-1β, rmTNF-α, or rmIFN-τ. Solubilized LAK granules at 4.4 μg granule protein/ml were incubated with 3 different concentrations of either rhIL-1β, rmTNF-α, or rmIFN-τ for 9 h. The macrophages were then washed and assayed for lytic activity against ^{131}IUdR labelled P815 tumor cells for 18 h. This experiment was repeated 4 times with similar results.

used 24-72 h activation time to detect IFN-γ or TNF-α activation of H_2O_2 production (27,28), we assayed the solubilized human LAK granules over a similar time period. After 24 h the solubilized LAK granules induced a large increase in PMA triggered H_2O_2 secretion in a dose dependent manner (Figure 10). Using optimal concentration of 25-50 U/ml rmIFN-γ as positive controls, in several experiments we observed that granule preparations increased H_2O_2 production 2 to 3 times more than rmIFN-γ. Activation of macrophages for 48 h with the granule-MAF resulted in the deterioration of the cells in increasingly acidic medium suggesting that prolonged LAK granule-MAF stimulation <u>in vitro</u> was toxic for the macrophages.

Cell surface Ia antigen expression. In their role as accessory cells in the immune response, macrophages present antigen in the context of class II MHC molecules (Ia antigens) for T cell activation (29). An increase in cell surface Ia antigens expression by peritoneal macrophages represents a state of activation in which they are able to perform such a function (30). To determine whether the human LGL granule-MAF could regulate this macrophage function, we measured the cell surface Ia antigen expression of mouse peritoneal macrophages previously primed as above after 24 h of incubation with the solubilized LAK granules. Unlike rmIFN-γ, the human LGL granule-MAF decreased macrophage Ia expression in repeated experiments.

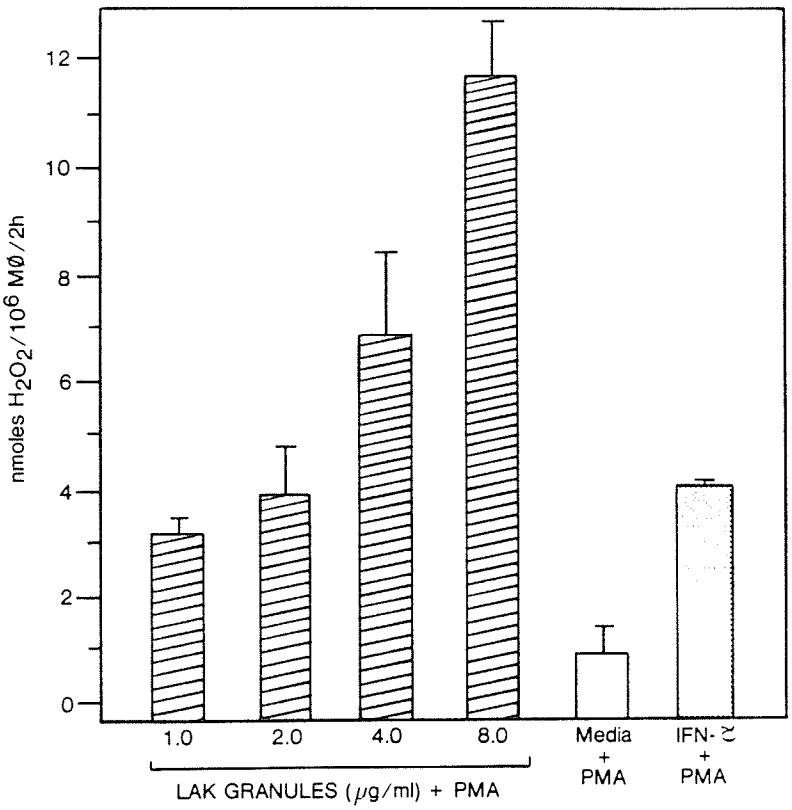

Figure 10. PMA triggering of H_2O_2 production after activation of peritoneal macrophages by human LAK granules. Mouse peritoneal macrophages were primed in vivo 4 days then incubated for 24 h in vitro with different concentrations of LAK solubilized granules, IFN-γ 25 U/ml, or media alone. At the end of the activation time, H_2O_2 production was triggered with PMA 30 ng/ml for 2 h and then measured with the scopoletin based assay as described in Materials and Methods. This experiment was repeated 4 times.

Moreover, coincubation of the solubilized human LGL granules with rmIFN-τ completely suppressed the IFN-τ effects on murine macrophage Ia expression, reducing the level below that seen with granule-MAF alone (Figure 11).

