MODULATION OF MACROPHAGE ANTITUMOR CYTOSTASIS BY ENDOGENOUS LEUKOTRIENES

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MODULATIE VAN MACROFAAG ANTITUMOR CYTOSTASIS DOOR ENDOGENE LEUKOTRIËNEN

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CONTENTS:

ABBREVIATIONS:

AA861 = 2,3,5-Trimethyl-6-(12-hydroxy-5,10-dedocadinyl)-1,4-benzoquinone
Ab = Antibody
ACTH = Adrenocorticotropic hormone
Ag = Antigen
APC = Antigen presenting cell
ATP = Adenosine triphosphate
A23187 = Calcium ionophore A23187
cAMP = Cyclic adenosine monophosphate
CF = Cytolytic factor
CG = Cysteinyl glycinase
cGMP = Cyclic guanine monophosphate
CO = Cyclooxygenase
CSF = Colony stimulating factor
CTL = Cytotoxic T lymphocytes
DAG = Diacylglycerol
DMEM = Dulbecco's modified Eagle's medium
DP = Dipeptidase
FBS = Fetal bovine serum
F_c = Crystallizable fragment of immunoglobulin
FCS = Fetal calf serum
GGT = τ-glutamyl transpeptidase
GM-CSF = Granulocyte-macrophage colony stimulating factor
GTP = Guanine triphosphate
HETE = Hydroxyeicosatetraenoic acid
HLA = Human leukocyte antigens
hMΦ = Human macrophage
HPETE = Hydroperoxyeicosatetraenoic acid
HPLC = High performance liquid chromatograph
I_a = Immune response associated antigens
IFN = Interferon
Ig = Immunoglobulin
IL = Interleukin
IP_3 = Inositol-1,4,5-triphosphate
i.v. = intravenous
LO = 5-Lipoxygenase
LPS = Lipopolysaccharide
LT = Leukotriene
M-CSF = Macrophage colony stimulating factor
MDP = Muramyldipeptide
MHC = Major histocompatibility complex
mMΦ = Murine macrophage
NDGA = Nordihydroguaiaretic acid
NSAID = Non-steroidal anti inflammatory drugs
NK = Natural killer cell
PAF = Platelet activating factor
PC = Phosphatidylcholine
PE = Phosphatidyl ethanolamine
PIP_2 = Phophatidylinositol-4,5-diphosphate
PG = Prostaglandin
PKC = Protein kinase C
PL = Phospholipase
PMA = Phorbol myristate acetate
PMN = Polymorphonuclear leukocytes

PS = Phosphatidylserine
P815 = P815 murine mastocytoma tumor cell
RIA = Radioimmunoassay
TDM = Trehalose dimycolate
TGM = RPMI-tumor cell growth medium
T_h = T helper cell
TNF = Tumor necrosis factor
TPA = 12-O-tetradecanoate phorbol-13-acetate
TX = Thromboxane

Figure 1.

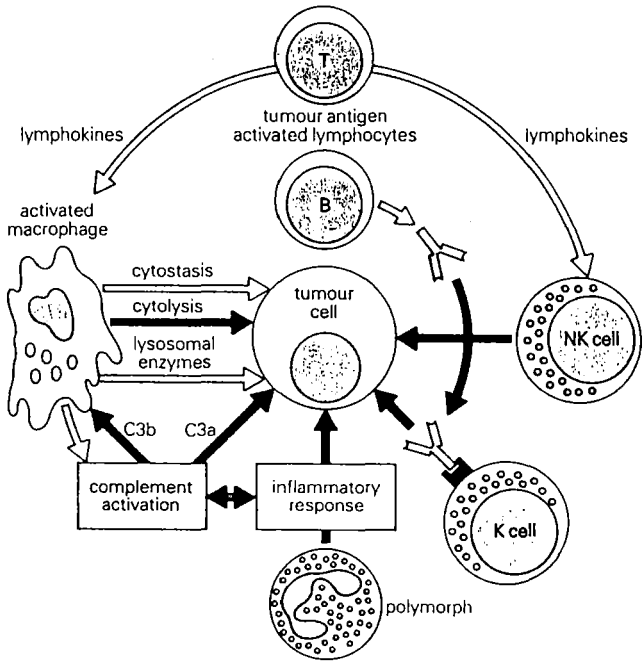


Figure 1.
Summary of the interactions between natural and adaptive immunity. Lymphokines activate macrophages and NK cells. Activated macrophages produce complement components locally which are involved in the development of the inflammatory response. C3a is cytolytic and chemotactic for neutrophils while C3b induces macrophage enzyme release. K cells are armed by antibody from tumor specific B cells. This scheme should be interpreted in the awareness that amplifying mechanisms only are shown (Roitt et al., 1988).

Chapter 1.

GENERAL INTRODUCTION

Macrophages play an essential role in natural resistance to infections, in mediation of specific immunological responses, in inflammatory processes and in expression of antitumor activity (Riott et al., 1988) (Figure 1). Expression of various macrophage functions, including antitumor cytostatic and cytotoxic events are usually related to a process defined as "macrophage activation". Activation is induced by various substances including products released from macrophages (cytokines and eicosanoids) and is characterized by several morphological, biochemical and metabolic changes (Adams and Hamilton, 1988).

Macrophage-mediated tumor cell destruction is predominantly a cell-to-cell contact mediated event involving secretion of effector substances from activated macrophages (Adams et al., 1980; and 1982). Activated macrophages can also inhibit growth of tumor cells (cytostasis) either by cell-to-cell contact (Ophir et al., 1987) or, by releasing soluble factors (Schiller et al., 1987; and Lovett et al., 1986). Among the many substances released by macrophages in response to inflammatory stimuli are eicosanoids (prostanoids and leukotrienes), which act as mediators of macrophage activation. Prostanoids (Prostaglandin E_2 and prostaglandin I_2) have negative effects whereas leukotrienes enhance macrophage/monocyte antitumor activity (Taffet and Russell, 1981a; Dinarello et al., 1984; and Rola-Pleszczynski and Lemaire, 1985a).

Using resident peritoneal macrophages, this study was focussed on the activating role of endogenous leukotrienes in the regulation of macrophage antitumor cytostatic activity in response to inflammatory stimuli. Inhibitors and inducers of leukotrienes synthesis were used to modulate the macrophage antitumor cytostatic function and to identify the essential leukotriene(s) and enzymes involved in inhibition of tumor cell growth by activated macrophages. Induction of high macrophage antitumor activity by modulation of leukotriene biosynthesis might represent an efficient immunotherapeutic tool.

Chapter 2.

REVIEW OF THE LITERATURE

I. MACROPHAGE FUNCTIONS IN THE IMMUNE RESPONSE TO TUMOR CELLS

Mononuclear phagocytes can lyse tumor cells as well as mediate the killing of microorganisms. The role of macrophages in the immune response to tumor cells was reviewed in the context of the cellular events occurring in the inflammatory reaction.

1. Macrophage activities in natural resistance and in immunity

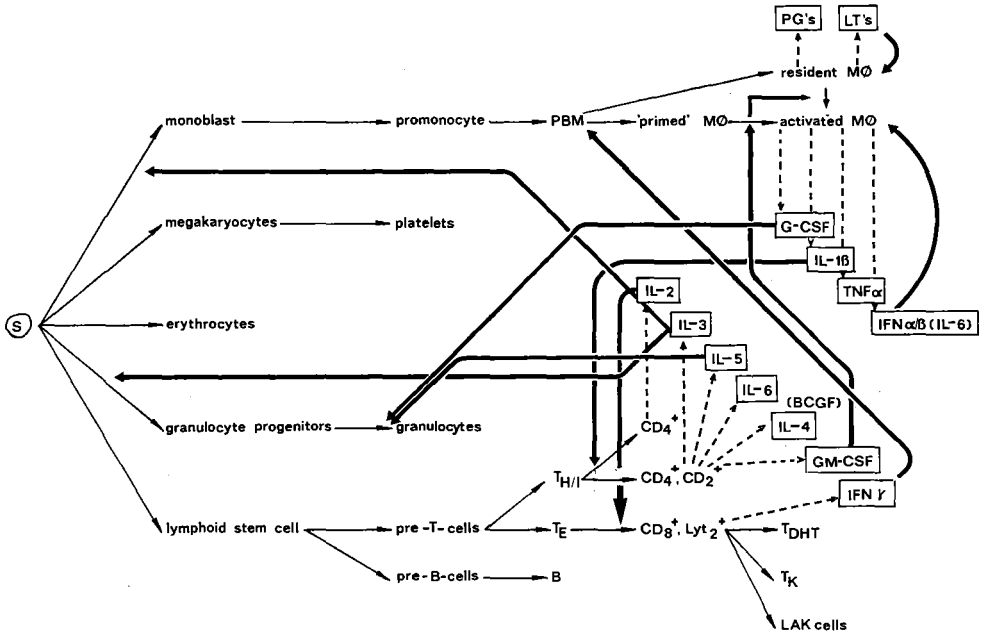
Macrophages have major functions in natural resistance and immune responses:

- F_c- (crystallizable fragment of immunoglobulin) receptor-mediated phagocytosis (associated with respiratory burst (H_2O_2 and O_2^- generation) protects against pathogens.
- secretion of products (tumor necrosis factor (TNF), interferon (IFN) α/β, interleukin-1β (IL-1β), prostaglandins (PGs), leukotrienes (LTs), platelet activating factor (PAF), proteases, lysozymes and components of the complements cascade) make crucial contributions to generation and control of inflammation.
- stimulation of both T- and B- lymphocytes, 'processing' and 'presenting' antigens (Ags) associated with "immune response- associated antigens" (I_a)- and IL-1β- expression (Beller and Unanue, 1981; and Unanue and Allen, 1987).
- inhibition of tumor cell growth (cytostatic activity) and destruction of microbes, viral-infected cells and tumor cells (cytotoxic activity) (Adams and Hamilton, 1988; and Johnston, 1988).

Mast cells and resident tissue mononuclear phagocytes involved in the cellular events during the immune response are already present in the tissues, while leukocytes (polymorphonuclear leukocytes (neutro-, eosino- and basophils) and mononuclear cells (monocytes and lymphocytes)) in the blood have to actively migrate through the blood vessel wall. The development of new mononuclear phagocytes starts in the bone marrow with differentiation of colony-forming-unit-granulocyte progenitors and monoblasts from self-renewing pluripotential hemopoietic stem cells (Figure 2.). Monoblasts develop into immature promonocytes, which differentiate further into monocytes and eventually develop into macrophages. In the steady state, peripheral blood monocytes (PBM) (less phagocytic and with fewer lysosomes than mature macrophages) migrate into the tissue at a constant rate and form the various pools of local resident tissue macrophages (Adams and Hamilton, 1988). Under these circumstances, small amounts of IL-1β may be released spontaneously (Martin and Resch, 1988) and little biologically active TNFα is present within macrophages (Michie et al., 1988). The primary function of macrophages is phagocytosis of damaged cells, of cellular debris and of foreign invaders. Thus, resident macrophages of peritoneal

Figure 2.

Hematopoesis of cells participating in immunological response, including derived cytokines, which regulate inflammatory reactions; ------> = release of cytokines
——— = differentiation/proliferation of cells
═══► = stimulation of cells



or alveolar space could provide a first line of (non)specific defense against microbial and parasitic infections.

At the site of an acute inflammation, many monocytes entering the tissue are confronted with a variety of inflammatory mediators (IL-1β, TNFα, PGE_2, Granulocyte /Macrophage-Colony Stimulating Factor (GM-CSF) and IFN-τ) while they mature and develop into large inflammatory macrophages (Adams and Hamilton, 1988). This network of polypeptides and eicosanoids influences macrophage inflammatory activities (phagocytosis, Ag-Antibody (Ab) presentation, eicosanoids-, TNFα-, and IL-1β release), increases recruitment of peripheral monocytes (by GM-CSF) and increases destructive activities of macrophages (increase in high affinity F_c receptors, generation of oxygen radicals (H_2O_2) release and I_a molecules expression) (Hogg, 1986; and van Furth and van Dissel, 1989). Increased plasma levels of TNFα and adrenocorticotropic hormone (ACTH)

Abbreviations used in Figures 2 and 3.

A23187	= The calcium ionophore A23187
BCG	= Bacillus Calmette-Guerin
BCGF	= B cell growth factors
BMDM	= Bone marrow derived macrophages
CF	= Cytolytic factor
G-CSF	= Granulocyte-colony stimulating factor
GM-CSF	= Granulocyte/Macrophage- CSF
IFN	= Interferon
IL	= Interleukin
LAK cells	= Lymphokine activated killer cells
LPS	= Lipopolysaccharide
LT	= Leukotrienes
MΦ	= Macrophage
PBM	= Peripheral blood monocytes
PG	= Prostaglandins
PHA	= Phytoheamaglutinin
PMA	= Phorbol myristate acetate
P815	= P815 murine mastocytoma tumor cells
S	= Stem cells
T_{DTH} cells	= Thymus derived delayed-type-hypersensitivity cells
T_E cells	= Thymus derived effector cells
TGL	= Thioglycollate
$T_{H/I}$ cells	= Thymus derived helper/inducer cells
T_K cells	= Thymus derived killer cells
TNF	= Tumor Necrosis Factor

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were detected in humans, 2 hours after administration of lipopolysaccharide (LPS) intravenously (i.v.), whereas the plasma levels of IFN-τ and IL-1β did not change (Michie et al., 1988). The mechanism by which stimulated macrophages increase and control TNFα- (Michie et al., 1988) and IL-1β- production and -release (Rola-Plezszynski and Lemaire, 1985a) was the topic of extensive research:

Stimulants that induce monocyte IL-1β synthesis and -release act on the plasma membrane: LPS (Lasfargues et al., 1987; and Bakouche et al., 1987), muramyldipeptide (MDP), Ag-Ab-complexes (Openheim et al., 1986), leukotrienes (LTs) (Rola-Pleszczynski and Lemaire, 1985a) or lymphokines (IFN-τ and GM-CSF) (Moore et al., 1980) are very potent IL-1β inducers. IL-1β release can also be elicited by nonspecific membrane perturbation, including exposure to cellular debris, adherence, phagocytosis or cell injury (Gery et al., 1981). Ca^{2+} ionophore (A23187) and phorbol esters are also potent IL-1β inducers, pointing to a role of protein kinase C (PKC) in the stimulus-response relationship for the synthesis of IL-1β (Matsushima and Oppenheim, 1985; Oppenheim et al., 1986; and Martin and Resch, 1988).

It was suggested that PKC- and calmodulin stimulated kinase-dependent pathways in macrophages are involved in the induction of IL-1 mRNA by LPS or muramyldipeptide (MDP) (Vermeulen et al., 1987), whereas TNFα mRNA expression was only PKC-dependent (Kovacs et al., 1988). LPS treatment was associated with enhanced phosphorylation of a characteristic set of proteins, similar to those induced by stimulating PKC with phorbol myristate acetate (PMA), by alteration of c-fos and c-myc oncogene expression (Introna et al., 1986; and 1987) and by enhancement of protein synthesis (Hamilton et al., 1986b). However, after second round of administration of LPS, macrophages failed to secrete TNFα in vivo and in vitro. This phenomenon, termed endotoxin tolerance, could be a mechanism of the host to desensitize its inflammatory response.

Each immune response requires the interaction of Ag-specific lymphocytes and accessory cells. Macrophages have a regulatory role in the immune response to proteins by the activation of an Ag-specific T helper (T_h) subset of lymphocytes ($CD4^+$). The activation of the $CD4^+$ cells initiates the diverse cellular interactions that result in B cell activation, development of inflammatory reactions and activation of $CD8^+$ positive cells to become cytotoxic T lymphocytes (CTL) (Unanue and Allen, 1987). The specificity of $CD4^+$ cell activity is restricted to recognition of products from the I region (in mice) of the major histocompatibility complex (MHC) expressed by macrophages. Therefore, this specific function of macrophages in activating T cells generates a much more effective and selective immune response for particular invading organisms. IL-1β production and -release from macrophages during the immune response appears also to be dependent on I_a products restricted - T cell contact (Oppenheim et al., 1986). Binding of Ag in association with MHC products to the helper/inducer subsets of T cells initiates the synthesis of the T cell-specific mitogen IL-2 (Gillis, 1983), a lymphokine that stimulates growth of, and IFN-τ production by $Lyt\text{-}2^+$ spleen cells (Johnson and Torres, 1984). Both lymphokines are essential for the development of cytotoxic ($CD8^+$) T cells (Maraskovski et al., 1989), incapable of self-I_a recognition (Oppenheim et al., 1986). Besides the advantage of activation and generation of specific T_h cell clones, one likely explanation for existence of this genetic control of the immune response, could be the cytotoxic effects of an unlimited lymphokine-induced activation of macrophages, which threatened survival of the host in the past. The toxic effects observed from IL-2 treated patients in the immune therapy towards malignancies (Rosenberg et al., 1987) supports this view.

M(macrophage)-CSF and GM-CSF (not found in normal serum) are regulators of tissue macrophage proliferation and differentiation (Chen et al., 1988). M-CSF can be considered as a regulator of new tissue macrophage production under normal steady state conditions, whereas locally produced GM-CSF acting with M-CSF hastes the production of new tissue macrophages (Lin et al., 1989). During inflammation, the concentration in the circulation of G(granulocyte)-CSF produced by macrophages

increases a 1000 times. It has been reported that this growth factor causes proliferation of immature monocytes (Hogg, 1986). Other lymphokines (TNF-β (lymphotoxin)) and B cell growth factors (IL-4 and 6) from T cell populations, which mediate important functions in the immune system has been reviewed by Mosmann (1987) (see Figure 2.).

2. Macrophage activities towards tumor cells

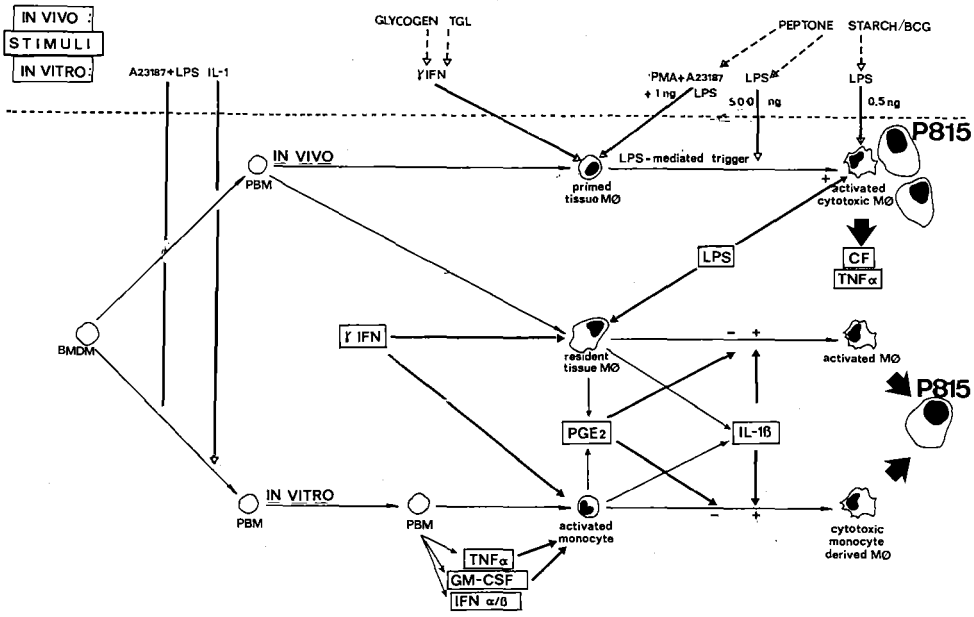
2.1 Cytotoxic- and cytostatic effects on tumor cells

Most results regarding antitumor cytotoxicity have been obtained by assays on the expanded release of ^{51}Cr- or ^{125}I incorporated into tumor cells, reflecting the degree of tumor cell lysis. The results of monocyte-mediated cytotoxicity against tumor cells were not always comparable from laboratory to laboratory because of considerable variation in the method of isolation of the cells, the choice of target cells, the duration of the cytotoxic phases of the response and the technique used for detecting the extent of cytotoxicity.

Carswell and coauthors first described in 1975 an activity in the sera of mice infected with BCG and subsequently injected with LPS. The active principle induced hemorrhagic necrosis of certain transplantable tumors in mice. The authors suggested that this activity was mediated by a macrophage derived product which they termed 'Tumor necrosis factor'(TNF). In 1986, TNF was purified from serum of mice, obtained at 2 hours after an i.v. injection with endotoxin (10 µg LPS) (Haranaka et al., 1986). Urban and coauthors (1986) demonstrated with recombinant TNFα and TNF-sensitive and -resistant tumor cells that TNFα is an important mediator of macrophage-dependent tumor cell cytotoxicity. Demonstrated with anti-TNFα-, anti-IL-1β- and anti-IFN-τ monoclonal Abs, TNFα appears to enhance and mediate monocyte-mediated antitumor cytotoxicity in response to LPS, IFN-τ , IL-1β and TNFα (Philip and Epstein, 1986) (Figure 3.). Weinberg and coauthors (1978) characterized macrophage differentiation in vivo (by infection of irritants) toward the tumoridal state, which parallels the responsiveness of macrophages to LPS in vitro: Less LPS is needed to trigger expression of antitumor activity in vitro by macrophages in a more differentiated stage (Figure 3.). Adams and coauthors (1980, 1982) recognized a cytolytic factor (CF) (1980) and selective tumor cell binding (1982) from BCG-activated macrophages in its effector function of tumor cell destruction. Ruco (1978) and Meltzer (1982) decribed a required completion of three phases of reactions for development of macrophage antitumor cytotoxicity activation; first, differentiation of immature blood-derived mononuclear phagocytes, second, a macrophage response to priming signals by IFN-τ (Pace et al., 1983) and third, primed macrophages respond to a trigger by LPS (Pace and Russell, 1981). Changes in protein expression by bone marrow derived macrophages has been described in traversing stages of macrophage differentiation to a macrophage tumoricidal state (MacKay and Russell, 1986) (Figure 3.).