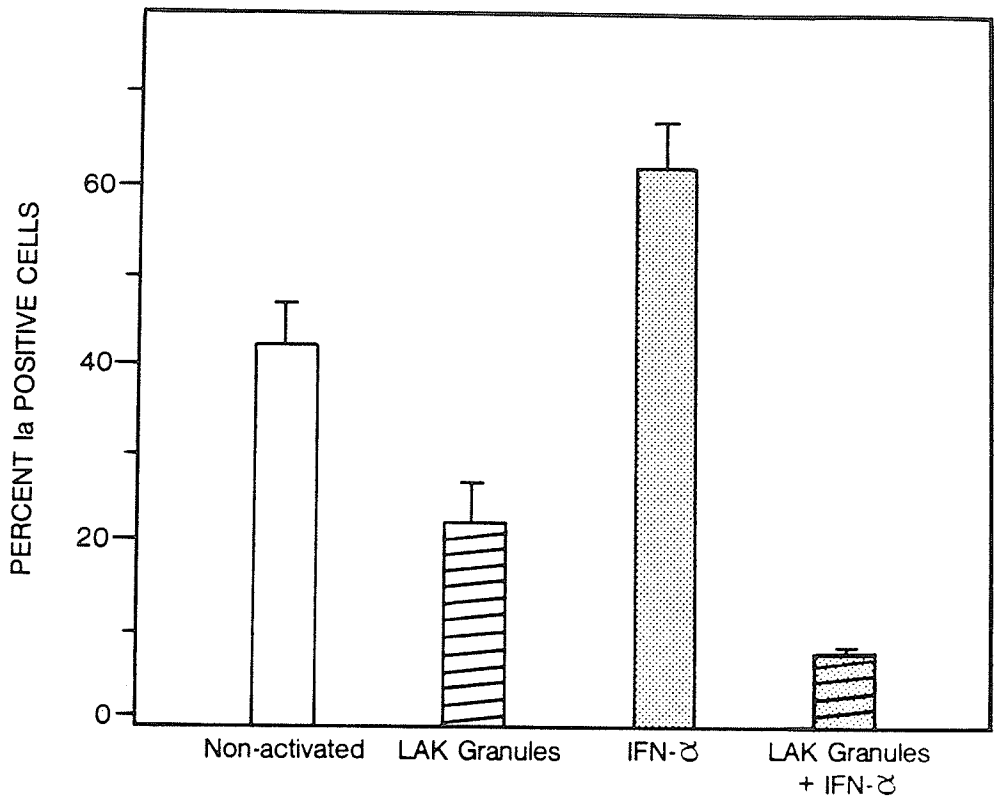

Figure 11. Percent peritoneal macrophages expressing Ia antigen after activation by LAK granule-MAF. Mouse peritoneal macrophages primed 4 days previously in vivo with proteose-peptone, were incubated 24 h with media alone, solubilized LAK granules (4.0 μg/ml), rmIFN-τ (50 U/ml), and rmIFN-τ with LAK granules. The macrophages were then detached and incubated with a mAb anti-Ia^k followed by goat anti-mouse Ig-FITC. Positive cells were detected by flow-cytometry analysis. Results shown are mean ± SEM percent of positive cells for two separate experiments.

DISCUSSION

This is the first report that presents evidence that human LGL granules contain a macrophage regulating factor. Macrophages can be activated to kill tumor target cells by two alternative pathways. One pathway involves two distinctive signals where a priming signal is necessary to render macrophages responsive to a triggering signal (22,24). The mechanism of killing used when macrophages are activated by this pathway is complex, the production of reactive oxygen intermediates (ROI) and H_2O_2 have been reported (31,32) and the production of nitric oxide from L-arginine acting alone and in synergy with TNF has recently been proposed (33,34). Cytotoxic factors released by monocytes that are distinct from TNF have also been reported (35). Macrophages activated by this two signals pathway lyse TNF-resistant as well as TNF-sensitive tumor cell lines (35). The other pathway activates macrophages through only one signal and the killing mechanism is mediated via production of macrophage-derived TNF (26,35, 36,37). Only TNF-sensitive tumor cells are lysed by this one signal pathway activated macrophages (35,36). Some lymphokines have been shown to activate macrophage tumoricidal activity through the one signal pathway, these include GM-CSF, IL-1, IL-4 and TNF (26,38,39). On the other side, IFN-τ has been well described as a priming signal in conjunction with

LPS or TNF as triggering signal in the 2 signal pathway macrophage activation (22,24,40). IL-2 is the only other lymphokine reported to induce lysis of TNF-resistant tumor cell by macrophages (33,41). The human solubilized granules showed unequivocally the ability to induce macrophage killing of the P815 tumor cells (Figure 8), commonly used as a TNF resistant target (40,42). This result indicated that human granules contain a factor able to activate the priming and triggering of the two signal macrophage tumoricidal pathway.

It is unlikely that LPS contamination in our granule preparations could account for these observations for the following reasons. All granules were prepared under sterile conditions. The Limulus Amebocyte Lysate assay for LPS in granule preparations have been negative. The human factor in LGL granules was active only after solubilization in high salt, suggesting that it was bound to a granule matrix that was dissociated by the high ionic strength buffer. An LPS contaminant should be soluble in 0.15M NaCl. The MAF biological activity was heat sensitive (100 C°,5 min) whereas LPS is known to be heat resistant (42). The size of the MAF (10-30 kD) determined by exclusion from Amicon ultrafiltration membrane was smaller than the high M.W. complex of hundred kD formed by LPS in aqueous solution (43). Moreover, it has been shown that LPS does not synergize with IL-1 or TNF (26) to increase macrophage tumoricidal activity unlike the human granule-MAF (Figure 9). Finally, LPS suppresses H_2O_2

production (44) whereas the human granule-MAF profoundly enhanced it. These observations with a pattern of biological properties for the human granule-MAF that are quite different from the LPS argue against a significant contribution by LPS.

There are seven cytokines that have been reported to activate macrophages, these are GM-CSF, M-CSF, TNF, IL-1, IL-4, IL-2 and IFN-τ (45,26). From these, only IFN-τ and IL-2 have shown the ability to activate the two signal pathway of macrophage tumoricidal activity (26,35,38,39,41). Moreover, GM-CSF does not cross species (46) unlike the human granule-MAF, and M-CSF has a M.W. between 45-50 kD (47) compared to 10-30 kD for the human granule-MAF. IL-1 does not stimulate H_2O_2 secretion (48) whereas the human granule-MAF was very potent and IL-4 induced Ia antigen expression (39) whereas human granule-MAF was suppressive. TNF does not induce the TNF-resistant Mϕ tumoricidal pathway (35) and it has been reported to synergize with IFN-τ in the induction of Ia antigen expression on Mϕ (50) whereas the human granule-MAF suppressed the action of IFN-τ. Finally, IFN-τ required LPS to induce the two signal pathway of macrophage activation (22,24) and it induced Ia antigen expression (27) unlike the granule MAF, and IL-2 does not stimulate H_2O_2 secretion in macrophages (48,49). Therefore, the behavior of the human solubilised LGL granules is different from known cytokines. However, we cannot rule out the possibility that the granules contain more than one active molecule thus accounting for the

described activities. With this possible exception, these observations suggest that the human LGL granule contain a unique factor with the ability to induce macrophage lysis of a TNF-resistant tumor, to strongly enhance macrophage H_2O_2 production and to suppress their Ia antigen expression.

This study supports our hypothesis that an immuno-regulatory factor is present in LGL granules and therefore, could be released through the granule exocytosis process after contact with target cells. In a tumor mass where tumoricidal activity is in demand, the role for such a CTL granule released MAF would be to regulate macrophages to be cytotoxic effectors and to augment their cytotoxic activity perhaps beyond what can be achieved by TNF-α, IL-1 or IFN-γ alone, rather than promote Ia expression on macrophage as antigen-presenting cells.

ACKNOWLEDGMENTS

We would like to thank Dr. Edward Rector for his expertise in performing the flow cytometry analysis.

REFERENCES

1. **Roussel, E., Gerrard, J.M., and A.H. Greenberg.** 1990. Long-term cultures of human peripheral blood lymphocytes with rhIL-2 generate a population of virtually pure $CD3^+$,$CD16^-$,$CD56^-$ large granular lymphocyte LAK cells.
Clin. Exp. Immunol. 82:416.

2. **Patel, S.S., Thiele, D.L.. and P.E. Lipsky.** 1987. Major histocompatibility complex-unrestricted cytolytic activity of human T cells. Analysis of precursor frequency and effector phenotype. J. Immunol. 139:3886.

3. **Ochoa, A.C., D.E. Hasz, R. Rezonzew, P.M. Anderson, and F.H. Bach.** 1989. Lymphokine-activated killer activity in long-term cultures with anti-CD3 plus Interleukin 2: Identification and isolation of effector subsets.
Cancer Res. 49:963.

4. **Berke, G., and D. Rosen.** 1987. Immunobiol.(Suppl.)3:145.

5. **Clark, W., Ostergaard, H., Gorman, K., and B. Torbett.** 1988. Molecular mechanisms of CTL-mediated lysis: a cellular perspective. Immunol. Rev. 103:53.

6. **Carpen, O., and E. Saksela.** 1988. Directed exocytosis

in the NK cell-mediated cytotoxicity. A review. Nat. Immun. Cell Growth Regul. 7:1.

7. **Young, J. D.-E.** 1989. Killing of target cells by lymphocytes: A mechanistic view. Physiol. Rev. 69:250.

8. **Müller-Eberhard, H.J.** 1988. The molecular basis of target cell killing by human lymphocytes and of killer cell self-protection. Immunol. Rev. 103:87.

9. **Henkart, P.A.** 1985. Mechanism of lymphocyte-mediated cytotoxicity. Ann. Rev. Immunol. 3:31.

10. **Podack, E.R., Lowrey, D.M., and M. Lichtenheld.** 1988. Immunol. Rev. 103:203.

11. **MacDermott, R.P., R.E. Schmidt, J.P. Caufield, A. Hein, G.T. Bartley, J. Ritz, S.F. Schlossman, K.F. Austen, and R.L. Stevens.** 1985. Proteoglycans in cell-mediated cytotoxicity. Identification, localization, and exocytosis of a chondroitin sulfate proteoglycan from human cloned natural killer cells during target cell lysis. J. Exp. Med. 162:1771.

12. **Schmidt, R.E., R.P. MacDermott, G. Bartley, M. Bertovich, D.A. Amato, K.F. Austen, S.F. Schlossman,**

R.L. Stevens, and J. Ritz. 1985. Specific release of proteoglycans from human natural killer cells during target lysis. Nature 318:289.

13. **Pasternack, M.S., C.R. Verret, M.A. Liu, and H.N. Eisen.** 1986. Serine esterase in cytolytic T lymphocytes. Nature 322:740.