Figure 3.

Macrophage differentiation toward tumoricidal state.



Abbreviations, see page 10.

It has been demonstrated that IFN-τ modulates the Ca^{2+}-dependent PKC in peritoneal macrophages (Hamilton et al., 1984; and Hamilton and Adams, 1987). Intracellular Ca^{2+} mobilization (Johnson and Torres, 1985 and Drysdale et al., 1987) and PKC activation (Hamilton et al., 1986a; and Celada and Schreiber, 1988) appears to be essential steps in the pathway of required signals for IFN-τ-dependent induction of tumoricidal activity during macrophage maturation. Human macrophages activated by IFN-τ induces specifically messenger (m)RNA for human leukocytes antigen (HLA)-DR. Regarding TNFα-mRNA, INF-τ did not induce expression of TNFα-mRNA in murine macrophages, but IFN-τ enhanced the accumulation of LPS-induced TNFα-mRNA (Koerner et al., 1987). It has been demonstrated that induction of macrophage-mediated tumor cytotoxicity could depend on the amount and type of INF (α/β or τ) used, the presence of a second signal and the type of tumor cell used (Koestler et al., 1987).

Monocytes activated by INF-τ contribute also to control of tumor cell growth by exerting cytotoxic responses (Fidler and Kleinerman, 1984; and Braun et al., 1989) (Figure 3.).

Resident peritoneal macrophages also express cytotoxic activity towards tumor cells. The magnitude of response to LPS and INF-τ appears to increase with cell size (Lee et al., 1981). The results from this study also suggests that macrophages can exist in a continuum of activated states, depending on the nature of the activation signals.

TNFα exerts similarly to IL-1β profound effects on several biological processes, including PGE_2 synthesis, endogenous pyrogen activity and proliferation of thymocytes (Ranges et al., 1988). Many investigators observed also a direct cytotoxic effect of IL-1β (Onozaki et al., 1985b; and Lachman et al., 1986) and TNFα on tumor cells in vitro (Ruggiero et al., 1987; and Nakano et al., 1986) and a cytotoxic effect of TNFα in vivo (Carswell et al., 1975 and Sohmura et al., 1986). It has been reported that IL-1β and TNFα released from monocytes have parallel and additive cytotoxic effects on tumor cells (Ichinose et al., 1988). TNFα binding and penetration through membranes in target cells appeared to be pH-dependent (Baldwin et al., 1988).

Mononuclear phagocytes and their cytokines appear also to play an important role in the control of the immune response to malignant cells by natural killer (NK) cells (de Boer et al., 1982, Rola-Pleszczynski et al., 1983; and Bloom and Babbitt, 1985), cytotoxic T lymphocytes (CTLs) (Schulof et al., 1981) and lymphokine activated killer (LAK) cells (Roth and Golub, 1988; and Chouaib et al., 1988). Recently, efforts were made to test lymphokines (IL-2 (Rosenberg et al., 1987; and Rosentahl et al., 1988,), and INF-τ (Laszlo et al., 1983)) in immunotherapy to cancer patients. But it remains to be seen whether this manipulation of immunity by biological response modifiers (Ruddon, 1981; Klein, et al. 1983; and Palladino and Finkle, 1986) will be of benefit to the patient.

Summarized, the expression of a macrophage antitumor function appears to depend on the signal(s) recieved from the polypeptide network of immunoregulators. Additionally, the presence or absence of receptors on macrophages and membranephospholipids containing arachidonic acid, could influence how macrophages express antitumor activity (Fidler, 1985; and Old, 1987).

Macrophage mediated cytostasis can be defined strictly as the inhibition of target cell division. Most results regarding studies of tumor cell cytostasis were obtained by the ability of tumor cells to incorporate ^3H-thymidine used in replication of their DNA. Inhibited uptake of label reflected thus reduced tumor cell growth. Tumor cell cytostasis requires a coculture of tumor cells and a relatively large number of macrophages (60% or more of the total cell population). Cytostasis appears

not to be selective for tumor cells, since both normal and transformed target cell types were susceptible (Adams and Hamilton, 1988). Macrophage cytostasis has also been effective across histocompatibility barriers, since targets from allogenic or xenogenic sources are sensitive. Because there is little evidence for effector cell-tumor cell contact, the actual cytostatic effect is likely carried out by soluble mediators that act upon proliferating cells. Candidate molecules from activated macrophages for such mediators are prostaglandins (Balazsovits et al., 1988), IL-1 (Lovett et al., 1986; and Tsai and Gaffney, 1987), TNFα (Schiller et al., 1987) thymidine (Adams and Hamilton, 1988) and unidentified released cytostatic product(s) (Lepoivre et al., 1988). The primary anti-proliferative effect appears to be at the level of DNA synthesis, preventing the target cell entering into S phase and/or, replicating its DNA. Therefore, it was suggested that cytostasis is associated with a differentiation step after which tumor cells no longer have the capacity to proliferate.

2.2 Mechanisms by which macrophages attack tumor cells

As mentioned before, macrophage antitumor cytotoxic activity is dependent on cell-cell contact (Adams and Hamilton, 1988). It was suggested that macrophage-tumor cell interactions are initiated by a recognition phase (Ruco and Melzer, 1978) that might result in extracellular release of lysosomes (containing proteolytic enzymes) through macrophage exocytosis into the cytoplasm of the tumor target cells (Bucana et al., 1976). Increasing evidence supports that proteolytic enzymes are involved in monocyte-mediated killing of tumor cells and that O_2^- or H_2O_2 release may not play a critical role in the mechanism of monocyte-mediated tumor cell killing (Colotta et al., 1985; and Adams and Hamilton, 1988). Additionally, it was demonstrated that injure of neoplastic cells by cytotoxic macrophages in cell culture was accompanied by inhibition of mitochondrial respiration (Kilbourn et al., 1984) at the level of NADH dehydrogenase and succinate dehydrogenase (Granger and Lehninger, 1982), and iron loss (Wharton et al., 1988). However, these injured tumor cells demonstrated inhibited thymidine incorporation, but they were not lysed.

II. REGULATION OF MACROPHAGE ACTIVATION BY PROSTANOIDS AND LEUKOTRIENES

1. Biochemistry of prostanoid and leukotriene synthesis

Arachidonic acid (AA) release is assumed to be the rate-limiting step in the synthesis by oxygenation of cyclooxygenase metabolites (prostanoids; prostaglandins (PG), prostacyclin (PGI_2) and thromboxanes (TX)), whereas oxygenation by 5-lipoxygenase requires Ca^{2+}. Prostaglandin was first discovered by von Euler in 1936 in human semen and he believed the prostate gland to be their major source. This conception is now known to be incorrect but the name prostaglandin has remained. Leukotrienes (5-lipoxygenase metabolites) recieved their name as being first found in leukocytes and containing a conjugated 'triene' structure (Samuelsson, 1982; and Borgeat et al., 1985). Prostanoids and leukotrienes are formed by oxydation of free AA, a polyunsaturated fatty acid released from membrane phospholipids.

1.1 Arachidonic acid release from phospholipids

Membrane phospholipids are the most imporant pool of AA. Phosphatidylcholine (PC) is the principal phospholipid of macrophages, with phosphatidylethanolamine (PE), -inositol (PI) and -serine (PS) present to a lesser extent. Macrophage stimulation by certain stimuli results in release of AA from phospholipids through activation of phospholipase(s) (PL) (Gerrard, 1988).

Concentrations of free AA are under control of numerous mechanisms (for review, see Irvine (1982)). Three general mechanisms have been implicated in the releasing process of AA: 1. Degradation of phopholipids by phospholipase A_2, which releases AA principally from PC, 2. Sequential methylation of PE to PC whereafter AA is liberated by PLA_2 and 3. Liberation of AA by PLC through the generation of diacylglycerol (DAG) (from phophatidylinositol-4,5- diphosphate (PIP_2)), from which AA is released by a diaglycerol lipase (Nishizuka, 1984a and b).

Exchange of free AA between immuno-competent cells can modulate the characteristic individual AA metabolism in a cell (Goldyne et al., 1982; Salari and Chang-Yeung, 1989). The availabilty of the eicosanoids precursor AA appears also to be regulated by the rate of reincorporation of the free fatty acids (Goppelt-Stuebe et al., 1986) in phospholipids (reacetylation). Activation of AA-releasing enzymes have been suggested to be dependent on the availibility of adenosine triphosphate (ATP) and cytosolic free Ca^{2+} in macrophages (Wightman et al., 1981a and b).

Phospholipases A_2 and C are found in macrophage lysosomes and in unidentified locations of macrophages (Hsueh et al., 1981). Release of AA from intracellular stores has been reported to be activated by many physiological and experimental

agents, including calmodulin (Wong and Cheung, 1979), 12-O-tetradecanoate phorbol-13-acetate (TPA) (Hoffmann et al., 1988), divalent cation ionophore A23187 (Emilsson and Sundler, 1985; and Hoffmann et al., 1988), IL-1β and TNFα (Chang, et al. 1986; Godfrey et al., 1987; and 1988), phagocytosing stimuli (Aderem et al., 1986b; Hoffmann et al., 1988) and immune complexes of IgG or E (Pawlowski et al., 1983). However, it is not always clear to what extent the stimulatory effect of these compounds on AA release is mediated by activation of PLA_2. Triggering of AA release in F_c receptor-mediated phagocytosis in resident macrophages requires Na^+ influx and protein synthesis. This receptor-mediated induction of PL activity can be bypassed by a Ca^{2+} influx mediated by A23187 (Aderem et al., 1986a).

1.2 Biosynthesis and metabolism of prostanoids

Liberated AA can be converted by an enzyme prostaglandin endoperoxide (PGH) synthetase / cyclo-oxygenase (CO) to the PG endoperoxide intermediate PGG_2 through insertion of two oxygen molecules in AA, and is further transformed by hydroperoxidase into PGH_2. Both of these activities require (probably the iron (Fe^{2+}) in) heme. PGH_2 is further transformed to various PGs (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2) (Johnson et al., 1976) or TXA_2 (Hamberg et al., 1975) (reviewed by Stenson and Parker, 1982) by PG endoperoxide isomerases (synthetases) or non-enzymatically.

Most non-steroid anti-inflammatory drugs (NSAIDs) inhibit oxygen insertion into substrate fatty acids by CO and are believed to interact with the substrate-binding site of the enzyme, although inhibitory effects on Ca^{2+} mobilization and increased cAMP levels in neutrophils have also been reported (Abramson et al., 1985).

The prostanoids PGI_2 and TXA_2 are intermediates which rapidly inactivate, when released from macrophages into aqueous solutions. They undergo a non-enzymatic degradation to less biologically active and stable products 6-keto $PGF_{1\alpha}$ and TXB_2, respectively. PGE_2 can be metabolized to $PGF_{2\alpha}$ by 9-ketoreductase or to PGA_2 and further to PGC_2 and PGB_2 enzymatically or non-enzymatically.

Most enzymes of CO pathway have been found in the microsomal subcellular fraction (Jackschik and Ko, 1983).

1.3 Biosynthesis and metabolism of leukotrienes

The biosynthesis and metabolism of leukotrienes have been extensively reviewed (Lewis and Austen, 1984; Borgeat et al., 1985; Samuelsson et al., 1987; and Levi and Krell, 1988). 5-Lipoxygenase (LO) catalyzes two reactions (Rouzer et al., 1986) in the biochemical pathway of the formation of the leukotrienes from AA. First the addition of oxygen resulting in 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE), which is further transformed to the unstable 5,6-oxido-7,9,11,14-

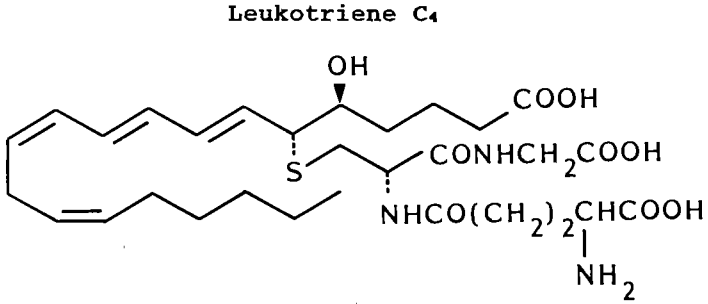
eicosatetraenoic acid (LTA_4) or conjugated to a triene (Pawlowski et al., 1982). LTA_4 can be converted either to LTB_4 by LTA_4 hydrolase, or with glutathione to LTC_4 by glutathion-S-transferase. LTB_4 transformes in cultured PMNs in vitro to 20-hydroxy- and further in 20-carboxy-LTB_4 by omega-oxydation. Non-enzymatic hydrolysis of LTA_4 results in biologically inactive isomers of LTB_4.

Recently, human LO, a single polypeptide chain of 75-80 K Daltons has been cloned and expressed in a osteosarcoma cell line (Rouzer et al., 1988a). It appears that maximal activity of LO requires Ca^{2+} (Lefer and Yanagisawa, 1987), ATP (Ahnfelt-Ronne and Bang OIsen, 1985; and Rouzer and Samuelsson, 1987) and a microsomal membrane preparation (Rouzer et al., 1985). A23187 treatment of intact leukocytes results in a Ca^{2+}-dependent translocation of 5-lipoxygenase (Rouzer and Kargman, 1988b) from the specific granules (Stuning et al., 1985) to a membrane bound site where it is utilized for LT synthesis and where it is consequently inactivated.

LTC_4 is a product of the conjugation of glutathione and LTA_4, by glutathione-S-transferase activity. Location of this enzyme appears to be the microsomal and cytosol subcellular fractions and not in supernatants of granulocytes (Raulf et al., 1985) and hepatocytes (Soderstrom et al., 1985). LTC_4 can be converted to LTD_4 by cleavage of a τ-glutamyl residue by τ-glutamyltranspeptidase (GGT) (Orning and Hammerstrom, 1980; and Morris et al., 1982) and metabolized further to LTE_4 by cysteinyl-glycinase (CG) by elimination of glycine. Both enzymes are present in supernatants of A23187-stimulated granulocytes (Raulf et al., 1985), in plasma and in granules of unstimulated granulocytes (Raulf et al., 1985) and PMNs (Lee et al., 1983). Formation of the cysteinyl LTs, LTC_4 and LTD_4 is also regulated by intracellular glutathione levels (Rouzer et al., 1982), probably by competition for GGT. The addition of glutathione and GGT to LTE_4 in vitro results in the formation of LTF_4 (Bernstrom and Hammarstrom, 1982).

12- and 15-HPETEs can be formed from free AA by 12- and 15-lipoxygenase, respectively. These metabolites can transform further into 12- and 15-hydroxyeicosatetraenoic acids (HETE).

Leukotriene C_4



It has been reported that 12- and 15-HETE can inhibit zymozan-induced LTC_4, -PGE_2 and -TXB_2 release from leukocytes (Vanderhoek et al., 1980) and resident macrophages (Camp and Fincham, 1985; Chang et al.,1985; and Humes et al., 1986). PGE_2 can inhibit stimulated macrophage LTB_4 release (Elliott et al., 1989) and exogenous LTC_4 stimulates PGE_2 release from macrophages (Feuerstein et al., 1981). This indicates a regulatory role of PGE_2 on macrophage -eicosanoids formation and -function (Schenkelaars and Bonta, 1986) and it appears that leukotrienes regulate their own production through a self-induced inhibitor, that is PGE_2. This proposal was launched several years ago (Bonta and Parnham, 1982).

Exogenous AA inhibits 5-LO metabolism in macrophages (Elliott et al., 1988; and Peters-Golden and Shelly, 1988), probably by ATP depletion. Additionally, there are reports of enhancement of A23187-increased LTB_4 formation (Docherty and Wilson, 1987; and Elliott et al., 1989) and activation of 15-LO (Vanderhoek and Bailey, 1984) by cyclooxygenase inhibitors.

Platelet- activating factor (1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine: PAF) induced production is associated with macrophage- (Albert and Snyder, 1983; and Elstad et al., 1988) and PMN- (Sisson et al., 1987) activation. This immunomodulating phospholipid appears to regulate macrophage (Huang et al., 1988) and T cell activity (Braquet and Rola-Pleszczynski, 1987; and Rola-Pleszczynski et al., 1988).

1.4 Arachidonic acid metabolism in resident peritoneal macrophages

Resident macrophages synthesize and release more eicosanoids in response to inflammatory stimuli or A23187 than in vivo elicited/activated macrophages. This could be due to the fact that a high (25% of the total) fatty acid content in the membrane phospholipid pool of resident macrophages is composed of AA (Scott et al., 1980). In resident peritonal macrophages two different phospholipases A_2 (Ca^{2+}-dependent and independent) and phospholipase C have been characterized, biochemically (Wightman et al., 1981a and b.) and it has been suggested that in resident macrophages the prostaglandin synthetase and 5-lipoxygenase can obtain substrate AA from two different sources (Humes et al., 1982). In other types of macrophages, like BCG-activated alveolar macrophages, free AA is derived from PE and PC only, whereas it has been suggested that lyso(bis)phosphatidic acid may also provide an additional source of AA in stimulated resident alveolar macrophages (Cochran et al., 1987).

A23187, immune complexes and phagocytosis of opsonized particles (serum-treated zymozan) induce both the release of leukotrienes (Rouzer et al., 1980; Bonney and Humes, 1984; Emilsson and Sundler, 1985; and Hoffmann et al., 1988) and prostanoids release (Bonney et al., 1979; Scott et al., 1980; and Chandler and Fulmer, 1987) in (alveolar and peritoneal) macrophages and monocytes, whereas TPA, PMA and LPS (largely

independent of calcium), induce prostanoid and 15-lipoxygenase metabolite synthesis (Humes et al., 1980; and Hoffmann et al., 1988). It has been observed that immune complexes and opsonized zymozan induce predominately prostanoid release, whereas A23187 induces AA metabolism in favor of leukotriene release by macrophages. Combined treatment of A23187 in addition to PMA (Tripp et al., 1985), TPA (Wey and Baxter, 1986) or LPS (Aderem and Cohn, 1988) synergistically stimulates LO metabolites release, but not the prostanoid release in macrophages (Wey and Baxter, 1986).

Results from these studies indicate that certain products (LPS, TPA and PMA) appears to stimulate AA release by a Ca^{2+}-insensitive pathway and products (A23187 and phagocytic stimuli), which cause Ca^{2+} influx and therefore stimulate PL activity and AA release of macrophages (Hoffman, et al., 1988).

Concerning the relation between Ca^{2+} mobilization and AA metabolism observed in macrophages exposed to certain stimuli, it could be suggested that resident peritoneal macrophages appear to use, like monocytes (Hoffmann et al., 1988); 1. a Ca^{2+}-dependent pathway of AA metabolism (LO- together with CO metabolite release) upon phagocytic stimuli (Rouzer et al., 1980) or dual Ab binding (Aderem et al., 1986a) and 2. a Ca^{2+}-independent pathway of AA -metabolism (CO- together with 15-lipoxygenase metabolite release) initiated by soluble products, like TPA-, LPS- and PMA- stimulation. Thus, stimulation of LO-metabolites release can be associated with CO- metabolites release from macrophages, but CO- metabolites release is not always associated with LO- metabolites release. This is because LO activity requires Ca^{2+} where a CO activity can be observed in the absence of measurable Ca^{2+} influx in macrophages.

2. Macrophage activation

2.1. Macrophage activation by A23187

Macrophage activation by Ca^{2+} ionophore A23187 is related with,
-increasing glucose consumption (Onozaki et al., 1983),
-IL-1β production (Matsushima and Oppenheim, 1985),
-secretion of lysosomal enzyme (Takenawa et al., 1982),
-reactive oxygen production (Lim et al., 1983),
-increased cAMP, prostanoid- and LT formation (Gemsa et al., 1979 and Gerrard, 1988),
-increased macrophage antitumor cytotoxicity (Wright et al., 1985),
-PKC activation and down regulation of macrophage transferrin receptor-phosphorylation and expression (May et al., 1985),
-induction of 90% macrophage antitumor cytostasis towards P815 cell growth by (trehalose dimycolate) TDM-elicited macrophage (Grand-Perret et al., 1986).

The calmodulin- (Ca^{2+}-binding protein) dependent activation of macrophages by A23187 may be mediated by AA metabolites, because calmodulin stimulates PLA_2 (Wong and

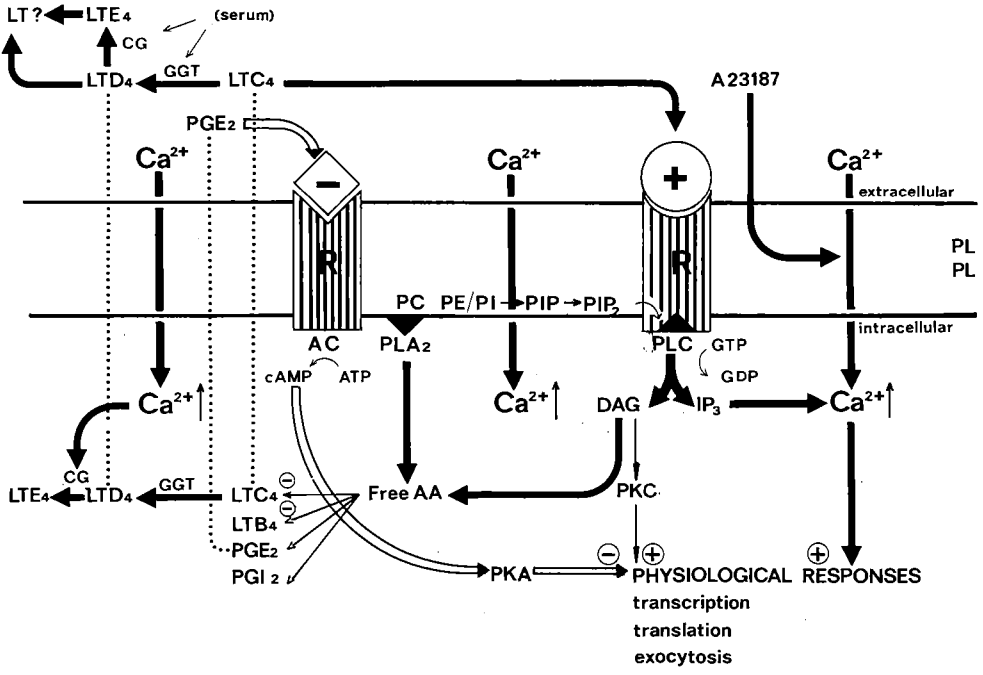
Figure 4.