14. **Takayema, H., G. Trenn, W. Hemphrey, J.A. Bluestone, P.A. Henkart, and M.V. Sitkovsky.** 1987. Antigen receptor triggered secretion of a trypsin-type esterase from cytotoxic T lymphocytes. J. Immunol. 138:566.

15. **Pohajdak, B., J.L. Gomez, J.A. Wilkins, and A.H. Greenberg.** 1984. Tumor-activated NK cells trigger monocyte oxidative metabolism. J. Immunol. 134:243.

16. **Gomez, J.L., B. Pohajdak, S. O'neill, J. Wilkins, and A.H. Greenberg.** 1985. Activation of rat and human alveolar macrophage intracellular microbicidal activity by a preformed LGL cytokine. J. Immunol. 135:1194.

17. **Roussel, E., and A.H. Greenberg.** 1989. Identification of a macrophage activating factor in granules of the RNK large granular lymphocyte leukemia. J. Immunol. 142:543.

18. **Millard, P.J., M.P. Henkart, C.W. Reynolds, and P.A. Henkart.** 1984. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors.
J. Immunol. 132:3197.

19. **Lee, K.-C., and M. Wong.** 1980. Functional heterogeneity of culture-grown bone marrow-derived macrophages.
I. Antigen-presenting function. J. Immunol. 125:86.

20. **Warren, M.K., and S.N. Vogel.** 1985. Bone marrow-derived macrophages: Development and regulation of differentiation markers by colony-stimulating factor and interferons. J. Immunol. 134:982.

21. **De la Harpe, J. and C.F. Nathan.** 1985. A semi-automated Micro-Assay for H_2O_2 release by human blood monocytes and mouse peritoneal macrophages.
J. Immunol. Meth. 78:323.

22. **Meltzer, M.S.** 1981. Macrophage activation for tumor cytotoxicity: Characterization of priming and trigger signals during lymphokine activation. J. Immunol. 127:179.

23. **Taramelli, D., H. T. Holden, and L. Varesio.** 1980. Endotoxin requirement for macrophage activation by lymphokines in a rapid microcytotoxicity assay.

J. Immunol. Methods. 37:225.

24. **Pace, S.L., and S. W. Russell.** 1981. Activation of mouse macrophages for tumor cell killing. I. Quantitative analysis of interaction between lymphokine and lipopolysaccharide. J. Immunol. 126:1863.

25. **Chen, L., Y. Suzuki, and E.F. Wheelock.** 1987. Interferon-τ synergizes with tumor necrosis factor and with interleukin 1 and requires the presence of both monokines to induce antitumor cytotoxic activity in macrophages. J.Immunol. 139:4096.

26. **Philip, R., and L.B. Epstein.** 1986. Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, τ-interferon and interleukin-1. Nature 323:86.

27. **Greenfield, R.S., P.A. Kiener, D.P. Chin, R.C. Curry, and G.L. Spitalny.** 1986. Biochemical analysis and biological activities of immunoaffinity purified natural murine gamma interferon (MuIFN-τ). J. of Leuk. Biol. 40:665.

28. **Hoffman, M., and J.B. Weinberg.** 1987. Tumor necrosis factor-α induces increased hydrogen peroxide production and

Fc receptor expression, but not increased Ia antigen expression by peritoneal macrophages.
J. of Leuk. Biol. 42:704.

29. **Unanue, E.R.** 1984. Antigen-presenting function of the macrophage. Ann. Rev. Immunol. 2:395.

30. **Beller, D.I., and E.R. Unanue.** 1981. Regulation of macrophage populations. II. Synthesis and expression of Ia antigens by peritoneal exudate macrophage is a transient event. J. Immunol. 126:263.

31. **Nathan, C.F., Silverstein, S.V., Bruckner, L.H., and Z.A. Cohn.** 1979. Extracellular cytolysis of activated macrophages and granulocytes. II. Hydrogen peroxides as mediators of cytotoxicity. J. Exp. Med. 149:100.

32. **Pabst, M.J., and R.B. Johnston Jr.** 1980. Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or LPS. J.Exp. Med. 151:101.

33. **Stuehr, D.J., and C.F. Nathan.** 1989. Nitric oxide. A macrophage product responsable for cytostasis and respiratory inhibition in tumor target cells.
J. Exp. Med. 169:1543.

34. **Higuchi, M., Higashi, N., Taki, H., and Osawa, T.** 1990. TNF and L-arginine-dependent mechanisms act synergistically as the major cytolytic mechanisms of activated macrophages. J. Immunol. 144:1425.

35. **Philip, R.** 1988. Cytolysis of TNF-resistant tumor target. Differential cytotoxicity of monocytes activated by the interferon, IL-2, and TNF. J. Immunol. 140:1345.

36. **Decker, T., Lohmann-Matthes, M.L., and G.E. Gifford.** 1987. Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. J. Immunol. 138:957.

37. **Feinman, R.D., Henrikson-DeStefano, D., Tsujimoto, M. and J. Vilcek.** 1987. TNF is an important mediator of tumor cell killing by human monocytes. J. Immunol. 138:635.

38. **Grabstein, K.H., Urdal, D.L., Tushinski, R.J., Mochizuki, D.Y., Price, V.L., Cantrell, S., Gillis, S. and P.J. Conlon.** 1986. Induction of macrophage tumoricidal activity by GM-CSF. Science 232:506.

39. **Crawford, R.M., Finbloom, D.S., Ohara, J., Paul, W.E., and M.S. Meltzer.** 1987. B cell stimulatory factor-1 (IL-4) activates macrophages for increased tumoricidal activity and expression of Ia antigens. J. Immunol. 139:135.

40. **Esparza, I., Mannel, D., Ruppel, A., Falk, W. and P.H. Krammer.** 1987. IFN-τ and lymphotoxin or TNF act synergistically to induce macrophage killing of tumor cells and schistosomula of Schistosoma mansoni.
J. Exp. Med. 166:589.

41. **Malkovosky, M., Loveland, B., North, M., Asherson, G.L., Gao, L., Ward, P., and W. Fiers.** 1987. Recombinant IL-2 directly augments the cytotoxicity of human monocytes. Nature 325:262.

42. **Hori, K., M.J. Ehrke, K. Mace, D. Maccubbin, M.J. Doyle, Y. Otsuka, and E. Mihich.** 1987. Effect of recombinant human tumor necrosis factor on the induction of murine macrophage tumoricidal activity. Cancer Res. 47:2793.

43. **Wright, A., and S. Kanegasaki.** 1971. Molecular aspects of lipopolysaccharides. Physiol. Rev. 51:748

44. **Rellstab, P., and A. Schaffner.** 1989. Endotoxin suppresses the generation of O_2^- and H_2O_2 by resting and lymphokine-activated human blood-derived macrophages.
J. Immunol. 142:2813.

45. **Balkwill, F.R., and F. Burke.** 1989. The cytokine network. Immunol. Today 10:299.

46. **Wong, G.G., and S. Clark.** 1988. Multiple actions of IL-6 within a cytokine network. Immunol. Today 9:137.

47. **Metcalf, D..** 1989. Haemopoietic growth factors 1. Lancet 8642:825.

48. **Nathan, C., Campanelli, D., Ding, A., Fuortes, M., Gabay, J., Kwon, N.S., Srimal, S., and D. Stuehr.** 1990. Three antimicrobial systems in phagocytes: Regulation by cytokines. J. Cell. Bioch. (Suppl.) 14B:16.

49. **Nathan, C.F., Prendergast T.J., and M.E. Wiebe.** 1984. Activation of human macrophages. Comparison of other cytokines with interferon-τ. J. Exp. Med. 160:600.

50. **Arenzana-Seisdedos, F., Morgenson, S., Vuiller, F., Fiers, W. and J.L. Virelizier.** 1988. Autocrine secretion of tumor necrosis factor amplifies synergistically interferon-τ induced HLA class II gene exression in human monocytes. P.N.A.S. (USA) 16:6087.

CHAPTER V

GENERAL DISCUSSION

The purpose of this chapter is to elaborate on the thinking that led to the conclusions proposed in each individual chapters. A critical analysis of the findings highlighted by the overall work is also presented with references to relevant work that has appeared in the literature since completion of the earlier studies.

The main point presented in chapter II was the identification of a rat LGL granule-MAF based on the presence in granules of several RNK LGL leukemia cell lines of a factor activating macrophage tumor lysis, on the necessity to solubilize these granules to recover the activity, and on the existence of a specific region with high activity corresponding to the cytoplasmic granules in the fractionated Percoll gradient of the cell homogenate. The reproduction of the results was shown by the recovery of MAF activity from the granules of the different RNK cell lines but our basic evidence to suggest that it was in the granules came from the follow up of MAF activity through all the gradient fractions. The first interpretation made from these results was that MAF might be granule associated as it copurified with the granules, but taking into account the observation that a second peak of activity was associated with the soluble proteins or cytosol fractions one could argue that the MAF was not always associated with the granules or was not in the granules at all. To this interpretation was added the fact

that in order to see MAF activity, the fractions had to be solubilized in high salt buffer. This requirement for solubilization also suggested that we were dissociating a non-covalent association with a granule structure. Subsequently, other laboratories reported that granules of LGL were made of a central core with smaller vesicles enclosed in a larger lipid bilayer (1). Proteoglycans were shown to be present and to interact with other proteins known to be contained in granules, like the serine esterases (2) or cytolysin (3). These observations supported the requirement for high salt concentration to solubilize granule fractions in order to release the MAF activity as high salt concentration was a usual method to dissociate proteoglycan interaction with other proteins, such as cytolysin (4). This observation argued for the presence of granule-contained MAF, and further studies done by our collaborators Henkart et al. (3) showed that they could clearly separate the second peak of activity for other biological active proteins by taking smaller fractions within the Percoll gradient of subcellular homogenate. Moreover, they showed that this second peak was made of what they called lighter granules. Their observation, together with our observation that the second peak needed to be solubilized in order to release MAF supported our interpretation that a granule contained MAF was present in a second peak of lighter granules. It was also found that the specific MAF activity/mg protein was higher in the first peak but the total

amount of activity was apparently greater in the second peak, therefore the dense Percoll granule fractions specific activity in our results appeared rather a weak point to argue that the MAF was granule contained. This could be explained by the relative dense granule enrichment obtained using the Percoll gradient and not the recovery of all granule types due to the heterogeneity of the granule population reported by Henkart (5). Our interpretation of the data to this point relied on the structure of the granules, the distribution of the granule population in Percoll gradient and the similarity of the MAF activity profile with other granule molecules rather than a direct demonstration within intact granules which was not yet possible with existing reagents. We also tried a different approach by attempting to block the MAF activity with an anti-granule antibody. If successful this would have argued strongly for the presence of a granule MAF. When we tried such experiments the results were not very clear due to background macrophage activation, probably related to the antibody preparations being contaminated with LPS and also the Fc portion of the antibodies which could be responsible for Mϕ stimulation.