Arachidonic acid metabolism-mediated macrophage activation stimulated by A23187.

→ :stimulated events by A23187 which enhance macrophage activation.

⇒ :stimulated events by A23187 which inhibit macrophage activation

..... :release of metabolites



Cheung, 1979), A23187 causes AA release from PC, PE and PI by PLA_2 deacylation and PLC mediates DAG generation from PI, PC and PE (Emilsson and Sundler, 1985). The phosphoinositol-derived messenger molecule DAG stimulates PKC activity (Majerus et al., 1986), which is involved in IFN-τ-mediated macrophage activation (Hamilton and Adams, 1987), in transmembrane signaling (Takai et al., 1985) and tumor promotion (Nishizuka, 1984a and b).

Ca^{2+} influx and not cyclic nucleotides (cAMP and cGMP) appeared primarily important in the activating effect of A23187 (Onozaki et al., 1983). It has been reported that A23187-induced macrophage leukotriene release is about 70% of the total induced eicosanoids release (Gerrard, 1988). Therefore, leukotrienes might be involved in transduction of signals required to induce macrophage activation in response to increased cytosolic [Ca^{2+}]. Nishizuka (1984) suggested that PI turnover results in a Ca^{2+} mobilization and increased turnover of AA. Metabolites of AA could than further modulate cell activation (Figure 4).

It has been reported that Ca^{2+} influx induced by A23187 (.1 -.4 μM) can enhance synergistically LO metabolite release by macrophages incubated with LPS (Aderem and Cohn, 1988), zymosan, or PMA (Tripp et al., 1985). A23187 also enhances lysosomal enzyme release from neutrophils incubated with DAG (Nishizuka, 1984).

Besides DAG, metabolism of PI can also give rise to

Abbreviations used in Figure 4.

A23187 = Calcium ionophore A23187
AA = Arachidonic acid
AC = Adenylate cyclase
ATP = Adenosine triphosphate
cAMP = cyclic adenosine monophosphate
CG = cysteinyl glycinase
DAG = Diacylglycerol
GDP = Guanine diphosphate
GGT = τ-glutamyl transpeptidase
GTP = Guanine triphosphate
IP_3 = Inositol triphosphate
LT = Leukotriene
PC = Phosphatidyl choline
PE = Phosphatidyl ethanolamine
PG = Prostaglandin
PI = Phosphatidyl inositol
PIP = Phosphatidyl inositol phosphate
PIP_2 = Phosphatidyl inositol diphosphate
PL = Phospholipid
PLA_2 = Phospholipase A_2
PLC = Phospholipase C
PK = Protein kinase
R = Receptor

inositol triphosphate (IP_3). IP_3 diffuses to endoplasmatic reticulum (Hirata et al., 1984 and O'Flaherty, 1987), where it releases Ca^{2+} and thus increases cytosolic [Ca^{2+}] (Berridge and Irvine, 1984 and Taylor, 1987). Increased concentrations of LTB_4, synthesized by stimulation of LO pathway in macrophages (Bonney et.al., 1985) could also affect the formation of IP_3 (Andersson, et al., 1986) and therefore the enhancement of cytosolic free Ca^{2+}.

An increase in intracellular Ca^{2+} in macrophages was observed after incubation with inflammatory stimuli; F_c-receptor mediated phagocytosis (Young et al., 1984; and di Virgilio et al., 1988), dual F_c receptor binding mediated by Abs (Aderem et al., 1986a), potentiation of PGE_1-induced increase in cAMP (Ishitoya and Takenawa, 1987) and PAF (Conrad and Rink, 1986). Regarding macrophage antitumor functions, a calcium-dependent process is involved in activation of macrophages tumoricidal state by LPS or A23187 following IFN-τ treatment (Wright et al., 1985).

2.2 Leukotriene receptors

There is no data of leukotriene (LT) receptors on peritoneal macrophages at this moment. Specific LTB_4 receptors (K_d = 10.9 nM) are found in PMNs (Goldman and Goetzl, 1982) and LTB_4 receptor activation results in an increase of IP_3 in HL60 cells. Binding studies of ^3H-LTB_4 in guinea pig alveolar macrophages demonstrated a specific high affinity LTB_4-receptor (K_d=3.85 nM) (Cristol et al., 1988) and with ^3H-LTD_4 a specific high affinity LTD_4-(K_d=3.8 nM) receptor was demonstrated in human alveolar macrophages, which has a relatively low affinity for LTC_4 (Opmeer and Hoogsteden, 1984).

It has been demonstrated that LTC_4 receptors are associated with membranes (one-third) and with lysosomal granules (two-third) in PMNs (Baud et al., 1987).

LTD_4 and E_4 receptors appear to be linked to the turnover of inositol phosphates via activation of PLC. Regulation of the activity of PLC appears to be coupled to the receptor via a guanine nucleotide binding protein (Halushka et al., 1989). Current data support that at least for LTD_4/LTE_4 receptors PIP_2 hydrolysis with subsequent Ca^{2+} mobilization and generation of AA metabolism constitute important transduction mechanisms (Halushka et al., 1989). A signal transduction system, which involved the LTD_4 receptor is postulated by Crooke and coauthors (1989).

3. Effect of prostanoids and leukotrienes on macrophage activation

3.1 Regulation of macrophage inflammatory activity

LPS primes resident macrophages for enhanced AA release in vitro (Aderem et al., 1986b) and the AA metabolites PGE_2 and PGI_2 down regulate macrophage functions in vitro (Cantarow et al., 1978; Snyder et al., 1982; Parnham et al., 1983; and Tripp et al, 1986b). Exogenous LTs B_4, C_4 and D_4 (> 2nM) are able to

replace the IL-2 requirement for IFN-τ production by Lyt-1$^-$,2$^+$ cells (Johnson and Torres, 1984) *in vitro* and LTs can enhance monocyte IL-1β release (Rola-Plezszynski and Lemaire, 1985a). IFN-τ decreases macrophage PG- and LT synthesis (Browning and Ribolini, 1987; and Boraschi et al., 1984a; and 1987) and stimulates I_a expression (Fertsch-Ruggio et al., 1988) and IL-1β production (Boraschi et al., 1984b; and Brandwein, 1986) *in vitro*. IFN-τ could so immunoregulate a specific immunological response (including T cell activation) mediated by macrophages *in vivo*. I.v. administration of LPS leads to macrophage activation, expressed by induction of increased I_a expression, and TNFα release (Michie et al., 1988). Monocytes migrate into acute inflammatory tissue (Issekutz et al, 1981) and diminished macrophage PGE_2- and PGI_2-, but not TXB_2- release appears to be a consequence of the *in vivo* activated state of recruited (elicited) mononuclear phagocytes (Stringfellow et al., 1978; Humes et al., 1980; Scott et al., 1982; and Tripp et al., 1986a). GM-CSF activates bone marrow-derived macrophages in synthesis and presentation of the transient expressed I_a molecule (Beller and Unanue, 1981; and Fischer et al., 1988). These events may constitute the mechanism by which macrophages could decrease PG release and therefore could decrease its mediator function during inflammation. Reduced eicosanoid release by activated macrophages could therefore play a role in resistance to infectious and neoplastic diseases (Stringfellow et al., 1978).

3.1.1 The role of cyclooxygenase metabolites

Macrophage release of PGE_2 is increased in response to acute inflammatory stimuli, like
- LPS (Stringfellow et al., 1978),
- bacteria *in vivo* (Edwards III et al., 1986),
- immune complexes (Bonney et al., 1979; Pawlowski et al., 1983; Chandler and Fulmer, 1986; and Ferreri et al., 1986),
- phagocytic stimuli (Scott et al., 1980; Pawlowski et al., 1983; Bonney et al., 1978; and Bonney and Humes, 1984; Chandler and Fulmer, 1986; Tripp et al., 1986a; and Balter et al., 1989),
- TNFα (Lehmmann et al., 1988),
- IL-1β (Browning and Ribolini, 1987).

LPS stimulates the hydrolysis of PIP_2 by macrophages, but did not cause substancial increases in intracellular Ca^{2+} (Prpic et al., 1987). IL-1β appears to provoke rapid increase in IP_3 and a decrease in PIP_2 in macrophages, which suggested a mechanism of IL-1β receptor activation by the generation of the second messengers IP_3 (Wijelath et al., 1988) and cAMP (Shirakawa et al., 1988).

PGE_2 and PGI_2 modulate the development of cell-mediated immunity (CMI) (Leung and Mihich, 1980) by inbiting macrophage inflammatory responses, like;
- I_a expression (Snyder et al., 1982; and Tripp et al., 1986b),
- hydrolase release (Bonney et al., 1978),
- Il-1β and TNFα release (Renz et al., 1988) and

- adhesion and spreading of the macrophages (Cantarow et al., 1978).

The inhibitory role of PGs in regulating macrophage activation appears to be mediated by an increase in the second messenger, cAMP, formation (Lim et al., 1983; Adolfs and Bonta, 1982; and Bonta et al., 1984) via the regulatory subunit of cAMP-dependent protein kinases (Yamamoto and Suzuki, 1987; Kammer, 1988; and Riabowol et al., 1988). In resident peritoneal macrophages, [PGE_2] >10 ng/ml reduces LPS-mediated mRNA for TNFα (Kunkel et al., 1988) and suppresses TNFα release (via cAMP), whereas lower dose stimulates TNFα release (Renz et al., 1988). Splenger and coauthors (1989) can desensitize the suppressive function of PGE_2 by PGE_2-pretreatment. This immunoregulatory role of PGE_2 could be important maintaining local TNFα levels during an inflammation. It has also been reported that GM-CSF primes macrophages for enhanced TNFα and PGE_2 release (Heidenreich et al., 1989) and that it modulates the functional state (F_c receptor expression and membrane bound IL-1β, but not I_a expression) of mature resident peritoneal macrophages (Morrissey et al., 1988).

PGE_2, cyclic nucleotides and INF-τ can regulate IL-1β production (Brandwein, 1986) and INF-τ appears to block IL-1β-induced PGE_2 release from monocytes (Browning and Ribolini, 1987; and Edwards III et al., 1986). The autocrine TNFα stimulates antitumor cytotoxicity, PGE_2 production, intracellular cAMP formation and metabolic activation in peritoneal macrophages (Lehmmann et al., 1988). Endogenous PGE_2 could down regulate TNFα release and therefore control macrophage antitumor cytotoxic functions. However, inflammation can be associated with decreased (in comparison with resident macrophages) stimulation of macrophage PGE_2 release (Humes et al., 1980; Scott et al., 1982; and Edwards III et al., 1986). This reduced PGE_2 release could be due to the prior stimulus in vivo, for example by IFN-τ, which appeared to reduce induced PG release, or may be affected by reduced incorporation of free AA in phospholipids of recruited cells (during maturation) in inflammation.

3.1.2 The role of Ca^{2+} and 5-lipoxygenase metabolites

Recent studies suggest that the LTB_4, C_4, D_4 and E_4 may be important mediators of inflammatory and allergic reactions (Barnes et al., 1988; Levi and Krell, 1988; and Ohuchi et al., 1988). It has been demonstrated that LO- metabolites are required in IL-1β induced IL-2 production, IL-2 mediated T cell proliferation and in INF-τ production (Dinarello et al., 1983; Farrar and Humes, 1985; and Russell et al., 1987). Theoretically, LO metabolites could 'bypass' MHC-restricted T cell mediated IFN-τ production (Rola-Plezszynski, 1985b). However, in vivo only LTB_4 remains likely, because τ-glutamyl-transpeptidase (GGT) and cysteinylglycinase (CG) are present in plasma (Lee et al., 1983), and can be released by activated granulocytes (Raulf et al., 1985). Thus, although LTs could accelerate inflammatory responses, the type of LT formed

appears to be dependent on the presence of GGT and CG and also on the stimulus and/or maturation stage of the cell:

In mice (Scott et al., 1983 and Locksley et al., 1985) and in porcine (Paterson et al., 1985), macrophages may be a major source of LTC_4 and LTD_4 release (upon opsonized zymosan challenge) in acute inflammation and of LTC_4 release in immediate-type hypersensitivity reactions (by Ag challenge with IgE Abs) (Scott et al. 1983; and Rankin et al., 1984).

Extensive comparative studies of 5-lipoxygenase metabolism in murine and human peritoneal macrophages (Du et al., 1983; Scott et al., 1983; and Laviolette et al., 1988), -lung macrophages (Hsueh et al., 1982; Fels et al., 1982; and Schonfeld et al., 1988) and -monocytes (Ferreri et al., 1986; Bigby and Holzman, 1987; and Balter et al., 1989) stimulated with A23187, Ags or aggregated Abs have been reported.

It has been demonstrated that different amounts of LTC_4 are released from alveolar macrophages and peritoneal macrophages stimulated under identical conditions (Rankin et al., 1984). A23187-stimulated lung macrophages release more 5-HETE and LTB_4 than stimulated peripheral blood monocytes. This indicates that enhanced 5-lipoxygenase product synthesis could be related to enhanced maturation (not accelerated by infection) of alveolar macrophages (Bigby and Holzman, 1987) in the alveolar space (Peters-Golden et al., 1990) or/and could be due to compartalization of endogenous AA and of 5-LO in blood monocytes (Balter et al., 1989).

Besides CO metabolites, human monocytes release LTB_4 and C_4 on stimulation of their phagocytic receptor (Williams et al., 1984; and Ferreri et al., 1986), although it is not clear whether eicosanoids stimulation by zymosan challenge in monocytes is in favour of CO metabolites release (Balter et al., 1989) or LO metabolites release (Ferreri et al., 1986). In response to exogenous AA, monocytes release more CO and LO metabolites than alveolar macrophages (Balter et al., 1989).

The role of LTB_4 in immunoregulation involves predominantly, chemotactive activity, endogenous activator in certain lymphocyte functions (Rola-Plezszynski, 1985b), activator of monocyte cytotoxicity (Gagnon et al., 1989) and macrophage IL-1β production (Rola-Plezszynski and Lemaire, 1985a).

Cysteinyl-LTs C_4 and D_4 have slow reacting substance of anaphylaxis activity, mediate blood flow, increase vascular permeability during inflammation (Samuelsson, 1982; and Ford-Hutchinson, 1985) and can replace mouse T_h cells or IL-2 in inducing the production of IFN-τ (Rola-Plezszynskiski and Lemaire, 1985a; and b). Endogenous LTC_4 and LTD_4 appear to be essential metabolites in T cell activation (Johnson and Torres, 1984; and Dornant et al., 1987), in CSF-stimulated myeloid colony formation (Miller et al., 1986; and Ziboh et al., 1986) and in macrophage IL-1β production (Dinarello et al., 1984).

Endogenous LTD_4 appears to stimulate $Fc\text{-}\gamma 2b$- receptor expression (Rhodes et al., 1985), which is involved in macrophage phagocytosis (Yamada and Suzuki, 1989). Thus, it can be suggested that reduced formation of the bioactive LTs could limit macrophage activation and maturation during inflammation. There are reports providing arguments that the activating role of endogenous LTs is mediated by GTP (Rola-Plezszynski, 1985b):

1. Macrophage growth is mediated by endogenous LTC_4 and LTD_4 (Miller et al., 1986; and Ziboh et al., 1986).
2. Colony stimulating factor (CSF)-1 induces activation of a GTP-binding protein in monocytes (Immamura and Kufe, 1988).
3. The LTD_4 receptor appears to be coupled to a guanine binding protein (Halushka et al., 1989).
4. PMA and a lymphokine macrophage mitogenic factor stimulate macrophages growth and increase macrophage cGMP levels in vitro (Hadden et al., 1982).

3.2 Regulation of macrophage antitumor activity

Inflammation can be related to decreased AA metabolites formation in macrophages. Resident macrophages were chosen to study the role of endogenous AA metabolites in mediating their ability to inhibit tumor cell growth, as their membrane phospholipids have a high content of AA and have a high eicosanoid release. If macrophage antitumor cytostasis is regulated by endogenous leukotrienes, stimulation of biosynthesis of these 'endogenous immunomudulators' should be most effective in resident macrophages. Additionally, size of resident macrophages is related to the degree of macrophage antitumor cytostatic activity (Lee et al., 1981). Based on the analogy to inflammation, that PGE_2 and PGI_2 inhibit and LTs enhance macrophage activity, we hypothesized that increased production of LTs could enhance macrophage antitumor cytostasis. Studies with calmodulin blockers and calcium channel blockers indicate that a calcium-dependent process is involved in activation of macrophages tumoricidal state by LPS with IFN-γ treatment (Wright et al., 1985).

3.2.1 The role of cyclooxygenase metabolites

PGE_2 has been reported to regulate CMI (Leung and Mihich, 1980) and it has been demonstrated that macrophage mediated antitumor cytotoxicity is regulated by PGE_2 (Schultz et al., 1978; and 1979; Taffet, 1982; and Adams and Hamilton, 1988).

Macrophage antitumor cytotoxic activity appeared to be regulated by the polypeptide network of immunoregulators: IL-1, IFN-γ, LPS, GM-CSF, TNF and eicosanoids (Grabstein et al.,1986; and Hamilton and Adams, 1987; and Adams and Hamilton, 1988). These immunoregulators affect macrophage eicosanoids release and therefore mediate macrophage antitumor activity:

-IFN-γ maintains macrophage cytotoxicity by inhibition of PGE_2 release (Edward III et al., 1988),
-prolonged PGE_2 administration could maintain macrophage functions by desensitization of PGE_2 receptors (Spengler et

al., 1989).
IFN-τ induced expression of macrophage antitumor cytotoxicity appears to be transient phenomenon, which can be be inhibited by 12 hr incubation with PGE_2, but not PGI_2 or 6-keto $PGF_{1\alpha}$ (Taffet and Russell, 1981a; and Taffet, 1982). IFN-τ could thus maintain macrophage antitumor cytotoxicity by inhibiting endogenous macrophage PGE_2 release (Edwards III et al., 1988).

Additionally, macrophage cytotoxicity mediated by TNFα can be inhibited by PGE_2 by inhibitory effect of PGE_2 on TNF-α release by macrophages (Renz et al., 1988).

Macrophages from mice transplanted with MC-16 fibrosarcoma show enhanced PGE_2 synthesis (Pelus and Bockman, 1979), although the PGE_2 production of macrophages from tumor-bearing mice had no direct effect on tumor cell killing in vitro (Shaw et al., 1979). Treatment with indomethacin or ibuprofen of tumor-bearing rats reduced tumor growth (Karmali and Marsh, 1986).

Impairment of cimetidine-induced transformation of monocytes from cancer patients was suggested (Giulivi et al., 1986), although IFN-τ and LPS- induced monocyte antitumor cytotoxicity in vitro from cancer patients does not differ from monocytes of normal subjects (Peri et al., 1981). It has been reported that exogenous and endogenous PGE_2 stimulate resident- and elicited macrophage cytotoxicity towards L cells in vitro (Snider et al., 1982).

PGE_2 appears to mediate macrophage cytostasis and to inhibit cytolysis a lens epithelial cell line (Mochizuki et.al., 1984) and 1 μM PGE_2 can reverse CSF-induced bone-marrow derived macrophage cytoxicity towards P815 cells (Meerpohl and Bauknecht, 1986).

LPS-induced cytostatic activity of granulocytes towards P815 cells can be prevented by indomethacin or by exogenous PGE_2 (Drapier and Petit, 1986). This could be due to the suggested direct inhibitory effect of PGs (by decreased cytosolic $[Ca^{2+}]$) on P815 cell proliferation (Balazsovits et al., 1988). Direct antitumor cytostatic effect towards several cell lines, including P815 cells (Balazsovits et al., 1988) was found with PGD_2 and PGJ_2 (Simmet and Jaffe, 1983; Narumiya and Fukushima, 1985 and 1987; Bregman et al., 1986; and Todo et al., 1986). Loss of PGE_2 receptors during progression of rat mammary tumors indicates differentiation of cells to autonomous growth (Abou-Issa and Minton, 1986) and increased PG production in breast cancer was associated with high metastatic potential (Rolland et al., 1980).

Toxic effects from TNFα, released by endotoxin administration in vivo could be prevented by CO inhibitors (Kettelhut et al., 1987), however PGs were not involved in TNF-induced weight loss (Mahony and Tisdale, 1989).

These results indicated that PGE_2 exerts a differential

role in controling macrophage antitumor function based on their prior state of activation and/or type of cocultured tumor cell.

3.2.2 The role of Ca^{2+} and 5-lipoxygenase metabolites

Macrophage-like tumor cells require Ca^{2+} influx for induction of their antitumor activity mediated by IFN-τ + LPS (Gorecka-Tisera et al., 1986). Macrophage activation mediated by LPS occurs without Ca^{2+} mobilization, but release of a cytolytic factor (Matthews, 1981) from activated macrophages does required Ca^{+} (Drysdale et al., 1987). 2 Hr. exposure of the calcium ionophore A23187 to trehalose dimycolate (TDM)-elicited macrophage induced antitumor cytostatic activity (Grand-Perret et al., 1986).