In summary, our conclusion that the MAF was granule associated was built upon the accumulation of evidence using the colocalization of MAF and other granule associated activity and biochemical properties associated with the dissociation of MAF activity from granules. However, a higher

level of purification would have provided a suitable immunogen for the production of specific antibody to help localize the MAF. This was not possible using the RNK granules because of serious problems with the stability of activity in the tumor cells. We decided to develop another method for generating the MAF using human LAK cells.

In chapter III we demonstrated that human PBL in long-term culture with IL-2 generates a LAK cell population of virtually pure $CD3^+$ LGL. The objective in this work was to develop a method for generating large quantities of LAK cells and characterize the resulting LAK cell population from these cultures.

There are three different issues that we want to discuss in this section regarding our findings and interpretation. The first point to clarify is how our low and high doses of IL-2 defined in International Unit (IU) compare with previous work where Cetus Units were used. The World Health Organization has established an International Standard for IL-2 (6). Cetus has adopted the International Standard calibration unit for human IL-2 since July 1989 and they have worked out their conversion factor as being 1 Cetus Unit of Proleukin (IL-2) = 6 IU (Sandra Patterson Kreuscher, director, Biological Therapeutics Developments, Cetus Corporation). Therefore in our work, we were really using low dose of IL-2 with 10 IU/ml equivalent to 1.7 Cetus Units. However our high

dose was equivalent to only 83.3 Cetus Units/ml, therefore what we did was to compare a difference of a 50 fold increase in IL-2 concentration rather than a specific 500 Cetus Units high dose of IL-2. We do not know if using 3 000 IU (500 Cetus Units) instead of 500 IU would have made any difference in the particular outcome of our results. From the previous work cited in chapter III, it is suggested that high dose of IL-2 triggers $CD16^+$ LAK cytotoxicity but there is no evidence that it is sufficient to trigger $CD16^+$ cell proliferation by itself. In any case this does not affect the interpretation of our basic results about the generation of a LAK cell population >90% $CD3^+$ from PBL long-term cultured with low dose of IL-2.

The second issue is about the possible explanation for our reported regression of the $CD16^+$ cells. We have considered that the $CD16^+$ cells could have been lost by adherence when the non adherent cells were recovered, or they could have been suppressed by a suppressor factor present in FCS, or simply died due to the absence of an appropriate cosignal with IL-2. The fact that we were able to induce the proliferation of the $CD16^+$ cells using coincubation with the RPMI 8866 cells in the exact same system suggested that the loss or suppression of the cells was less probable and that the requirement for a cosignal was more likely. The existence of a suppressor factor for $CD16^+$ cells in the FCS was considered after we found that when the PBL were grown in AIM-V

media without sera, the regression of the $CD16^+$ cells did not occur and even a certain amount of proliferation was obtained (Lisa Selin personal communication). This possibility was rejected as we were able to induce $CD16^+$ cell proliferation by coincubation with the RPMI 8866 cells and IL-2. This phenomena is probably related to the AIM-V media designed specifically to grow NK cells rather than a FCS NK suppressor agent. We have also considered the possibility that the FCS could have supplied some antigenic stimulation which would be responsible for the T cell proliferation with our low dose of IL-2, however we think that this is less probable as our cells were triggered for non MHC restricted LAK activity rather than a T cell specific cytotoxicity. From these considerations and the work reported, it appeared to us that FCS had mainly a role of growth support as the cells did not proliferate in RPMI with 5% FCS but did well in 10 or 15%, and that the best explanation for the regression of the $CD16^+$ cells was the absence of an appropriate cosignal with IL-2. We cannot exclude the fact that this regression might have been amplified by a combination of a slight dilution effect associated to the replating procedure and some $CD16^+$ cell death.

The third issue that we want to discuss relates to the resulting LAK effector population from these cultures. We have considered the fact that a remaining non $CD3^+$ cells could be responsible for the LAK activity but it appears rather

unlikely as the effector:target ratio of this population would be unusually low and as far as we know no such thing was ever reported. An other possibility was that only the $CD3\gamma\delta^+$ cells would mediate this LAK activity. Our methodology cannot exclude this totally because we did not FACS sort the $CD3^+CD4^+$ and the $CD3^+CD8^+$ cells and tested their activity. However, this possibility supposes that no LAK activity would be exerted by the $CD3^+CD4^+$ and the $CD3^+CD8^+$ cells. There are several reports in the literature as mentioned in the chapter III which would argue against that. These are the main reasons why we concluded that our LAK activity appeared due to the whole population of $CD3^+$ cells.