It has been reported that exogenous LTB_4 augments macrophage antitumor activity (Gagnon et al., 1989), NK and CTL antitumor cytotoxicity (Rola-Plezszynski et al., 1983 and 1985b). Additionally, LTB_4 release from T cells involved in allograft rejection, is also related to their allograft cytotoxicity (Jordan et al., 1988). 5-Lipoxygenase inhibitors decrease macrophage production of IL-1β (Dinarello et al., 1984) and thus expression of cytostatic- (Lovett et al., 1986 and Tsai et al., 1987) and cytotoxic (Onozaki et al., 1985a and Lachman et al., 1986) activity towards certain tumor cells.

Nordihydroguaiaretic acid (NDGA), a LO/CO inhibitor, does not inhibit antitumor cytostatic activity ex vivo (Mochizuki et al., 1984). No effects were found on macrophage tumoricidal activity towards P815 cells in vitro cultured with various inhibitors of AA oxygenation (Schultz et al., 1985). However, in these studies LO/CO inhibitors were used (NDGA, E.T.Y.A or BW755C).

Inhibition (by 12-HETE) of lipoxygenase metabolites synthesis reduced neuroblastoma- (Werner et al., 1985) and (by Nafazatrom) B16a melanoma growth (Honn et al., 1982).

III. PHARMACOLOGY OF PROSTANOID AND LEUKOTRIENE SYNTHESIS

1. Prostanoid synthesis inhibitors

Anti-inflammatory steroids, like dexamethason, prevents prostanoid and leukotriene synthesis by induction of the biosynthesis of a PLA_2 inhibitor (Flower and Blackwell, 1979).

The term non-steriodal anti-inflammatory drug (NSAID) is related to their ability to reduce (mostly) acute inflammatory effects (pain, oedema) in vivo. Their role is may be related to inhibition of CO metabolite formation in acute inflammation. These drugs could affect vascular effects by TXA_2 released from macrophages and could inhibit the effect of PGE_2 on T_s cells (Goodwin and Ceuppens, 1983). However, the role of NSAIDs in chronic inflammation is controversial. Stimulated prostanoid synthesis in macrophages can be inhibited by the cyclooxygenase inhibitors, indomethacin, benoxaprofen, ibuprofen and aspirin, whereas BW755C demonstrated to be an inhibitor of both CO and LO (Yoshimoto et al., 1982 and Humes et al., 1983).

2. Leukotriene synthesis inhibitors

Nordihydroguaiaretic acid (NDGA), reported to be an inhibitor of LO, also inhibits PGE_2 release (> 3 µM) (Humes et al., 1983). AA861 inhibits 5-LO from elicited peritoneal leukocytes (ID_{50} = .8 µM). The inhibition is of a competitive type and 12-lipoxygenase and CO are not affected by [AA861] <10 µM (Yoshimoto et al., 1982). Also specific inhibition by AA861 of A23187-induced LTB_4- (IC_{50} = .3 µM) and LTC_4- (IC_{50} = .08 µM) release has been reported in PMN (Mita et al., 1986). L-Serine has been reported to bind the active τ-glutamyl site of GGT (Thompson and Meister, 1977), while a serine-borate-complex has been reported to inhibit the conversion of LTC_4 to LTD_4 (Orning and Hammerstrom, 1980).

IV. AIM OF THE PRESENT STUDY

Increased cytosolic [Ca^{2+}] in macrophages and enhanced leukotriene release from macrophages have been described in response to certain inflammatory stimuli (Rouzer et al., 1980; Young et al., 1984; Aderem et al., 1986a; Ferreri et al. 1986; and di Virgilio et al., 1988). It has also been demonstrated that increased 5-lipoxygenase metabolite (leukotrienes) release from macrophages requires Ca^{2+} (Lefer and Yanagisawa, 1987). Additionally, it has been reported that leukotrienes enhance macrophage antitumor activities (Dinarello et al., 1984; and Rola-Pleszczynski and Lemaire, 1985a). In view of the above quoted reports, we hypothesized that endogenous leukotrienes, released by increased cytosolic [Ca^{2+}] in macrophages, could have an activating role in the regulation of macrophage antitumor cytostatic activity.

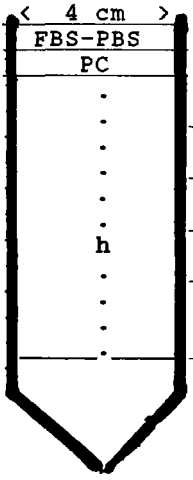
Calcium ionophore (A23187) stimulates leukotriene release from resident peritoneal macrophages (Humes et al., 1982). Therefore, an A23187-induced macrophage antitumor cytostatic coculture-assay (P815 tumor cells and resident peritoneal macrophages) was developed and served as a model to study the role of endogenous leukotrienes in macrophage activation by increased cytosolic [Ca^{2+}]. Agents which affect the macrophage leukotriene biosynthesis stimulated by A23187 were studied. The aim of this study was to gather more information concerning the activating role of a specific leukotriene released from macrophages, in enhancing macrophage activity against tumor cell growth in vitro. This modulation of macrophage antitumor cytostasis could be a tool for new applications of immunotherapy to cancer.

Chapter 3.

METHODOLOGY:

I. Separation of peritoneal macrophages:

Resident peritoneal cells were harvested by rinsing twice the peritoneal cavity from BALB/c mice with 5 ml Dulbecco's Modified Eagle Medium (DMEM). Cells were resuspended in 2.5 ml of 4 % FBS (Fetal Bovine Serum)-PBS (Filtered Dulbecco's Phosphate Buffered Saline) and viability was determined by trypan blue exclusion. Macrophages were separated on the basis of cell size by sedimentation at unit gravity at 4° C on a discontinuous FBS-gradient column (r= 2 cm) according to a modification of the method described by Miller and Phillips (1969): The gradient maker, (three connected columns (r= 1 (8%), 2 (16%) and 2 cm (30%FBS-PBS)), including stirring-rods, was placed 1 meter above the connected, autoclaved and silliconized separation-column. It filled slowly the column by unit gravity at a constant rate, approximately. A 35 ml buffered step discontinuous gradient of FBS (8-30%)-PBS, was thus made under the 2.5 ml peritoneal cell suspension in the column and was used to stabilize the layers of separated cells during 2 hours sedimentation. This separation method of macrophages was developed in order to obtain nonadherent peritoneal macrophages, because it has been demonstrated that adherence and incubation of macrophages in conditioned medium could affect macrophage eicosanoids release (Bockman, 1981). 2.5 and 1.25 ml cell fractions were collected, after two hours sedimentation, at a rate of 0.5 ml/min. and sampled in samples I (v>6). II (v>5), III (v>4) and IV (v>3 mm/hr). (Chapter 8 ; Figure 5). Velocity sedimentation (v) {v = h / sedimentation time (h = sedimentation volume / 3.14 x (2 cm)^2)} of each sample was calculated accordingly:



Sample	sed.Vol.	h	sed. t.	v (mm/hr)
	10 ml	.80 cm	120'+50' =2.83 hr.	2.8
IV				
	12.5 ml	.99 cm	120'+45' =2.71 hr.	3.7
III				
	16.25 ml	1.32 cm	120'+37.5'=2.63 hr.	5
II				
	18.75 ml	1.48 cm	120'+32.5'=2.54 hr.	5.8
I				
	23.75 ml	1.88 cm	120'+22.5'=2.38 hr.	7.9
	35 ml (Total Vol.)		2 hrs.	

II. Separation of elicited peritoneal macrophages:

Murine macrophages were elicited by in vivo administration of .5, .2, or .05 mg carrageenan (Thomson et al., 1979) (1 mg/ml) in peritoneal cavity. After three days the mice were killed and peritoneal cells were obtained by two 5 ml lavages with DMEM. Macrophages were isolated using 1500 g centrifugation (30 min.) on a Percoll gradient (Lymphoprep^{R}) (Ulmer and Flad, 1979; and Gmelig-Meyling and Waldmann, 1980).

III. Characterization of peritoneal macrophages:

Cells separated by velocity sedimentation were characterized using morphological criteria (May-Grunewald Giemsa staining) and immuno-β-galactosidase staining (Leenen et al., 1987), using MAC-1 (Springer et al., 1979; and Garner et al., 1987) and MAC-2 (Ho and Springer, 1982; and Holmes and Morse III, 1988) monoclonal antibodies. This immunochemical technique involved briefly, incubation of cells with either MAC-1 (affinity for macrophages, granulocytes and NK cells) or MAC-2 (affinity for macrophages) monoclonal antibodies and with a second antibody (affinity purified rabbit anti-rat IgG and IgM) conjugated with β-galactosidase (E. Coli). After two hours incubation, positive identification of cell surface antigens stained the cells green. Additional DNA staining according to Feulchen (see Leenen et al., 1987) was performed to visualize the negative cells.

Based on experience of more than 100 separations a representative characteristic pattern of cells/ml obtained in each fraction with its corresponding velocity sedimentation ($3 < v < 8$ mm/hr) was observed (Figure 5).

IV. Preparation of supernatants and determinations of eicosanoids release

Cells from samples I,II and/or III were washed and resuspended in serum free DMEM. Supernatants were taken out in triplicate after various minutes of incubation at 37°C in DMEM or serum containing tumor cell growth medium ('TGM') with or without the calciumionophore A23187 in a microwell (in vitro). Supernatants for TXB_2-, 6-keto $PGF_{1\alpha}$- or LTB_4- determinations by radioimmunoassay (RIA), were centrifuged and stored separately at -70°C.

In the first series of experiments, cells were preincubated (5 min. in DMEM) with or without the inhibitors AA861 or NDGA. Triplicate supernatants were obtained after an additional 60 min. incubation in DMEM in the presence of A23187 (Chapter 4).

In the second series of experiments, cells from samples I, II and III were pooled before kinetic studies of A23187-induced macrophage eicosanoids release in serum free DMEM . Triplicate supernatants were obtained after 5, 10, 20 and 40 min. incubation with or without A23187 at 37°C (Chapter 7).

In the third series of experiments, cells from samples I and II were pooled and incubated either during 20 minutes in the presence of A23187 (with or without AA861 or L-serine preincubation), or during 24 hrs. with or without L-serine in

DMEM or TGM (Chapter 5 and 6). Supernatants were collected and applied to a Sep-Pak C_{18} cartridge, diluted in ethanol and stored in -70 °C, dried and filtered through a .45 µM filter before separation of leukotrienes by high-performance liquid chromatography and the absorbtion was measured at 280 nm (Zijlstra and Vincent, 1984).

V. Target cell growth and macrophage antitumor cytostatic activity in vitro:

P815 murine mastocytoma tumor cells were gifts from Dr. T.A. Hamilton (Cleveland, U.S.A.) and Prof. Zan-Bar (Tel Aviv, Israel). They were cultured in vitro at 37° C in TGM of 10% FBS and RPMI 1640 with 2 mM L-glutamine, 2 g/l $NaHCo_3$, 5 x 10^{-5}M β-mercaptoethanol and 100 IU/ml penicillin / streptomycin in 7.5% CO_2 air. 10^5 cells were transfered each third day of culture (viability > 95%).

6 x 10^3 P815 cells were cultured and assayed in vitro for macrophage cytostatic activity (Gyongyossy et al., 1979; and Lee and Barry, 1977; and Lee et al., 1981) in RPMI+10% FBS tumor cell growth medium ('TGM'). NDGA-, AA861- and L-serine pretreated macrophage samples were assayed simultaneously in TGM. The macrophage cytostatic assay involved 24 hrs coculture of tumor cells with the separated peritoneal cell samples I, II, III and IV in a effector cell (macrophage) : target cell (P815) ratio 2:1 with of without A23187 in a total volume of 100 µl TGM. After an additional 16 hrs. incubation with ^{3}H-thymidine (.5 µCi), cells were collected onto filtermats using a cell harvester. ^{3}H-thymidine uptake by target cells on dried filtermats was measured using a scintillation β-counter and expressed as c.p.m.. Cytostatic activity was calculated in terms of c.p.m., as follows;

$$\frac{(P815_{A/S/A+S} - R_{A/S/A+S}) \times 100\%}{P815_{A/S/A+S}}$$

P815= c.p.m. of target cells.
R = c.p.m. of target cells and separated peritoneal cells in cocultured ratio 2.
A = c.p.m. of target cells or in cocultured ratio 2 in the presence of A23187.
S = c.p.m. of target cells or in cocultured ratio 2 in the presence of L-serine.
A+S= c.p.m. of target cells or ratio 2 (with serine-(200 mM) pretreated effector cells) in the presence of (100 mM) serine and A23187.

Inhibition of cytostatic activity was calculated with;

$$\frac{(P815_A - R_A) - (P815_{A+AA/N} - R_{A+AA/N}) \times 100\%}{P815_A - R_A}$$

A+AA/N = c.p.m. of target cells or ratio 2 (with AA861- or NDGA-pretreated effector cells) in the presence of A23187.

VI. Statistical analysis:

Data are represented as means $\pm$ S.D. of triplicate samples and are identical to two other experiments. All data regarding % cytostatic activity are represented as mean of three or six experiments each from triplicate determinations and were statistically analysed by Mann-Whitney U - or unpaired Student's -t test ($p < 0.05$ and 0.01).

Chapter 4.

SPECIFIC LIPOXYGENASE INHIBITION REVERSES MACROPHAGE CYTOSTASIS TOWARDS P815 TUMOR CELLS *in vitro* INDUCED BY THE CALCIUM IONOPHORE A23187

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PROSTAGLANDINS LEUKOTRIENES AND ESSENTIAL FATTY ACIDS

Prostaglandins Leukotrienes and Essential Fatty Acids (1988) 34, 187–192

Specific Lipoxygenase Inhibition Reverses Macrophage Cytotasis Towards P815 Tumor Cells *In Vitro* Induced by the Calcium Ionophore A23187

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Abstract — A23187-stimulated cytostatic activity of peritoneal macrophages towards P815 tumor cells served as a model for macrophage activation: A macrophage enriched preparation, separated on the basis of cell size in a discontinuous FCS gradient column, expressed cytostatic activity when stimulated by A23187. This was inhibited dose-dependently, by AA-861 but not by nordihydroguaiaretic acid (NDGA). AA-861 inhibited 5-lipoxygenase specifically, NDGA inhibited both 5-1ipoxygenase- and cyclooxygenase activity. The ratio cyclooxygenase/lipoxygenase products increased with AA-861 but not with NDGA. These results show that lipoxygenase products are necessary for expression of cytostatic activity of these arachidonic acid metabolite-producing macrophages and that the ratio cyclooxygenase/lipoxygenase metabolites plays an important role in macrophage activation.

Introduction

Resident macrophages (MPs) have a high concentration of arachidonic acid (AA), present in membrane phospholipids (1) and a characteristic pattern of AA metabolism (2–4): The calcium ionophore (A23187) stimulates phospholipase activity (5–8) and hence oxygenation of the released AA via the cyclooxygenase and lipoxygenase pathway (9–12). A23187 stimulation in resident MPs is thought to prime MPs for tumor cell cytolysis (13, 14) and stimulating MP glucose consumption and migration inhibition (15, 16). Leukotrienes (lipoxygenase products) can also enhance interleukin-1 (IL-1), β-glucuronidase and prostaglandin release (17–19) and cytostatic activity of MPs towards syngeneic tumor cells (20). It is therefore conceivable that lipoxygenase metabolites could play an important role in the mechanism by which A23187 activates MPs. We studied the effect of 2 lipoxygenase inhibitors AA-861 (21) and NDGA (22) on A23187 activated MP cytostasis towards P815 tumor cells. Murine resident peritoneal cells were separated into MP — rich and poor

samples, based on differences in cell sizes, using a discontinue fetal calf serum (FCS)-gradient column (23, 24). The cytostatic activity of each cell fraction was compared with its ability to release eicosanoids.

Materials and Methods

Materials

The calcium ionophore A23187 (diluted in ethanol: 4×10^{-3}M) was purchased from Calbiochem and nordihydroguaiaretic acid (NDGA) (diluted in ethanol: 10^{-2}M) and lipopolysaccharide (LPS, 0111:B4) from Sigma Chem. Co. Leukotriene 84(LTB_4) antiserum was ordered from Wellcome Diagnostics and 6-keto $PGF_{1\alpha}$ antisera from Seragen. ^{3}H-LTB_4, ^{3}H-6-keto $PGF_{1\alpha}$ and ^{3}H-thymidine were obtained from Amersham. AA-861 (2, 3, 5-trimethyl-6-(12-hydroxy-5, 10-dodecadinyl)-1, 4-benzoguinone) (diluted in ethanol: 10^{-2}M) was a gift from Dr. S. Terao (Takeda Chemical Research Division, Osaka, Japan). RPMI 1640 and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco Europe BV. L-Glutamine, F.C.S., penicillin/streptomycin from Flow Laboratories and β-mercaptoethanol was purchased from Merck. Pathogen free female BALB/c ByJIco mice were bought from Iffa-credo, Lyon, France. Filtermats (Skatron) for a 12 well cell harvester (Colinca, Tel Aviv, Israel) and cell culture flasks were ordered from Costar. The 96-microwell flat-bottom trays were purchased from NUNC.

Macrophages and target cells

Resident peritoneal cells were obtained from 6–8 week old female BALB/c mice. They were harvested by two peritoneal lavages of 5 ml DMEM. 10–12.5 × 10^6 cells were pooled from 4 mice, washed, resuspended in 2.5 ml 3% FCS-PBS (Dulbecco's Phosphate Buffered Saline) and viability was determined by trypan blue exclusion . Vigorous resuspension of cells with a 5 ml syringe and 19G needle was essential to disrupt cell clusters. P815 mastocytoma target tumor cells (gift from Dr. T. A. Hamilton, Cleveland, U.S.A.) were cultured *in vitro* at 37°C in complete medium (10% FCS RPMI with 2mM L-glutamine, 2 g/l $NaHCO_3$, 5 × 10^{-5}M β-mercaptoethanol and 100 IU/ml penicilin/streptomycin) in 7, 5 % CO_2 air. For cytostasis experiments P815 cells were resuspended at an amount of 2.4 × 10^5/ml.

Fractionation of MPs by discontinue gradient sedimentation

Fractionation was performed by a modification of the method of Miller and Phillips (21): Separation of 10^7 cells (2, 5 ml) on the basis of size by velocity sedimentation at unit gravity was performed at 4°C in a siliconized and autoclaved column (4 cm diameter). A buffered step discontinue gradient of FCS (8–30%) diluted in filtered PBS (40 ml) was used to stabilize the layers of separated cells (Fig 1). After a sedimentation time of 2 hours, 2.5 and 1.25 ml fractions were collected at a rate of 0,5 ml/min.. The cell number in each fraction was determined (Fig. 1b) and pooled in 4 samples (I–IV). Total recovery was >70% with a viability >98%. Cells were washed twice in DMEM and characterizsed using morphological criteria (May-Grunewald Giemsa) and immuno-β-galactosidase staining (25) , using the MAC-1 antibodies. Briefly, this involved incubating cells with MAC-1- (affinity for macrophages, granulocytes and NK cells) monoclonal antibodies (Ab's) (gift from drs. P. J. Leenen, Erasmus University, Rotterdam) and with a second Ab (affinity purified rabbit anti-rat IgG+IgM) conjugated with β-galactosidase (E.coli) (Sigma). After 2 hrs. incubation the MAC-1^+ cells stained green. The MAC-1^+ cell population was 41% of the total resident peritoneal cells. MP enriched — (morphological criteria: >85% MPs) and MAC-1^+ (85–95%) phenotype enriched peritoneal cell populations were obtained in sample I as well as in sample II (Fig. 1b). Sample III was a mixture of MPs , granulocytes and lymphocytes (morphological criteria) and 70% MAC^+ cells. Sample IV represented lymphocytes (≧90%) and 30% MAC^+ cells.

Assay for cytostatic effect of samples I-IV

The cytostatic effect of cells from samples I-IV on P815 cells was manifested as inhibition of ^{3}H-thymidine uptake, a measure of tumor cell growth. 25 μl (5×10^5/ml) of samples I–IV were incubated with 25 μl A23187 and 25 μl P815 cells (Ratio = MP:P815 = 2:1) in a total volume of 100 μl complete medium for 24 hours. The cells were incubated for an additional 18 hours with ^{3}H-thymidine (0.5 μCi/100 μl) and were collected onto filtermats using a Colinca cell harvester. The filtermat was dried and ^{3}H-thymidine uptake was measured using a Beckman scintillation B-counter (c.p.m.). ^{3}H-Thymidine uptake of each peritoneal cell sample

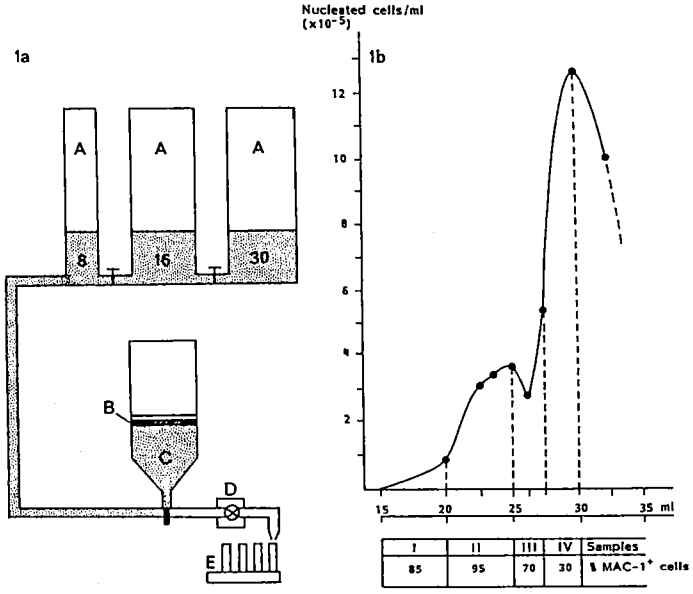

Fig 1a Separation of resident peritoneal cells by sedimentation in a discontinue FCS-gradient column. A = Three gradient makers with 8, 16, and 30% FCS, respectivily, B = peritoneal cell band, C = Discontinue FCS column, D = Flow rate regulator, E = 4 sampled fractions.

Fig 1b Characterization of cells separated from FCS-gradient column by immuno-β-galactosidase staining, expressed as % MAC-1⁺ cells (variations from the means of duplicate samples were <10%) of sampled cell number.

(12, 5×10^3 cells) was <500 c.p.m. Calculation of cytostatic activity was as follows:

$$\frac{[(\text{c.p.m.P815}_z) - (\text{c.p.m.R}_z)] \times 100\%}{\text{c.p.m. P815}}$$

Inhibition of cytostatic effect of A23187 was calculated with:

$$\frac{[(\text{c.p.m.P815}_z)-(\text{c.p.m.R}_z)]-[(\text{c.p.m.P815}_{z+x/y})-(\text{c.p.m.R}_{z+x/y})] \times 100\%}{\text{c.p.m.P815}_z - \text{c.p.m.R}_z}$$

x = AA- 861
y = NDGA
z = A23187
R = Ratio 2

R.I.A. of supernatants

During an incubation period of 60 min. at 37°C the peritoneal cells from samples I,II,III and IV (5×10^5/ml) and tumor cells (2, 5×10^5/ml) were exposed in DMEM to different concentrations of NDGA or AA-861 in addition of A23187. At the end of the incubation, the 100 µl and 20 µl supernatants were taken out and frozen at −70°C for assay of LTB4 and 6-keto $PGF_{1\alpha}$, respectively, by direct radioimmunoassay (R.I.A.).

Statistical analysis

Data are represented as means ± S.D. of triplicate samples and are typical of two other experiments. Significant values were assessed using the Mann-Whitney U-test (*= $p<0.05$)

Results

Cells from samples I,II and III were cocultured with P815 tumorcells in a non-cytostatic ratio 2:1 and stimulated with non-cytostatic 3, 5×10^{-7}M A23187. This could induce the expression of cytostatic activity with 49%, 37% and 0% of

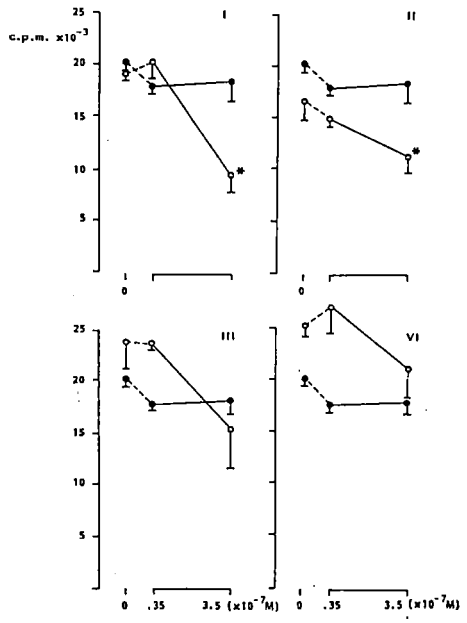

Fig 2 Effect of A23187 on ^{3}H-thymidine uptake of P815 cells, cocultured with peritoneal cell samples I,II,III and IV (ratio: peritoneal-/tumor cell = 2) was expressed as mean c.p.m. ± S.D. of triplicate wells. (•) = Control, ^{3}H-thymidine uptake by P815 cells alone, (○) = Ratio 2. (* =P < 0.05).

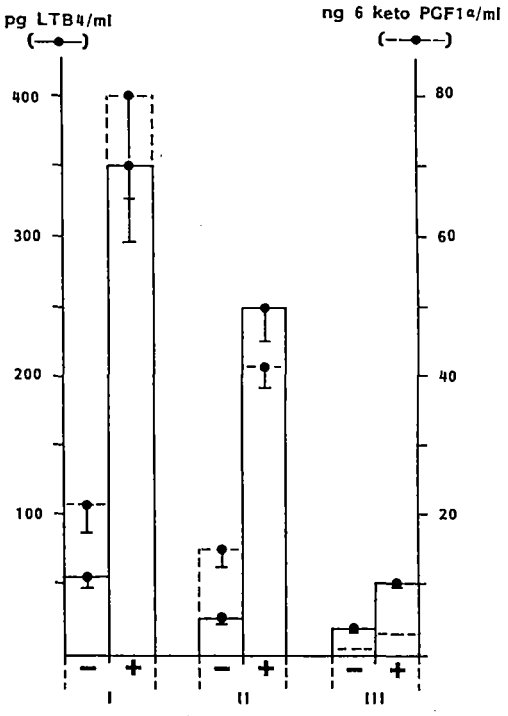

Fig 3 The basal and A23187 induced LTB_4 (pg/ml) and 6-keto $PGF_{1\alpha}$ (ng/ml) release of peritoneal cell samples I–III (5×10^5/ml). Results are means ± S.D. of triplicate supernatants of each sample. −= control, + = 3.5 × 10^{-7}M A23187.

samples I, II and III, respectively (Fig. 2). Sample IV promoted cell growth (Fig. 2). Supernatant studies of AA metabolites of samples I–III stimulated with 3, 5 × 10^{-7}M A23187 showed increased lipoxygenase (LTB_4 release) and cyclooxygenase (6-keto $FGF_{1\alpha}$ release) activity (Fig. 3). AA metabolites release of cell sample IV and P815 cells was less than 16 pg LTB_4/ml and 0.8 ng 6-keto $PGF_{1\alpha}$/ml (unpublished data). 3.5 × 10^{-8}M A23187 and LPS (<12.5 μg/ml, unpublished data) had no significant cytostatic effect in this MP/tumor cell model. NDGA did not inhibit this A23187 induced cytostatic effect (Fig. 4). Inhibition of LTB_4 release >57% and inhibition of 6-keto $PGF_{1\alpha}$-release >15% was caused by NDGA (≧1.25 μ,M) (Fig. 5). Comparative studies with AA-861 showed an increasing cytostatic inhibition upto max. 66% (sample I) and 25% (sample II) with 0.6 μM, while the thymidine uptake by P815 cells alone was elevated (Fig. 4).

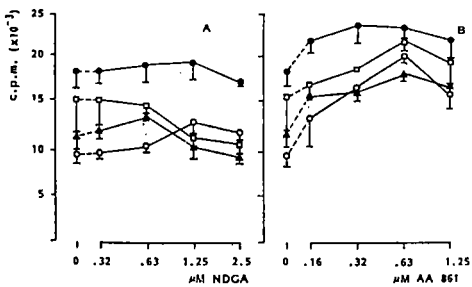

Fig 4 Comparative inhibition of A23187-induced cytostatic activity by NDGA and AA-861. P815 cells were cocultured with samples I,II and III (Ratio = 2) in addition of 3, 5 × 10^{-7}M A23187 and NDGA (Fig. A), or AA-861 (Fig. B) for 24 hr.. ^{3}H- Thymidin uptake by P815 cells (•) and ratio 2 (I = ○, II = ▲, III = □) was expressed as mean c.p.m. ± S.D. of triplicate wells.

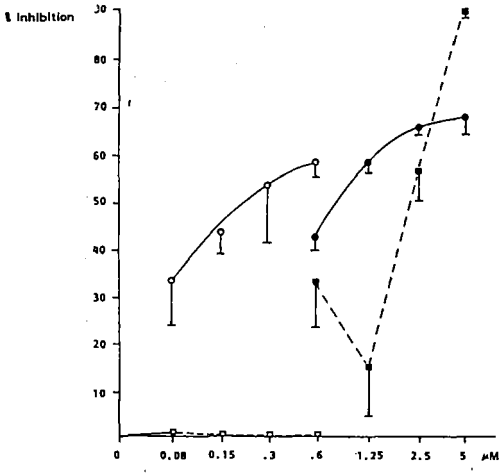

Fig 5 Inhibition of cyclooxygenase- (6-keto $PGF_{1\alpha}$) (--) and lipoxygenase- (LTB_4) (—) metabolites release from pooled cell samples I,II and III (5×10^5/ml) with NDGA (closed symbols) and AA-861 (open symbols). Stimulation with A23187 (3.5×10^{-7}) increased 6-keto $PGF_{1\alpha}$ (19 ng/ml) and LTB_4 (250 pg/ml) release. Results are expressed as means ± S.D. of triplicate samples.

0.6 μM AA-861 inhibited LTB_4 release (57%) and did not inhibit 6-keto $PGF_{1\alpha}$ release of the pooled samples I, II and III (Fig. 5).

Discussion

A23187 activates several apparently distinct phospholipid degrading processes (12). Once AA is released from phospholipids, it is available for conversion by cyclooxygenase and lipoxygenase (14). Our studies showed that the large peritoneal MAC-1^+ cell populations are the major source of both types of AA metabolites. A23187 also stimulated the cytostatic activity of MP enriched samples (I, II>85% MP). AA-861 (0.6 μM) inhibited generation of LTB_4 (IC_{50} = 0.27 μM) in pooled cell samples I, II and III, which confirms with the finding of Mita et. al. (27) in human polymorphonuclear leukocytes . AA-861 also reversed the MP cytostatic function. It is generally accepted that the cyclooxygenase product PGE_2 inhibits MP activity (18) and it has been shown that leukotrienes activate MPs (20). Our studies with AA-861 indicate that a 5-lipoxygenase product is required for expression of MP cytostatic activity by A23187. NDGA, a non specific inhibitor had no effect on MP cytostasis (Fig. 4). However, lipoxygenase inhibition by NDGA (>0.3 μM) was associated with cyclooxygenase inhibition (Fig. 5). Thus synthesis of potentially inhibitory cyclooxygenase metabolites was prevented (i.e. the ratio cyclo-/lipoxygenase metabolites remained low) in A23187 activated MPs, exposed to NDGA. This emphasizes the importance of the balance between cyclo-/lipoxygenase eicosanoids in regulating MP functions. The mechanism by which A23187 increases cytostatic activity towards P815 cells remains speculative; reactive oxygen production (25), tumor necrosis factor (T.N.F.) release and cell-cell contact could be important. Our results clearly demonstrate however that 5-lipoxygenase products play an important role in Ca^{2+}-mediated activation of MP cytostasis.

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Chapter 5.

LEUKOTRIENE C_4 IS AN ESSENTIAL 5-LIPOXYGENASE INTERMEDIATE IN A23187-INDUCED MACROPHAGE ANTITUMOR CYTOSTATIC ACTIVITY AGAINST P815 CELLS

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LEUKOTRIENE C_4 IS AN ESSENTIAL 5-LIPOXYGENASE INTERMEDIATE IN A23187-INDUCED MACROPHAGE CYTOSTATIC ACTIVITY AGAINST P815 TUMOR CELLS

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ABSTRACT

Resident peritoneal macrophages incubated with 3.5 x 10^{-7} M Calciumionophore A23187 in tumor cell growth medium (TGM) release large amounts of leukotriene (LT)E_4 and an unidentified 5-lipoxygenase product, whereas A23187-stimulated macrophages produce in serum free medium LTD_4, predominately. LTC_4 and ^{3}H-LTC_4 incubated for 20 minutes at 37°C in serum containing TGM, convert into LTE_4 and ^{3}H-LTE_4, respectively. Thus, LTC_4 released from A23187-stimulated macrophages is an intermediate in TGM which rapidly converts into LTE_4 probably because of presence of gamma-glutamyl transpeptidase and cystenyl-glycinase in TGM. Macrophages express antitumor cytostatic activity towards P815 cells (49-53 %) in a cocultured ratio (macrophage: tumor cell) 2:1 when stimulated with 3.5 x 10^{-7} M A23187 in TGM. The 5-lipoxygenase inhibitor AA861 reverses the cytostatic activity by 42-58% and it inhibits also the formation of A23187-induced 5-lipoxygenase products from macrophages. Restoration of 38% macrophage- antitumor cytostatic activity by exogenous LTC_4 (10^{-8}M) indicates that LTC_4 is an essential 5-lipoxygenase intermediate in the pathway of required signals underlying A23187-induced macrophage antitumor cytostatic activity. Macrophages not stimulated by A23187 do not express cytostatic activity in the presence of LTC_4. This implicates that besides LTC_4, increased cytosolic [Ca^{2+}] is required for A23187 induction of macrophage cytostatic activity.

INTRODUCTION

Activated macrophages can selectively destroy neoplastic cells (1) and increased cytosolic [Ca^{2+}] appear to be an important feature in macrophage antitumor cytostatic activity (2) and in priming macrophage antitumor cytotoxic functions (3,4) at a certain stage of macrophage maturation. It has also been demonstrated that the development of macrophage antitumor activity towards P815 cells <u>in vitro</u> is regulated by eicosanoids (5). Therefore, many investigators gave great attention to elucidate the signal transduction pathway in macrophage activation that involves increased cytosolic [Ca^{2+}] and eicosanoids release.
We assayed previously Calciumionophore A23187-stimulated peritoneal macrophages for their cytostatic activity and

eicosanoids release. Macrophages were separated on the basis of cell size in a discontinuous fetal bovine serum (FBS) gradient column and cocultured with A23187 and P815 tumor cells in vitro. Under these circumstances macrophages expressed antitumor cytostatic activity and showed an increased 5-lipoxygenase and cyclooxygenase metabolites release (2). By preincubation of macrophages with inhibitors of eicosanoids release, this assay could serve as a model to study the role of endogenous AA metabolites in regulation of resident macrophage activation by increased cytosolic [Ca^{2+}]. Our previous work demonstrated that the expression of resident macrophage cytostatic activity by A23187 in a fetal bovine serum (FBS) conditioned-RPMI tumor cell growth medium (TGM) towards P815 tumor cells required endogenous 5-lipoxygenase metabolites synthesis (2).

The cascade of signals involved in the mechanism by which A23187 (increased cytosolic [Ca^{2+}]) could induce macrophage activation are undefined but in the presence of sufficient Ca^{2+} and an intact energy metabolism, liberated AA released from phospholipids can be further metabolized by 5-lipoxygenase (6), into 5-hydroperoxy eicosatetraenoic acid (5-HPETE) and leukotriene (LT) A_4. There are reported data, showing that A23187 generates 5-lipoxygenase- and cyclooxygenase metabolites release also in other tissue macrophages (7-9) and it has been reported that A23187 induces cytosolic 5-lipoxygenase translocation to a membrane bound site where it will be utilized and consequently inactivated (10). The sulfidopeptide LTC_4 can be catalyzed by cytosolic glutathione transferases and glutathione from LTA_4 (11) and it is now widely appreciated that LTC_4 rapidly undergoes sequential peptide cleavage reactions to LTD_4 and LTE_4 in the presence of gamma-glutamyl transpeptidase and cysteinyl-glycinase (12), respectively. Supernatants of other cells like polymorphonuclear granulocytes stimulated with A23187 revealed these two enzymes activities (13). LTE_4 can be further converted by N-acetyl-transferase and oxidated by omega-oxidation in hepatocytes (14), or LTF_4 can be derived from LTE_4 by transpeptidase and glutathione (15).

The aim of the present work was to determine the cascade of LTs formation from A23187-stimulated macrophages as related to expression of cytostatic activity towards P815 tumor cells.

MATERIALS AND METHODS

Materials

A23187 (Antibiotic A23187, Calciumionophore), free acid (diluted in ethanol absolute: 1 mM) was purchased from Calbiochem-Behring, U.S.A.. LTB_4 and LTD_4 were bought from Sigma U.S.A.. LTE_4 and LTC_4 were gifts from Dr. J. Rokach (Merck Frost, Canada). AA-861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dedocadinyl)-1,4-benzoguinone) (diluted in ethanol absolute 5 mM) was a gift from Dr. S. Terao (Takeda Chem. Reseach Div., Osaka, Japan). ^{3}H-LTC_4 and ^{3}H-thymidine were obtained from Amersham (England). RPMI 1640 and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco Europe BV. L-Glutamine, penicillin/streptomycin were ordered from

Flow Laboratories, β-mercaptoethanol was purchased from Merck (F.R.G.). 96-Microwell flat-bottom trays were purchased from NUNC (Denmark) and Fetal Bovine Serum (FBS 1064) was bought from Sanbio BV biological products (The Netherlands). Filtermats (Skatron) for a 12 well cell harvester (Colinca, Tel Aviv, Israel) and cell culture flasks were ordered from Costar (The Netherlands). Sep-Pak C_{18} cartridges were purchased from Waters Assoc.(U.S.A.) and 0.45 µm disposable filters acro LC3A were obtained from Gelman Sci.(The Netherlands). HPLC-solvent filters HVLP (0.45 µm) were purchased from Millipore Corp.. Prepacked HPLC columns Nucleosil $5C_{18}$ (250 x 3 mm) were from Chrompack (The Netherlands). A 1082B high-performance liquid chromatograph (Hewlett-Packard, U.S.A.) was used, consisting of double-head pump, temperature-controlled column compartment, variable-volume injector and variable-wavelength detector. LTD_4/C_4 antibodies were obtained from Advanced Magnetics Inc. U.S.A..

Macrophage separation and target cells

Resident peritoneal cells were obtained from 6-8 week old female BALB/c mice (ordered from Iffa-Credo, Lyon, France), killed by cervical dislocation. Cells were harvested by two peritoneal lavages using 5 ml DMEM. Approximately 10 x 10^6 cells were obtained from 4 mice and resuspended in 2.5 ml 3% FBS-PBS (Dulbecco's Phosphate Buffered Saline) and viability was determined by trypan blue exclusion.. The separation and identification of macrophages have been described previously (2); briefly, peritoneal macrophages were separated on the basis of cell size by velocity sedimentation on a discontinue 8-30% FBS gradient in a siliconized and autoclaved column (4 cm diameter) column. Fractions were collected after 2 hrs. of sedimentation, the cell number in each fraction was determined and velocity sedimentation (v as mm/hr) of each fraction was calculated. The fractions containing macrophages were sampled and numbered I-III; $v_I > 7$, $v_{II} > 5$, $v_{III} > 4$ mm/hr. Cells were washed and characterized by morphological criteria (May-Grunewald Giemsa) and by identification of cell surface antigens with immuno-β-galactosidase staining, using MAC-1 (2) antibodies. Samples I and II are macrophage enriched fractions (>85%). Sample III represents a mixture of macrophages, granulocytes and lymphocytes (70 % $MAC\text{-}1^+$) and cells with a v< 4 mm/hr are lymphocytes (>90%). The incubation experiments with A23187 were performed with pooled samples I and II. P815 tumor cells were a gift from Prof. I. Zan-Bar, Dept. Human Microbiology, Tel Aviv University, Israel and were cultured in vitro at 37° C in TGM (stored at 0-4°C) of 10% FBS and RPMI 1640 with 2mM L-glutamine, 2 g/l $NaHCO_3$, 5 x 10^{-5}M β-mercaptoethanol and 100 IU/ml penicilin/streptomycin in 7,5 % CO_2 air. For cytostasis experiments P815 cells were resuspended at an amount of 2,4 x 10^5/ml and 10^5 cells were transfered each third day of culture (viability >95%).

Macrophage antitumor cytostasis assay.

The cytostatic activity of cells from macrophage enriched

samples I and II on P815 cells was manifested as inhibition of ^{3}H-thymidine uptake by tumor cells. Twentyfive µl (5 x 10^5/ml) of samples I or II were preincubated with 25 µl TGM or AA861 diluted in TGM and subsequently exposed to 25 µl A23187 and 25 ul P815 cells (ratio macrophage:P815= 2:1) in a total volume of 100 µl TGM for 24 hours. LTC_4 and LTE_4, were diluted in a AA861/TGM on ice. Biological activity of LTC_4 was checked on smooth muscle contraction of human bronchiolar segments (16). The cells were incubated for an additional 18 hours with ^{3}H-thymidine (0.5 µCi/150 µl) and were collected onto filtermats using a cell harvester. The filtermat was dried and ^{3}H-thymidine uptake was measured using a scintillation β-counter (c.p.m.). Data are expressed as means ± S.D. and represents one out of two other experiments. ^{3}H-thymidine uptake of 1,25 x 10^4 macrophages was < 350 c.p.m. and cytostatic activity was calculated in terms of c.p.m., as follows:

$$\frac{(P815_A - R_A) \times 100\%}{P815}$$

R= ratio 2
A= A23187

Inhibition of cytostatic activity was calculated with:

$$\frac{(P815_A - R_A) - (P815_{A+AA} - R_{A+AA}) \times 100\%}{P815_A - R_A}$$

AA= AA861

Preparation of supernatants from macrophages and P815 cells

2-3 x 10^6 cells from separated samples and P815 cells were preincubated for 5 min. with and without AA861 and subsequently exposed to A23187 (3,5 x10^{-7}M) at 37°C in 600 µl DMEM (pooled samples I-III: 20 min.) or in 600 µl TGM (pooled samples I and II:20 min.). At the end of the incubation, the cells were centrifuged (1 min.;12000 g) and supernatant was collected and applied to a Sep-Pak C_{18} cartridge. The cartridge has been prewashed with ethanol and water. The eluates experiments with AA861 and/or A23187 stimulated cell samples were pooled at -70°C and dried in vacuum. The dried samples were dissolved in 100 µl methanol and filtered through an Acro LC3A filter (.45 um). Sampled volumes of 40-75 µl were injected onto the HPLC column. Supernatants of ^{3}H-LTC_4 incubated in TGM were prepared identically.

Separation of LTs by reversed phase HPLC

Reversed-phase HPLC of LTs of the methanol-sampled volumes were carried out on a Nucleosil 5 C_{18} column, using the solvent system: Tetrahydrofuran-methanol-water-acetic acid (25:30:45:0.1) adjusted to pH 5.5 with ammonium hydroxide filtered by vacuum filtering through a Millipore filter and degassed with helium (17). The flow rate was .35 ml/min. and the column was equilibrated with the mobile phase at an oven

temperature of 37°C and the absorption was measured at 280 nm. Fractions were collected for LTs detection by R.I.A.. After each run the column was rinsed with methanol for at least 30 min. The elution position of the LTs was defined, before and after the runs of the samples, by using the chemically identical standards of LTB_4, LTC_4, LTD_4 and LTE_4. Radioactivity in the ^3H-labeled substances were counted on-line in a Berthold radioactivity flow-cell, type LB506C.