The important aspect of this work for our subsequent studies on MAF was the development of a method to generate large number of virtually homogeneous $CD3^+$ LAK cells without stimulation by anti-CD3 antibodies. The method could also prove useful for studies on the mechanisms of LAK activity.

Our main conclusion from chapter IV was that, like the RNK cells, a macrophage regulating factor(s) was present in the granules of human LGL. This was based on the observation that at least three different macrophage functions could be modulated when Mϕ were incubated with solubilized $CD3^+$ LAK cell granules. One approach was to obtain the best data out of these experiments and compare them with published work in order to get a clue about the critical issue of the human

granule-MAF identity. Although it would have been a valuable information to know if known cytokines were stored in human $CD3^+$ LGL granules, we have not screened them using specific Elisa or specific cytokine biological assays as we did with the rat LGL granules. Instead, as a first attempt in this process, we considered that the most stringent assay for MAF was cytotoxicity and examined this alone and in synergy experiments with IL-1ß, TNF-α and IFN-τ in the bone marrow Mϕ tumor cytotoxicity assay. Then, we continued by searching for regulation by granules of other macrophage functions in different MAF assays. The particular outcome of the activation pattern obtained with the granules when we compared the results from all the different assays with the literature suggested that no one known cytokine could explain the data. Although we would like to suggest that this regulating activity could be due to one molecule, we did not present any other evidence in this work to support this hypothesis. The unique pattern of macrophage regulation exerted by the solubilized granules, which was different from the pattern of all cytokines known so far to activate macrophages could have been due to more than one cytokine. At this point, we cannot exclude the fact that any of the known cytokines could be present in the granules neither that a combination of any of them with an other cytokine or a different type of molecule would be responsible for this activity. It is obvious that our methodology for identification is inadequate. Our

synergy experiments between the human granule-MAF and the cytokines could have been counter tested using antibodies against the specific cytokines in order to block the synergistic effect attributed to the cytokines. We could also have tried to block the MAF action with specific antibodies against the different cytokines known to activate Mϕ in order to see if any of these cytokines were involved in this granule activity. This type of method using specific antibodies for help in the identification was not our first choice because of the background problems mentioned previously, although some others (7) have reported good results with such a methodology. The best approach and the one considered for the subsequent step was the purification of the molecule responsible for at least one activity based on the H_2O_2 biological assay. During the purification, activity peaks would then be assayed in the two other biological assays, possibly also with an attempt to neutralize the activity with different anti-cytokines antibodies. Absence of inhibition with neutralizing antibodies, and coincident profiles of activity during purification would strongly argue for a novel single molecule, and justify higher level purification for sequencing and further identification.

Analysis of the thesis overall conclusions

The main conclusion from this entire work is that there is a potential for an immunoregulatory activity exerted on macrophages through the LGL degranulation process. Our results with LGL granules from rat and then human demonstrated unequivocally that a macrophage regulating activity exists in the granules. An interesting issue is how the rat and human granule activities compare to each other, to see to which extent the rat granule MAF could relate to a possible homologous one responsible for the human granule activity. A summary of this comparison is presented in Table X. Both granule factors were able to activate Mϕ tumoricidal activity against TNF resistant tumor cells which is an important point if you consider that only IFN-γ and IL-2 are known so far for such capability. A difference was that the rat granule-MAF needed the copresence of LPS in order to be effective whereas the human one was active on its own. The optimum activation time appeared quite similar. We also got stimulation of Mϕ H_2O_2 production in some preliminary experiments with the rat granules. The most striking difference between the two sources of activity came from the absence of synergy with IFN-γ for the rat granule-MAF whereas the human one showed synergy with IFN-γ in the Mϕ cytotoxicity assay as well as IFN-γ synergized with the human granule-MAF in its antagonistic

TABLE X

Comparison Between the Rat and Human LGL Granule-MAF Activities[a]

	rat	human
Mϕ cytotoxicity against TNF-resistant tumor cells	+	+
Required copresence of LPS	+	-
Optimum activation time for Mϕ tumor lysis	12 h	9-12 h
Stimulated Mϕ H_2O_2 production	+	+
Modulated Ia expression	ND	+
Synergized with IFN-τ	-	+
M.W.	<10kD	10<30kD
Protease sensitivity	+	ND
Heat resistance	+	-

[a] ND: Not done

effect of suppressing Ia antigen expression. We do not have any direct explanation for this observation. It should be noted that there are also difference in the M.W. and we like to suggest the possibility that the rat factor may have been a fragment of a larger molecule. One would need to postulate that only the intact larger molecule had multiple functional sites and activities like we detected in human, otherwise it is probable that there was more than one molecule responsible for all these activities in human granules. An other interesting possibility is that regardless of the M.W. difference that we detected, the specific activity of the human molecule is greater than the rat MAF, at least in terms of Mϕ activation for tumor lysis of TNF-resistant targets. This last observation is supported by the identification of a murine T cell derived lymphokine with the rare ability to prime Mϕ for cytotoxicity against TNF-resistant tumor cells that was recently reported by Kern et al. (8). This factor was found different from IFN-γ and IL-2. Their factor had a M.W. of 30 kD which is similar to our findings with the human granule-MAF. Their factor was LPS dependent similarly to what we observed with the rat granule-MAF except that ours was a small peptide but we already mentioned that we suspected to have detected only a fragment of the entire molecule. In any case, this is an observation which supports our work in suggesting that T cells produce a unique cytokine different from IFN-γ and IL-2 that is able to activate Mϕ tumoricidal

activity against TNF-resistant tumor cells.