Radioimmunoassay of leukotrienes

Fractions collected between 7-8, 10-11 and 12-13 minutes from the HPLC run were evaporated and dissolved in radioimmunoassay

Figure 1.

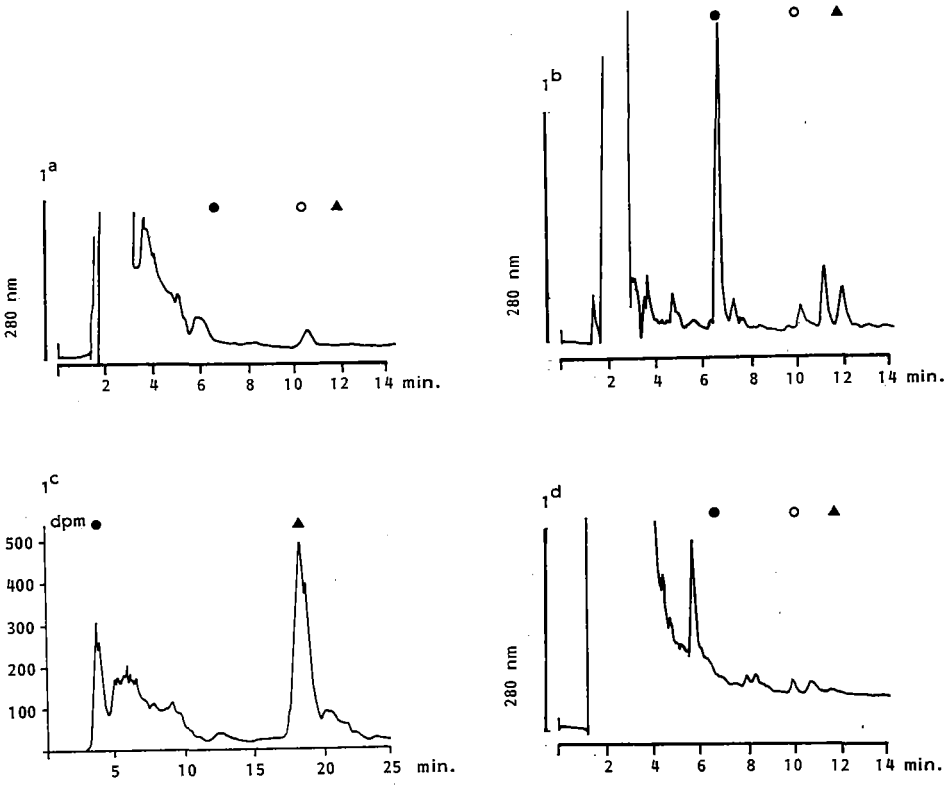


Figure 1
Determinations of leukotrienes after 20 min. incubation at 37°C in conditioned 'tumor cell growth' medium (TGM). Eluates from cell free TGM (Figure 1a), 100 ng LTC_4 incubated in TGM (Figure 1b) and ^3H-LTC_4 incubated in TGM (Figure 1c, separate experiment, not corrected for time-delay). Figure 1d represents eluates from supernatant from non stimulated macrophages ($2x10^6$ cells from pooled Samples I and II). Elution positions:
●= LTC_4, O= LTD_4, ▲= LTE_4, or ^3H-labeled metabolites.

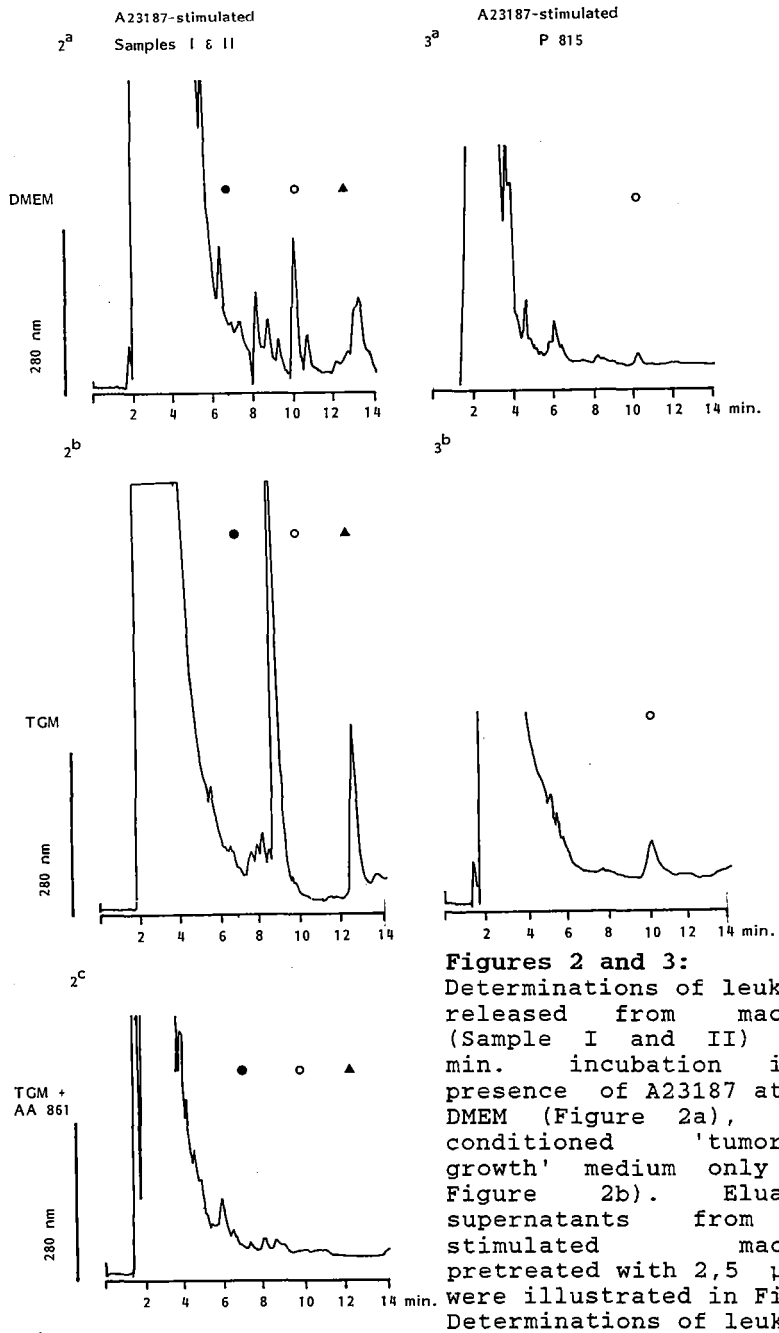
(R.I.A.) buffer for assay of LTC_4, LTD_4 and LTE_4 by direct R.I.A. with LTC_4/D_4 antibodies (cross reactivity: (LTD_4 100%, LTC_4 64% and LTE_4 7.3%).

Statistical analysis

All data regarding % cytostatic activity are represented as mean of three or six experiments each from triplicate determinations and were statistically analysed by unpaired Student's -t test ($p< 0.01$).

RESULTS

In the first series of experiments the effect of incubation of TGM (without macrophages) at 37°C for 20 min. on LT concentrations was determined. As shown in Figure 1a, fresh TGM contains detectable amounts of LTD_4 at elution time (t_R) of 10.5 min. when incubated for 20 min. at 37°C. Under similar conditions, LTC_4 (100 ng) incubated in TGM induces increase in formation of LTD_4 and LTE_4 (t_R 10.5 and 12.0 min., respectively: Figure 1b). This finding has been confirmed by the formation of ^3H-LTE_4 from ^3H-LTC_4 incubated at 37°C for 20 min. in TGM (Figure 1c). Macrophages nonstimulated with A23187 of pooled samples I and II, do not release detectable amounts of LTC_4, LTD_4 and LTE_4 over the amounts released in TGM alone (Figure 1d). Macrophages from pooled samples I and II stimulated by A23187 in DMEM release LTC_4 (Measured by RIA: 400 pg in fraction 6-7 min.), mainly LTD_4, a small amount of LTE_4 and an unidentified 5-lipoxygenase product at t_R 9 min. (Figure 2a). On the other hand, A23187 stimulated macrophages from pooled samples I and II, release in TGM large amounts of LTE_4 at t_R 12.0 min. and of the unidentified 5-lipoxygenase product at t_R 9 min. (Figure 2b). Incubation of macrophages with 2.5 µM AA861 (17) before stimulation with A23187 in TGM resulted in inhibition of release of LTE_4 and of the unidentified 5-lipoxygenase product (Figure 2c).
It seemed to be of interest to determine if P815 tumor cells release leukotrienes when incubated with A23187. As shown in Figure 3, P815 tumor cells incubated with A23187 release detectable amounts of LTD_4 when kept either in DMEM (Figure 3a) or in TGM (Figure 3b) for 20 min. at 37°C. We found that a concentration of $3,5 \times 10^{-7}$M Ca.ionophore A23187 was not cytostatic by itself against P815 tumor cells, but induced cytostatic activity in macrophages from sample I (53% $\pm$ 5) and sample II (49% $\pm$ 10, n=6). We reported previously (2), that this cytostatic activity induced in macrophages by stimulation with A23187 was significantly inhibited by preincubation with AA861. We confirmed these data with 5 µM AA861 (Sample I: 19% $\pm$ 6 and Sample II: 17% $\pm$ 12 cytostatic activity, n=3, $p< 0.01$, representative experiment: Figure 4). Exogenous LTC_4 (10^{-8}M) restores the AA861 (5 µM) - inhibited cytostatic activity towards P815 cells of macrophages treated with A23187 (38% cytostatic activity, n=3, $p<0.01$, representative experiment: Figure 4a), whereas a similar treatment with LTE_4 was not effective (Figure 4b). Macrophages not stimulated with A23187 but incubated with LTC_4 (< 10^{-7}M) do not express antitumor cytostasis towards P815 cells (Figure 5).



Figures 2 and 3:
Determinations of leukotrienes released from macrophages (Sample I and II) after 20 min. incubation in the presence of A23187 at 37° C in DMEM (Figure 2a), and in conditioned 'tumor cell growth' medium only ("TGM": Figure 2b). Eluates of supernatants from A23187 stimulated macrophages pretreated with 2,5 µM AA861 were illustrated in Figure 2c. Determinations of leukotrienes after incubation of P815 cells in the presence of A23187 at 37° C in DMEM (Figure 3a) and in TGM (Figure 3b). Elution positions: ● = LTC_4, O = LTD_4, ▲ = LTE_4.

4a Figure 4 4b

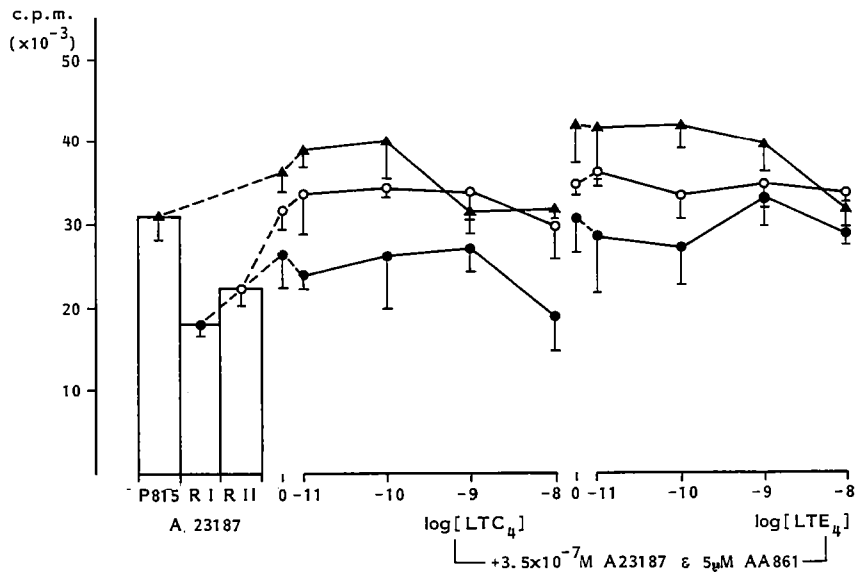


Figure 4a: ^{3}H-Thymidine uptake of P815 cells alone (▲) cocultured with AA861 (5μM) + LTC_4 - pretreated macrophages from Samples I (●) and II (o) (Ratio 2), in the presence of $3.5x10^{-7}$M A23187. The bar-diagrams represent ^{3}H-thymidine uptake of P815 cells alone and in coculture with Sample I (R_I) and II (R_{II}) in addition of A23187. Figure 4b: Similar experiment as illustrated in Figure 4a but with AA861 (5 μM) + LTE_4-preincubated macrophages from Samples I (●) and II (o) and P815 cells (▲). Data are presented as mean c.p.m. ± S.D. of triplicate determinations.

DISCUSSION

We reported previously (2),that A23187 stimulated the cytostatic activity of murine peritoneal macrophages towards P815 tumor cells. This activity was correlated with induced release of 5-lipoxygenase products by A23187 from macrophages, because a specific 5-lipoxygenase inhibitor, AA861 (18) prevented generation of this antitumor cytostatic activity by the Ca. ionophore (2).

The aim of the present work was to determine the type of LTs responsible for expression of cytostatic activity towards P815 tumor cells by A23187 stimulated macrophages. The presence of LTs was determined in medium alone, medium with unstimulated macrophages and medium containing macrophages stimulated with A23187. We looked also on the effect of a 5-lipoxygenase inhibitor, AA861, on the release of LTs. The data obtained were evaluated in terms of correlation between various situations of LT release or inhibition of LT release and the expression of cytostatic activity towards P815 tumor cells.

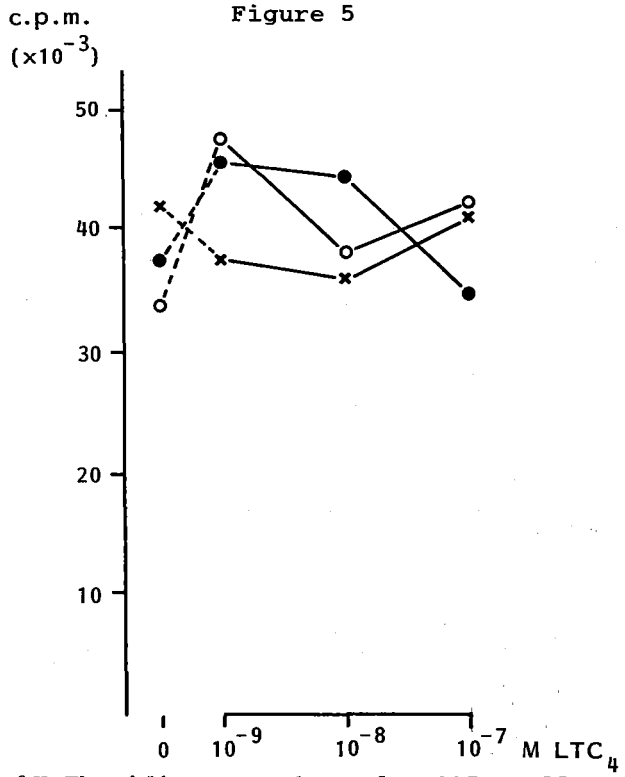


Figure 5. ^{3}H-Thymidine uptake of P815 cells alone (x) cocultured with macrophages from Samples I (●) and II (o) (Ratio 2) in the presence of LTC_4. S.D. of triplicate determinations was less than 10%.

The TGM itself (without macrophages) contained detectable amounts of LTD_4 probably present in FBS. In view of the fact that LTC_4 added to TGM was converted into LTE_4, it seems likely that gamma-glutamyl transpeptidase and cysteinyl-glycinase are present in TGM, because these enzymes are required for the formation steps of LTC_4 into LTD_4 (19) and further into LTE_4 (20). This assumption was further strenghtened by the difference observed in LTs release by A23187 stimulated macrophages in DMEM (serum-free medium) in comparison with A23187 stimulated macrophages in TGM. Thus, incubation of macrophages with A23187 in DMEM resulted in accumulation of released LTD_4 whereas incubation in TGM led to release of high amounts of LTE_4 and of an unidentified 5-lipoxygenase product probably by rapid conversion of released LTC_4.
Macrophages stimulated by A23187 in TGM were cytostatic towards P815 tumor cells. The cytostatic activity was prevented by treatment with AA861, a 5-lipoxygenase inhibitor. It appears therefore, that release of 5-lipoxygenase products is correlated with occurence of antitumor activity in macrophages. The fact that LTC_4 is the 5-lipoxygenase product involved in

macrophage antitumor activity was indicated by the finding that treatment with LTC_4 in addition to AA861 treatment, restored antitumor cytostatic activity of A23187-stimulated macrophages. This observation suggest that endogenous LTC_4 is an essential intermediate in the pathway of required signals in induction of macrophage antitumor cytostasis by increased cytosolic $[Ca^{2+}]$. However exogenous LTC_4 could not induce antitumor cytostasis towards P815 cells in untreated macrophages (not incubated with A23187 and AA861). This implicates that besides LTC_4, also increased cytosolic Ca^{2+} is required for induction of A23187-stimulated macrophage antitumor cytostatic activity.
Detection of LTD_4 in supernatants of P815 cells exposed to A23187 in TGM suggests that the formed LTC_4 (21) rapidly converts into LTD_4 by gamma-glutamyl transpeptidase present plausibly in TGM. We also observed that A23187 - induced macrophage 5-lipoxygenase metabolites release is enhanced in TGM (Figure 2a and 2b). This could confirm the observations of Kouzan et.al.(22), who demonstrated a shift of alveolar macrophage AA metabolism towards 5-lipoxygenase metabolites release by FBS.
Increasing LTC_4 release (23,24) from macrophages and/or increased cytosolic $[Ca^{2+}]$(25,26) in macrophages have been described in response to many inflammatory stimuli. This study with A23187 demonstrated the importance of these increases in macrophage antitumor cytostasis.

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Chapter 6.

ADDITIVE EFFECT OF L-SERINE AND CALCIUM IONOPHORE A23187 ON INDUCTION OF MACROPHAGE ANTITUMOR CYTOSTASIS: RELATION TO ACCUMULATION OF LEUKOTRIENE C_4 FORMED IN MACROPHAGES

J.A. van Hilten, S. Ben Efraim, F.J. Zijlstra and I.L. Bonta

(Submitted for publication)

Additive effect of L-serine and calcium ionophore A23187 on induction of macrophage antitumour cytostasis: Relation to accumulation of leukotriene C_4 in macrophages

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Running title:
Macrophage antitumour cytostasis & -LTC_4 formation

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Summary

1. Murine peritoneal macrophages are cytostatic towards P815 murine mastocytoma tumour cells when treated with 100 mM L-serine: 62-78% inhibition of ^{3}H-thymidine incorporation.
2. Murine peritoneal macrophages are cytostatic towards P815 tumour cells when incubated with A23187 Calcium Ionophore: 49-53% inhibition of ^{3}H-thymidine incorporation by 0.35 µM.
3. Combination of L-serine and A23187 had an additive effect: 91-95% inhibition of ^{3}H-thymidine incorporation.
4. The following patterns of leukotrienes (LT) release was observed; small amounts of LTC_4 and LTE_4 by unstimulated macrophages, large amounts of LTE_4 and of unidentified LT by A23187-treated macrophages and large amounts of LTC_4 by either L-serine treated or L-serine plus A23187 treated macrophages.
5. Our results indicate that accumulation of LTC_4 in macrophages is correlated with high antitumour cytostatic activity.

Introduction

Macrophage antitumour activity in vitro was found to be regulated by eicosanoids (Drapier & Petit, 1986), so that exogenous leukotrienes (LTs) (5-lipoxygenase metabolites) augment (Gagnon et al., 1989; Ophir et al., 1987) and prostanoids (cyclooxygenase metabolites) reduce (Schultz et al., 1978; Taffet et al., 1981; Renz et al., 1988; Lehman et al., 1988) macrophage antitumour activity.
Stimulated by the calcium ionophore A23187, murine macrophages release increased quantities of 5-lipoxygenase and cyclooxygenase products (Du et al., 1983; Laviolette et al., 1988; Balter et al., 1989) and acquire cytostatic activity against P815 tumor cells (van Hilten et al., 1988). Under our assay conditions, macrophages stimulated by A23187 induced the biosynthesis of LTC_4, which converts rapidly into LTE_4 and in an unidentified LT (van Hilten et al., 1990).
It has been reported that L-serine binds to the active (gamma-glutamyl) site of gamma-glutamyl transpeptidase (Thompson & Meister, 1979), which is important in the peptide cleavage of LTC_4. Accordingly, high concentrations of L-serine can inhibit the conversion of LTC_4 into LTD_4, by competitive binding of gamma-glutamyl transpeptidase (Orning & Hammerstrom, 1980), and could so induce accumulation of LTC_4 in macrophages. Conceivably, accumulation of LTC_4 in macrophages might be related to induction of antitumor cytostatic activity of these cells. The aim of the present research was to investigate this possibility by determination of the effect of L-serine alone or in combination with A23187, on induction of macrophage antitumour cytostatic activity against P815 murine mastocytoma cells.

Methods

Materials. A23187 (Antibiotic A23187, Calciumionophore), free acid (diluted in ethanol absolute: 1 mM) (Calbiochem-Behring, U.S.A.), ^{3}H-thymidine (Amersham, England), RPMI 1640 and

Dulbecco's Modified Eagle Medium (DMEM) (Gibco Europe BV), L-Glutamine, penicilin/streptomycin (Flow Laboratories, The Netherlands) and β-mercaptoethanol (Merck F.R.G.) were used. 96-Microwell flat-bottom trays were purchased from NUNC (Denmark) and Fetal Bovine Serum (FBS 1064) was bought from Sanbio BV biological products (The Netherlands). Filtermats (Skatron) for a 12 well cell harvester (Colinca, Tel Aviv, Israel) and cell culture flasks were ordered from Costar (The Netherlands). Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (U.S.A.) and 0.45 µm disposable filters acro LC3A were obtained from Gelman Sci. (The Netherlands). HPLC-solvent filters HVLP (0.45 µm) were purchased from Millipore Corp.. Prepacked HPLC columns Nucleosil $5C_{18}$ (250 x 3 mm) were from Chrompack (The Netherlands). A 1082B high-performance liquid chromatography (Hewlett-Packard, U.S.A.) was used, consisting of double-hed pump, temperature-controlled column compartment, variable-volume injector and variable-wavelength detector. Leukotrienes were a gift from Dr. J. Rokach (Merck Frost, Canada) and L-serine was bought from Aldrich-Europe (Belgium).

Macrophage separation and target cells. The separation and identification of macrophages have been described previously (van Hilten et al., 1988): Briefly, murine BALB/c peritoneal cells were separated on the basis of cell size by velocity sedimentation on a discontinue 8-30% FBS gradient in a siliconized and autoclaved column. Macrophage enriched samples I and II (> 85%), were collected after 2 hrs. of sedimentation. Leukotrienes were determined in supernatants from pooled samples I and II. Exposure of 2 hrs. to 200 mM L-serine did not affect macrophage viability. P815 tumour cells were cultured in 10% serum containing tumor growth medium ('TGM': stored at 4°C), as described (van Hilten et al., 1988).

Macrophage antitumour cytostasis assay. The cytostatic activity of macrophage samples I and II cocultured with P815 tumour cells, was manifested as inhibition of ^{3}H-thymidine uptake by tumour cells (van Hilten et al., 1988). Twentyfive µl ($5x10^5$/ml) of samples I and II were preincubated (5 min.) either with 25 µl L-serine (400 mM) diluted in tumour growth medium (TGM, pH 7.2), or with TGM only. Twentyfive µl of macrophage samples were subsequently mixed without washing, with 25 µl P815 cells (Effector cell: P815 cell ratio= 2:1) and cultured for 24 hrs. with or without 25 µl A23187 (1.4 µM) in a total volume of 100 µl TGM. Data in Figs. 1 and 2 are expressed as means ± S.D. and represent one out of two similar experiments. Mean % cytostatic activity was calculated ± S.D. of three or six experiments. Calculations of % inhibition of tumour cell growth was performed as described (van Hilten et al., 1988).

Preparation of supernatants from macrophages. The macrophage pooled samples I and II (2×10^6 cells/ 250 µl) were incubated for either 20 min. or 24 hrs at 37°C. Supernatants collected after 20 min. incubation originated from macrophages first incubated for 5 min. with (Fig. 4B) or without (Fig. 4A) 250 µl L-serine (400 mM L-serine in TGM) and subsequently diluted and incubated without washing in the presence of A23187 (250 µl of 1.4 µM). Supernatants collected after 24 hrs. incubation

originated in a similar way from macrophages first incubated with (Fig. 3B) or without (Fig. 3A) L-serine for 5 min. and subsequently incubated without washing for 24 hrs in a total volume of 1 ml TGM. The final L-serine concentration in the serine pretreated macrophage suspensions was 100 mM. At the end of the incubation, the cells were centrifuged (5 min.,1500 g) and supernatants were collected and applied to a Sep-Pak C_{18} cartrige. The cartridge was prewashed with ethanol and water. The eluates were stored at -70° C and dried in vacuum. The dried samples were dissolved in 75 µl methanol, filtered through an Acro LC3A filter (0.45 µm) and injected onto the HPLC column.

Separations of LTs by reversed phase HPLC: Reversed-phase HPLC of LTs of the methanol-volumes were carried out on a Nucleosil 5 C_{18} column, using the solvent system: Tetrahydrofuran-methanol-water- acetic acid (25:30:45:0.1) adjusted to pH 5.5 with ammonium hydroxide filtered by vacuum filtering through a Millipore filter and degassed with helium (Zijlstra & Vincent, 1984). The flow rate was 0.35 ml/min. and the column was equilibrated with the mobile phase at an oven temperature of 37° C and the absorption was measured at 280 nm. After each run the column was rinsed with ethanol for at least 30 min.. The eluation position (T_R) of the LTs was defined, before the runs of the samples, by using the chemically identical standards of LTC_4, LTD_4 and LTE_4. Results shown in Fig. 4 were obtained separately and results shown in Fig. 3 were subsequently performed with new columns.

Figure 1

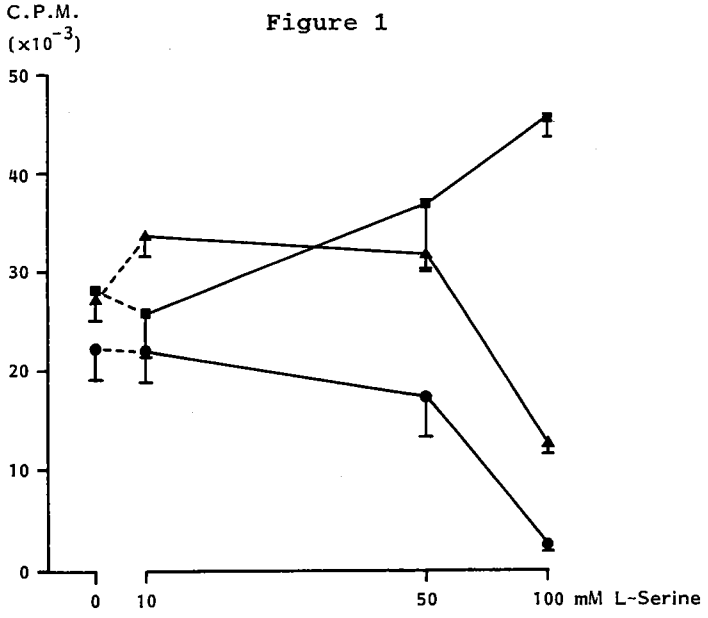

Figure 1. The effect of L-serine on ^{3}H-thymidine uptake of P815 cells alone (■) or cocultured with macrophages from sample I (●) and II (▲). Data are presented as mean c.p.m. ± S.D. of triplicate determinations.

Figure 2.

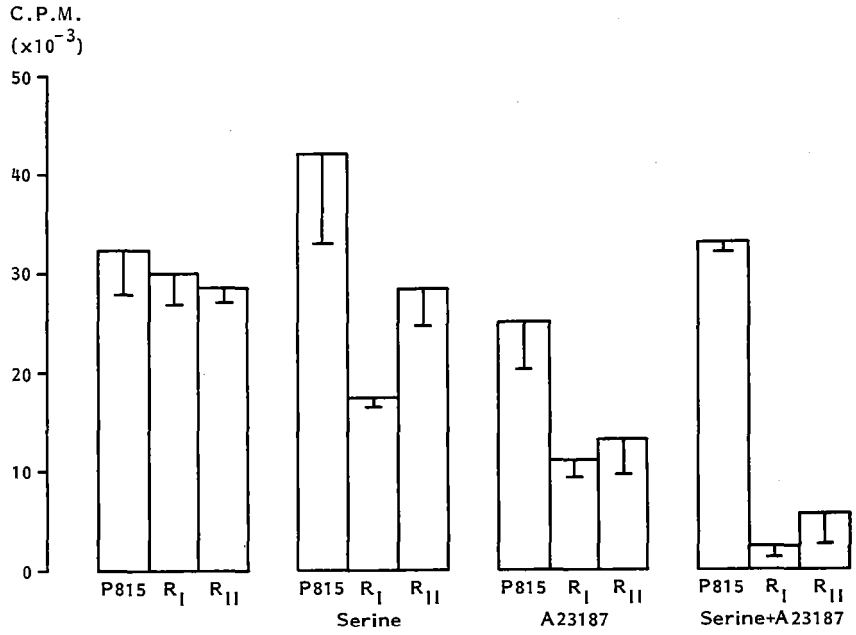

Figure 2. The effect of L-serine (100 mM), A23187 (0.35 µM) and L-serine + A23187 on ^{3}H-thymidine uptake of P815 cells alone (P815), or cocultured with sample I (R_I) and with sample II (R_{II}). Data are presented as mean c.p.m. ± S.D. of triplicate determinations.

Results

The highest concentration of L-serine used (100 mM), had no cytostatic activity towards P815 tumour cells. On the contrary, L-serine concentration > 10 mM promoted P815 cell growth (Fig 1). Macrophage preparations from samples I and II expressed antitumor cytostatic activity (78% ± 18 and 62% ± 25 inhibition of thymidine incorporation by P815 cells, respectively, n=3), when preincubated with 200 mM L-serine and subsequently exposed (by dilution) to 100 mM L-serine during 24 hrs in coculture at a ratio of 2:1 (macrophage/tumor cell) (Fig. 1 and Fig. 2). In confirmation with previous results (van Hilten *et al.*, 1988; 1990), macrophages from samples I and II stimulated with A23187 (0.35 µM) expressed antitumour cytostatic activity of 53% ± 5 and 49% ± 10 inhibition of thymidine incorporation, respectively (n=6). L-serine preincubated macrophages expressed enhanced antitumour cytostasis (sample I: 95% ± 2 and sample II: 91% ± 6, n=3) when subsequently exposed to 0.35 µM A23187 in addition to 100 mM L-serine. Untreated macrophages were not cytostatic (Fig. 2).

Figures 3 and 4

Figure 3.

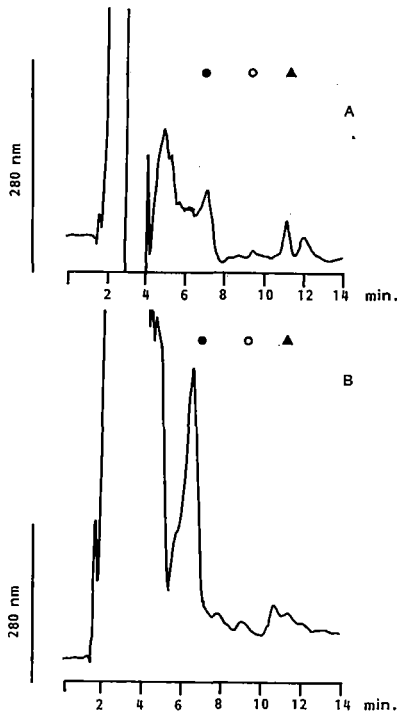

Figure 4.

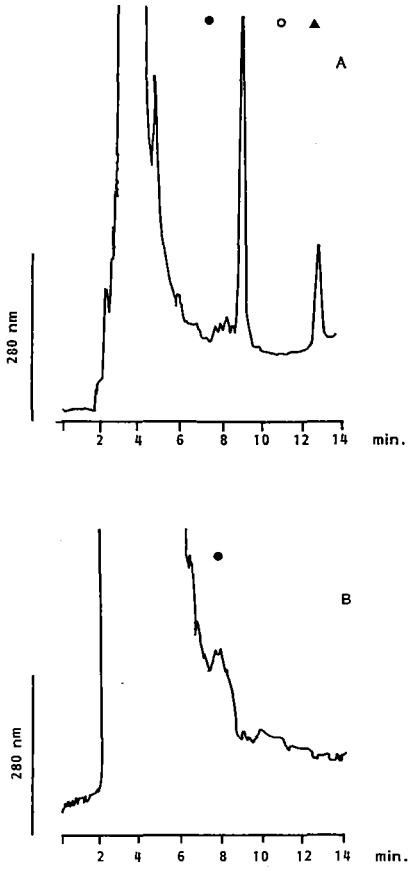

Figure 3. Determinations of leukotrienes released from macrophages (2 x 10^6 cells from pooled sample I and II) without pretreatment (3A) and with serine pretreatment (3B) after 24 hours incubation at 37° C.
Figure 4. Determination of leukotrienes released from macrophages (2 x 10^6 cells from pooled sample I and II) after 20 min. incubation with 0.35 µM A23187 alone (Figure 4A). Figure 4B represents the determination of leukotriene released from serine (200 mM)-pretreated macrophages after subsequently incubation during 20 min. with 0.35 µM A23187 and 100 mM serine. ● =LTC_4 O =LTD_4 ▲ =LTE_4 .

Small amounts of detectable LTC_4 at T_R 7 min. and LTE_4 at T_R 11 min. have been observed in supernatants of unstimulated macrophages from pooled sample I and II (Fig. 3A), whereas serine-treated macrophages released a large amount of LTC_4 and

a small detectable amount of LTE_4 (Fig 3B) after 24 hrs. incubation at 37°C (we noticed a small shift from both LTC_4 and LTE_4 in Fig. 3B). Shown previously (van Hilten et al., 1990) and in Fig 4A, A23187 stimulated macrophages from pooled sample I and II release large amounts of LTE_4 at T_R 12.5 min. and an unidentified LT at T_R 9 min., whereas macrophages treated with A23187 and L-serine (100 mM) release only LTC_4 at T_R 7.5 min. after 20 min. incubation at 37°C (Fig 4B).

Discussion

Previous research indicated that increased [LTC_4] and increased cytosolic [Ca^{2+}] are essential in A23187-induced macrophage antitumour cytostasis (van Hilten et al., 1990). Based on the implication that the rapid conversion of LTC_4 into the not effective LTE_4 (van Hilten et al., 1990), could limit A23187-induced macrophage cytostatic activity, we used L-serine (inhibitor of LTC_4 conversion) for further studies of cytostatic activity and LTs formation. We demonstrated that L-serine induced antitumour cytostatic activity in macrophage samples I and II was related to increase in LTC_4 formation by comparison with macrophages nonstimulated by L-serine. In confirmation with previous results (van Hilten et al., 1990), A23187 induced both macrophage cytostatic activity and LT formation. Neither L-serine, nor A23187, were cytostatic for tumour cells in absence of macrophages. Serine pretreatment of macrophages and subsequent exposure to A23187 induced a cytostatic effect much higher than either L-serine or A23187 alone. The enhanced cytostatic activity by macrophages treated with both L-serine and A23187 was again related to increase in LTC_4 accumulation. Moreover, we found recently (results not shown here), that a lower concentration of A23187 (0.3 µM), which by itself, did not induce significant macrophage cytostatic activity, was still able to enhance the cytostatic activity of L-serine. The findings presented here favour the assumption that endogenous LTC_4 accumulation in macrophages induced by L-serine treatment, is related to induction of macrophage antitumour cytostasic activity by the agent. It is also suggested that low [A23187] treatment in combination with L-serine could increase macrophage cytostatic activity by increased macrophage 5-lipoxygenase activity (van Hilten et al., 1988) and rapid accumulation of formed endogenous LTC_4 in macrophages (fig. 4).

In conclusion, using serine and A23187 we developed a stimulus for immunomodulation of resident peritoneal macrophage functions expressed by strong inhibition of tumour cell growth. In context to our observed relation of mature resident macrophage cytostatic activity to increased [Ca^{2+}] and LTC_4 biosynthesis, the enzyme gamma-glutamyl transpeptidase affects macrophage activation by decreasing macrophage endogenous [LTC_4].

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Chapter 7.

CALCIUM IONOPHORE A23187 INDUCES DUAL CHANGES IN THE RELEASE OF 5-LIPOXYGENASE AND CYCLOOXYGENASE PRODUCTS BY MACROPHAGES

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Calcium Ionophore A23187 Induces Dual Changes in the Release of 5-Lipoxygenase- and Cyclooxygenase Products by Macrophages

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Abstract

A23187-treated murine peritoneal macrophages release increased quantities of the immunoreactive 5-lipoxygenase metabolite leukotriene B_4 (LTB_4) and the immunoreactive cyclooxygenase products 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) and tromboxane B_2 (TXB_2) during a 40 minutes incubation period. The increase in release of LTB_4 was marked already after 5 minutes of incubation and was maximal after 20 minutes incubation. The increase in release of 6-keto $PGF_{1\alpha}$ and TXB_2 started in most cases after 5 minutes of incubation and augmented gradually up to 40 minutes of observation period of incubation. The ratio of increase of LTB_4/6-keto $PGF_{1\alpha}$ and of LTB_4/TXB_2 revealed an increase in favour of LTB_4 in the first 5 minutes of incubation.

Running title: A23187-induced macrophage eicosanoids release.

Introduction

Certain inflammatory stimuli as τ-Interferon (Celada and Schreiber, 1986), or dual F_c receptor binding (Aderem et al., 1986) generate Ca^{2+} mobilization in macrophages (MΦs). This could induce MΦ activation as expressed by interleukin-1 release (Matsushima and Oppenheim, 1985), tumor cytostatic- (van Hilten et al., 1988), or cytotoxic effects (Celada and Schreiber, 1986), superoxide release (Sakata et al., 1987), lysosomal release (Takenawa et al., 1982), phagocytosis (Young et al., 1984) and transferrin receptor expression (Weiel et al., 1985). Ca^{2+}- mobilization in MΦs by the calciumionophore A23187 increases 5-lipoxygenase- and cyclooxygenase metabolites release from MΦs (van Hilten et al., 1988). This increase may account for induction of antitumor activity in MΦs by A23187, because increase in leukotrienes (5-lipoxygenase products) production by MΦs during incubation with A23187 was correlated to occurrence of cytostasis against P815 tumor cells (van Hilten et al. 1988). These authors concluded that the balance between lipoxy-/cyclooxygenase products release is important in regulating the antitumor MΦ function. The aim of the present work was to determine the relative (with respect to baseline production) changes induced in release of lipoxygenase and cyclooxygenase products in murine MΦs as related to the period of incubation with A23187. Determinations of leukotriene B_4 (LTB_4), of 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) and of thromboxane B_2 (TXB_2) release from murine MΦs stimulated with A23187 revealed a marked increase of LTB_4 in the first 5 min. and a gradual increase in the cyclooxygenase products 6-keto $PGF_{1\alpha}$ and TXB_2 up to 40 min. of observation period.

Materials and Methods

Materials

The calcium ionophore A23187 (diluted in ethanol: 1.5 mM) was purchased from Calbiochem and Dulbecco's Modified Eagle medium (DMEM) was ordered from Gibco Europe BV. 96-Microwell flat-bottom trays were bought from NUNC. LTB_4-, 6-keto $PGF_{1\alpha}$- and TXB_2-antisera were ordered from Advanced Magnetics Inc., Cambridge, Massachusetts. ^{3}H-LTB_4, ^{3}H-6-keto $PGF_{1\alpha}$ and ^{3}H-TXB_2 were obtained from Amersham Laboratories, England. Fetal Bovine Serum (FBS: heat inactivated by 30',56° C.) was ordered from Flow Laboratories, England. LTB_4, 6-keto $PGF_{1\alpha}$ and TXB_2 were obtained from Sigma (St. Louis, U.S.A.).

Separation and characterization of peritoneal MΦs

The method has been descibed in detail elsewhere (van Hilten et al., 1988). Briefly, resident peritoneal mΦs obtained from BALB/c ByJIco mice were harvested in DMEM and resuspended in 3% FBS-PBS (Dulbecco's Phosphate Buffered Saline supplied with fetal bovine serum). MΦs were separated on the basis of size by velocity sedimentation (Miller and Phillips, 1969; van Hilten et al.,1988). Four samples, namely I, II, III and IV were separated. Samples I.II and III contained respectively 80%, 63% and 15% MΦs as identified by use of anti MAC-2 monoclonal antibodies. Sample IV included mostly lymphocytes.

For kinetic determinations of the effect of incubation with A23187 on eicosanoids release, the fractions I, II and III were pooled in order to obtain all the MΦs present in the peritoneal cell population. As reported previously (Paubelle et al., 1987), lymphocytes show no detectable LTB_4 and 6-keto $PGF_{1\alpha}$ release. Accordingly, contamination of MΦ containing fractions with lymphocytes could not interfere with the effect of A23187 on MΦ eicosanoid release.

Eicosanoids determinations
Cells from samples I,II and III were gathered, washed, resuspended in DMEM (5×10^5/ml), and exposed to 3.5×10^{-7} M A23187 in a microwell. Supernatants were taken out in triplicate after 5, 10, 20 and 40 minutes incubation at 37°C. followed by 12000 g centrifugation (1 min.) to remove cells and stored separately at -70°C. for each eicosanoid determination. Eicosanoid determinations of 6-keto $PGF_{1\alpha}$, TXB_2 and LTB_4 in supernatants were performed by R.I.A.. The detection limits of the R.I.A. for these eicosanoids were 0.8 ng/ml, 20 pg/ml and 10 pg/ml, respectively. The recovery of these cyclooxygenase products is > 97% and of LTB_4 is > 90%. (Measured after 20 minutes incubation at 37°C of these ^3H-labelled eicosanoids). The A23187-induced net release of eicosanoids is:

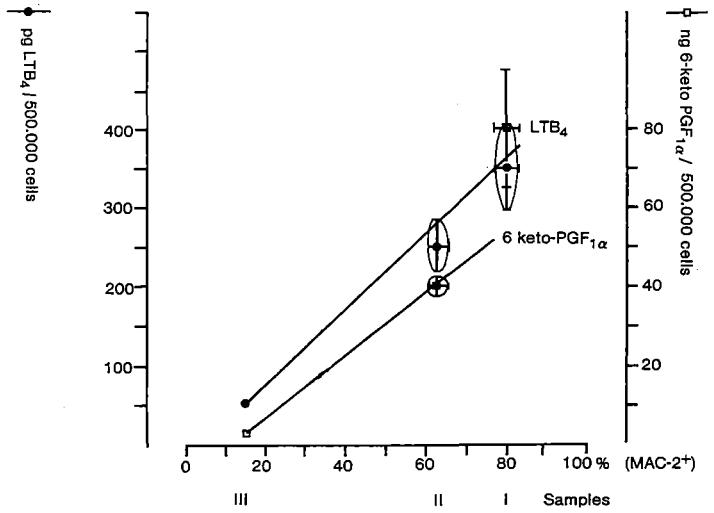
Eicosanoid release stimulated by A23187 minus non-stimulated eicosanoid release

Statistical analysis
Eicosanoid release data are presented as mean $\pm$ S.D. of three experiments in triplicate and statistically analysed by unpaired Student's -t test ($P<0.05$ and $P< 0.01$).