Our overall conclusion from this thesis brought up two mains issues; one was the possibility for the presence of a unique cytokine in the LGL granules and we have already discussed it. The second issue is to consider if our conclusion suggesting the presence of immunoregulatory proteins in the LGL granules has any other relevant observations in different biological systems. It has been reported earlier by Greenberg et al. (9) that rat LGL RNK cell granules contained a chemotactic factor for leukocytes. Moreover, recent observations suggest that granule membrane proteins would be involved as integrins in leukocytes (10). Integrins are also considered a type of immunoregulatory molecules (11). Therefore our conclusion are in accordance with other findings involving leukocyte granule proteins in immunoregulation.

A final point in our general conclusion assumes that this granule-MAF would have a physiological role once it would be secreted through the degranulation process. We did not present experiments of granule-MAF recovery in supernatants of LGL after contact with tumor cells. These experiments were reported previously from our laboratory and were at the origin of the basic hypothesis of this work (12). Secondly, evidence of degranulation by LGL after tumor contact have been well documented by others (1,4). We presented evidence for the presence of the postulated Mϕ regulatory factor(s) in the

granules of rat and human LGL. Taken together we consider that this work represents a respectable amount of evidence in support of our basic hypothesis that LGL may use granule exocytosis to exert macrophage immunoregulatory functions.

Concluding remarks

The original scientific contribution presented in this thesis has three features: 1) Chapter II was the first paper indicating the presence of Mϕ tumoricidal activating factor in rat LGL granules. 2) Chapter III was the first study showing that long-term cultures of PBL in presence of IL-2 generate a pure population of $CD3^+$ LAK cells, with an original explanation for the literature ambiguity in the past several years about the nature of LAK cell effectors generated from human PBL. 3) Chapter IV was the first study showing that human LGL granules contain a Mϕ regulating factor, which might be a novel cytokine. This work brings also a better comprehension on the mechanism by how LGL can activate Mϕ. Moreover, the identification of a possibly new cytokine raises all kinds of interesting questions about its effect on other cell types in order to define its range of action. Finally, this monokine might offer in the future a therapeutic application for enhancing immune response at local sites specially because of its property of Mϕ activation and synergy with other relevant cytokines.

REFERENCES

1. **Peters, P.J., Geuze, H.J., van der Donk, H.A. and J. Borst.** 1990. A new model for lethat hit delivery by cytotoxic T lymphocytes.
Immunol. Today 11:28.

2. **Kamada, M.M., Michon, J., Ritz, J., Holldack, J., Serafin, W.E., Austen, K., MacDermott, R.P. and R.L. Stevens.** 1989. Identification of carboxypeptidase and tryptic esterase activities that are complexed to proteoglycans in the secretory granules of human cloned natural killer cells.
J. Immunol. 142:609.

3. **Henkart, P.A., G.A. Berrebi, H. Takayama, W.E. Munger, and M.V. Sitkovsky.** 1987. Biochemical and functional properties of serine esterases in acidic cytoplasmic granules of cytotoxic T lymphocytes.
J. Immunol. 139:2398.

4. **Henkart, P.A. and C.C. Yue.** 1988. The role of cytoplasmic granules in lymphocyte cytotoxicity.
Prog. Allergy 40:82.

5. **Millard, P.J., M.P. Henkart, C.W. Reynolds, and P.A. Henkart.** 1984. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors.
J. Immunol. 132:3197.

6. **Gearing, A.J.H. and R. Torpe.** 1988. The international standard for human IL-2: Calibration by international collaborative study.

J. Imm. Methods 114:3.

7. **Chen, L., Y. Suzuki, and E.F. Wheelock.** 1987. Interferon-τ synergizes with tumor necrosis factor and with interleukin 1 and requires the presence of both monokines to induce antitumor cytotoxic activity in macrophages.

J.Immunol. 139:4096.

8. **Kern, D.E., Grabstein, K.H., Okuno, K., Schreiber, R.D. and P.D. Greenberg.** 1989. Identification of a unique T cell-derived lymphokine that primes macrophage for tumor cytotoxicity. J. Immunol. 143:4308.

9. **Greenberg, A.H., Khalil, N., Pohajdak, B., Talgoy, M., Henkart, P., and F.W. Orr.** 1986. NK-leukocyte chemotactic factor (NK-LCF): a large granular lymphocyte (LGL) granule-associated chemotactic factor.

J. Immunol. 137:3224.

10. **Melder, R.J., Walker, E., Herberman, R.B., and T.L. Whiteside.** 1991. Adhesion characteristics of human interleukin-2 activated natural killer cells.

Cell. Immunol. 132:177.

11. **Larson, R.S. and T.A. Springer.** 1990. Structure and function of leukocyte integrins.

Immunol. Rev. 114:181.

12. **Pohajdak, B., J.L. Gomez, J.A. Wilkins, and A.H. Greenberg.** 1984. Tumor-activated NK cells trigger monocyte oxidative metabolism.
J. Immunol. 134:243.