Results

Incubation for 60 minutes with 3.5×10^{-7}M A23187 increased markedly the release of immunoreactive LTB_4 and immunoreactive 6-keto $PGF_{1\alpha}$ (Fig. 1). The quantity released of these products was in correlation with the percentage of MAC-2^+ cell population in fractions I,II and III (Fig. 1). The kinetics of eicosanoid-induced release by A23187 was determined in pooled samples of fraction I,II and III. The A23187-activated cells showed a markedly enhanced release of LTB_4 which was already significant ($p<0.01$) after incubation for 5 min. with A23187 (Fig. 2a). Incubation with A23187 induced also a gradual increase in release of 6-keto $PGF_{1\alpha}$ at up to the end of 40 min. observation period (Fig 2b). A gradual increase in immunoreactive TXB_2 release was also evident in 3 experiments performed with A23187 (Table). In one of the 3 experiments the induced increase by A23187 of TXB_2 was observed only after 10 min. of incubation by comparison with the release from cells not incubated with A23187 (Exp III., Table). The results presented in Fig. 2a and 2b and in Table I indicated that incubation with A23187 induced in the first 5 min. relatively enhanced increase in LTB_4 release than in 6-keto $PGF_{1\alpha}$ and in TXB_2 release. Accordingly, the net ratios of LTB_4/6-keto

Figure 1



Relations between A23187 - induced Leukotriene B_4 - (LTB_4) (●) or 6 - keto Prostaglandin $F_{1\alpha}$ release (6-keto $PGF_{1\alpha}$)(□)/5 x 10^5 sampled cells ± S.E.M.) during 60 minutes (of samples I,II,III and pooled sample I-III) and % MAC-2^+ cells of the sampled cell number ± S.E.M..

$PGF_{1\alpha}$- and of LTB_4/TXB_2- release were in the first 5 minutes of incubation in favour of LTB_4 release. This fact is exemplified in Fig. 3 in which calculated ratios of these relative (with respect of baseline line eicosanoid production of nonstimulated cells) net LTB_4/6-keto $PGF_{1\alpha}$- and of LTB_4/TXB_2- release are presented.

Discussion

Stimulation of murine MΦs by the Ca^{2+} ionophore A23187 induces a relativily enhanced increase of a immunoreactive 5-lipoxygenase product LTB_4 followed later on by more marked increase in the immunoreactive cyclooxygenase products 6-keto $PGF_{1\alpha}$ and TXB_2. Although the population studied still includes other types of cells besides MΦs, it seems most likely that the MΦ is the type of cell stimulated by A23187, based on the relationship of A23187-induced eicosanoids release and the presence of MAC-2^+ cells studied. Non stimulated cells released also LTB_4, 6-keto $PGF_{1\alpha}$ and TXB_4 after 40 min. of incubation, however the quantities released were significantly less than A23187 stimulated cells. It is plausible to consider that the initial production of eicosanoid release observed in non - stimulated MAC-2^+ cells could be due to the increase in temperature between separation (t=0, 4° C) and

Figure 2 and Table

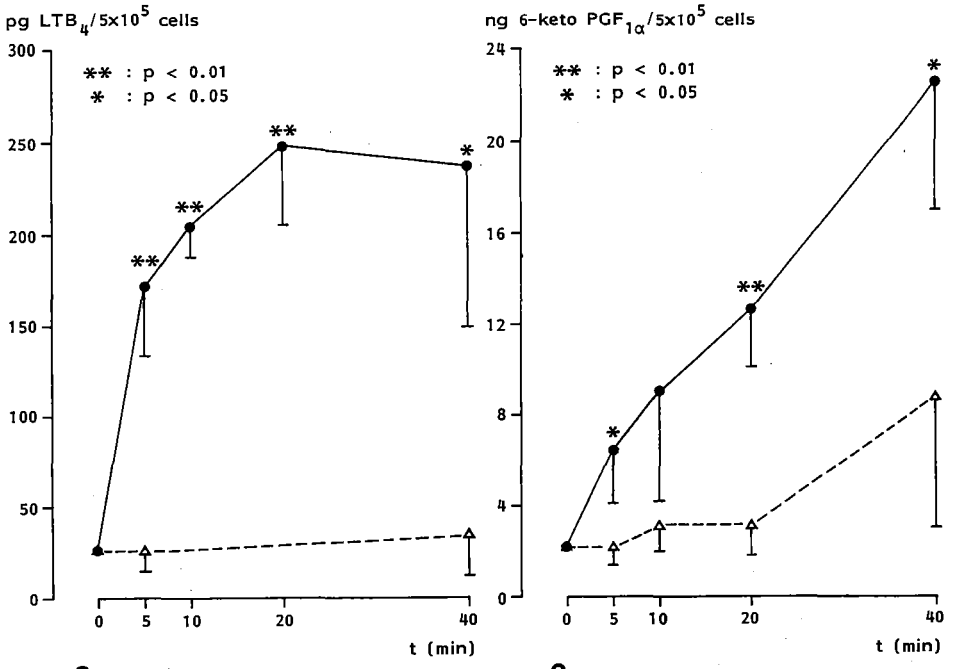


Figure 2a Figure 2b

	t=5		t=10		t=20		t=40	
+/- A23187	+	-	+	-	+	-	+	-
EXPERIMENT I	241 ± 6	126 ± 14	346 ± 36	96 ± 8	403 ± 35	124 ± 30	494 ± 70	150 ± 5
EXPERIMENT II	527 ± 93	125 ± 12	582 ± 59	97 ± 19	720 ± 66	122 ± 23	901 ± 86	219 ± 28
EXPERIMENT III	57 ± 5	52 ± 5	64 ± 15	23 ± 10	119 ± 10	38 ± 10	345 ± 89	136 ± 20

Figure 2: The mean lipoxygenase- and cyclooxygenase products release ± S.D. of pooled cell samples I-III (5 x 10^5 /ml). The data were statistically analysed by unpaired Student's -t test (**P< 0.01 and *P< 0.05).

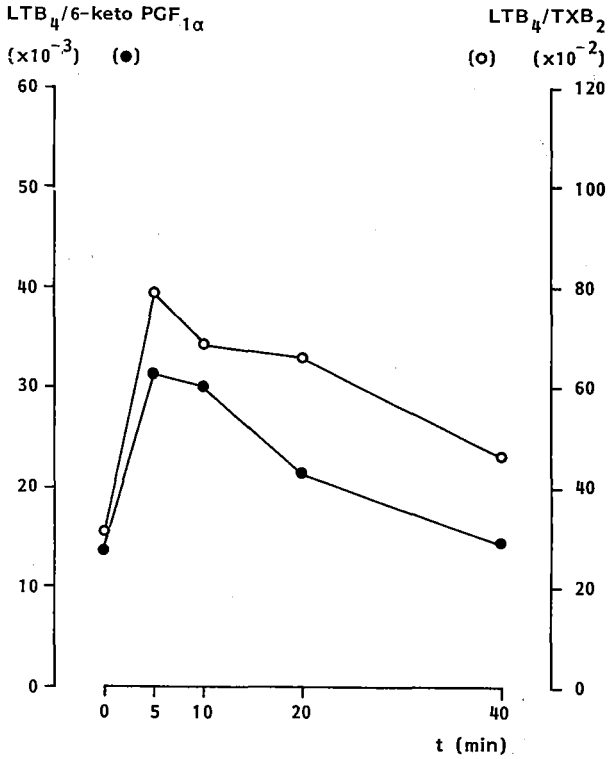
2a: Leukotriene B_4 (LTB_4)-release of non stimulated (Δ) and with A23187 stimulated (●) cells during 40 minutes.

2b: 6-keto Prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$)-release of non stimulated (Δ) and with A23187 stimulated (●) cells during 40 minutes.

Table:

Thromboxane B_2 (TXB_2)-release (pg/ml) of A23187- (+) and non-stimulated (-) pooled samples I-III (5 x 10^5 cells/ml) during 40 minutes from three individual experiments ± S.D. of triplicate incubations.

Figure 3



The mean A23187-induced net LTB_4 / TXB_2 -(O) and net LTB_4 / 6-keto $PGF_{1\alpha}$ (●) release ratios within 40 minutes of pooled cell samples I-III. Data were obtained from Figure 3a (mean net LTB_4 release), 3b (mean net 6-keto $PGF_{1\alpha}$ release) and Table I (calculated means of TXB_2 release from the three experiments, followed by mean net TXB_2 release). The $ratios_{t=0}$ were represented as $ratios_{t=5}$ of unstimulated eicosanoids release.

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incubation (t=5 min., 37° C). Lack of continuation of eicosanoids release could be explained by the absence of the necessary requirements (Ca^{2+} mobilization) for continuation of enzymatic reactions, which are characteristic for the feature of MΦ activation.

The dual changes of MΦ eicosanoids release induced by A23187 might represent a crucial event in the process of calmodulin dependent activation of MΦs (Wright et al. 1985). Lipoxygenase products are involved in expression of antitumor activity by MΦs (van Hilten et al. 1988) and cyclooxygenase products deactivate MΦs (Bonta et al. 1984). Conceivably, the initial increase in LTB_4 release by A23187 overcomes the deactivating effect by PGI_2 (precursor of 6-keto $PGF_{1\alpha}$). The decrease in the 5-lipoxygenase/cyclooxygenase products ratio is caused by the subsequent increase in of the cyclooxygenase products. The marked increase in release of cyclooxygenase products by A23187 might have induced a decrease in LTB_4 generation, because PGE_2

was shown to inhibit A23187-induced LTB_4 release with MΦs (Elliott et al.1989).

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Chapter 8.

OUTLINE OF THE RESULTS

1. Cell separation by velocity sedimentation in a discontinuous FBS gradient: Purification of peritoneal macrophages by cell size without adherence:

Many investigators use a technique to purify a macrophage population by the ability of macrophages to adhere to a microwell bottom or to glasswool and subsequently vigerously wash the microwells to remove non adherent cells (Adams, 1979). By modification of a method of cell separation described by Miller and Phillips (1969), we obtained macrophage- enriched samples separated on the basis of cell size in a discontinuous FBS gradient. With this technique, highly purified macrophage preparations were obtained with 1. a high viability 2. exact amounts of purified macrophages 3. a high recovery (number of total purified cells / peritoneal cells from lavages x 100%) and 4. no eicosanoids loss upon cell adherence. Determination of macrophages by Ab recognition of MAC-1 and -2 markers and May Grunwald Giemsa staining were performed in order to obtain a most accurate specific macrophage identification.

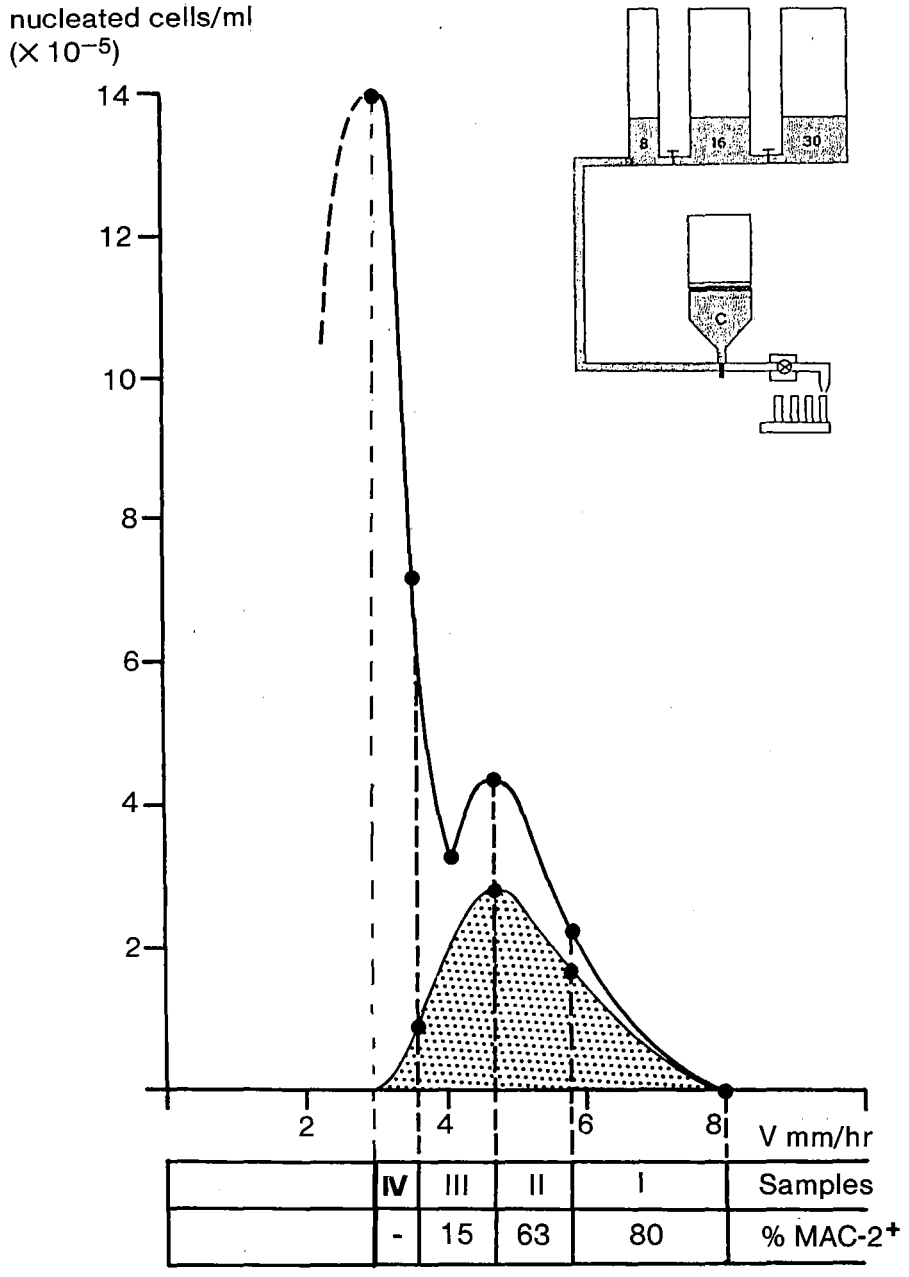
Macrophage-enriched (>85% by morphological criteria) samples I and II from peritoneal lavages (erythrocytes containing lavages (visually red) were excluded) were collected after 2 hrs sedimentation from the discontinuous FBS gradient. Sample I (8 > v > 6 mm/hr) contained 85% phenotypical MAC-1^+ (Chapter 4) and 80% phenotypical MAC-2^+ cells (Figure 5). Sample II (6 > v > 5 mm/hr) contained 95% MAC-1^+ (Chapter 4) and 63% MAC-2^+ cells (Figure 5). The latter difference might be due to contamination of granulocytes in Sample II. The viability of cells from both samples was > 99%. This could be explained by the observation earlier by Miller and Phillips (1969), that death cells either don't sediment in the FBS gradient or adhere to the tube during collection of cells. Samples I and II were the major source used in eicosanoid release- and antitumor cytostasis studies.

Sample III (5 > v > 3.5 mm/hr) represents a mixture of macrophages, granulocytes and lymphocytes (morphological criteria). It contained 70% MAC-1^+ (Chapter 4) and 15% MAC-2^+ cells (Figure 5). The difference in phenotypical MAC-1^+- and MAC-2^+ expression of cells in sample III can be explained by the presence of granulocytes. This sample was used in some eicosanoids release- and antitumor cytostasis studies (Chapters 4 and 8).

Sample IV (3.5 > v > 3 mm/hr) is the lymphocyte-sample (> 95% by morphological criteria). It contained 30% MAC-1^+ (Chapter 4) and no MAC-2^+ cells (Figure 5). This lymphocyte-enriched sample incubated with or without A23187 release no detectable LTB_4 (Chapter 4), which confirmed the data from Paubelle et al. (1987). 41% of the total viable separated peritoneal cells were MAC-1^+ and 26% MAC-2^+. This suggests that <15% of the peritoneal cells were granulocytes.

Figure 5.

Velocity sedimentation and identification of peritoneal cell-samples separated on a FBS-gradient column.



2. Macrophage eicosanoids release detected by RIA and HPLC

2.1 Non-stimulated macrophage eicosanoids release under *in vitro* conditions:

Separated peritoneal cell from samples I, II and III release detectable concentrations of eicosanoids in DMEM at 37°C in the initial 60 minutes: Leukotriene B_4 (LTB_4): 50, 25, 20 pg/5 x 10^5 cells and 6-keto Prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$): 22, 17 and 1.5 ng/5 x 10^5 cells, respectively (Chapter 4).

Cells from pooled samples I-III release under these circumstances in the sequential 5, 10, 20, 40 and 60 minutes: LTB_4: 20-25 (t=5-40 min.) and 45 pg/5 x 10^5 cells (t=60 min.), 6-keto $PGF_{1\alpha}$: 1.8, 3, 3, 7.5 and 4 ng/5 x 10^5 cells and Thromboxane B_2 (TXB_2): 90 (t=5-20), 100 and 208 pg/5 x10^5 cells, respectively (Chapter 7).

This emphasizes that nonstimulated macrophages cocultured under these "steady state" *in vitro* conditions in DMEM, have an initial eicosanoids production (t=5 min.), with a "stable" baseline (no significant increase) of eicosanoids concentrations in the additional 35 min (Chapter 7). After 60 min. of incubation in DMEM nonstimulated macrophages show an significant increase of eicosanoids production. This could be due to the effect of cell adherence to the microwell bottom.

The stable base line (t=5-40 min.) of eicosanoids concentration was extended theoretically to t=0, because this reflects, by approximation, the most real steady state condition of nonstimulated macrophage eicosanoids release *in vitro*.

2.2. A23187-induced macrophage eicosanoids release.

2.2.1 6-keto $PGF_{1\alpha}$-, TXB_2- and LTB_4 release:

Samples I,II, and III stimulated with .35 µM A23187 show increased eicosanoids release: LTB_4: 350, 250 and 50 pg/5 x 10^5 cells, 6-keto $PGF_{1\alpha}$: 80, 42, and 4 ng/5 x 10^5 cells, respectively (Chapter 4).

Cells from pooled samples I-III stimulated with A23187 show also significant ($p<0.05$ and 0.01, n=3) increased eicosanoids release under these circumstances in the sequential 5, 10, 20, 40 and 60 min.: LTB_4: 170, 205, 245, 240 and 220 pg/5 x 10^5 cells, 6-keto $PGF_{1\alpha}$: 6, 8.5, 12, 22 and 20 ng/5 x 10^5 cells and TXB_2 (not significant): 210, 310, 400, 600 and 518 pg/5 x 10^5 cells, respectively (Chapter 7).

The net A23187-induced macrophage eicosanoids release (A23187-induced release less baseline release) was used for further study of ratios.

2.2.2 Ratio of A23187-induced net 5-lipoxygenase/cyclooxygenase metabolites release:

A23187-induced 5-lipoxygenase/cyclooxygenase products release ratio could be of importance in regulation of A23187-induced macrophage activation, because certain released

cyclooxygenase metabolites are described in down-regulation of macrophage activation and some released 5-lipoxygenase metabolites are suggested to activate macrophages. Using the net A23187-induced LTB_4, 6-keto $PGF_{1\alpha}$ and TXB_2 release, the A23187-induced 5-lipoxygenase/cyclooxygenase products release ratio could be demonstrated by calculation of A23187-induced LTB_4/6-keto $PGF_{1\alpha}$ and LTB_4/TXB_2. Determination of the time course of this ratio could help to understand how leukotrienes activate macrophages, although A23187 stimulated macrophages release much more 6-keto $PGF_{1\alpha}$ or TXB_2 than LTB_4. Using these net induced release ratios, with respect of the steady state situation of nonstimulated macrophages, the change in steady state of macrophages eicosanoids formation by A23187 could be demonstrated:

Cells from pooled samples I-III stimulated with .35 µM A23187 revealed an increase in both LTB_4/6-keto $PGF_{1\alpha}$- and LTB_4/TXB_2 ratios in the initial 5 minutes, which decreased in the additional 35 minutes of incubation with A23187 (Chapter 7). This indicates an A23187-induced macrophage eicosanoids release first in favor of 5-lipoxygenase metabolites and in the additional 35 minutes in favor of cyclooxygenase metabolites.

2.2.3 Effect of NDGA and AA861 on A23187-induced eicosanoids release from resident peritoneal macrophages:

Two 5-lipoxygenase inhibitors, nordihydroguaiaretic acid (N.D.G.A.) and 2, 3, 5-trimethyl-6-(12-hydroxy-5, 10-dodecadinyl)-1, 4-benzoquinone (AA861) were used for eicosanoids release inhibition studies:

[NDGA] > .6 µM inhibited both A23187-induced cyclooxygenase metabolites (6-keto $PGF_{1\alpha}$: 32% and TXB_2: 39%) and 5-lipoxygenase metabolites (LTB_4: 42%) release of resident cells from pooled Samples I-III. The IC_{50} of N.D.G.A for cyclooxygenase metabolites release is 1.25-2 µM and the IC_{50} for 5-lipoxygenase metabolites release is 1 µM (Chapter 4).

.04 µM < [AA861] < 10 µM inhibited A23187-induced 5-lipoxygenase metabolites (LTB_4) release of resident cells from samples I-III, specifically; IC_{50}= .3 µM (Figure 6A and Chapter 4).

2.2.4 Effect of AA861 on A23187-induced eicosanoids release from elicited peritoneal macrophages:

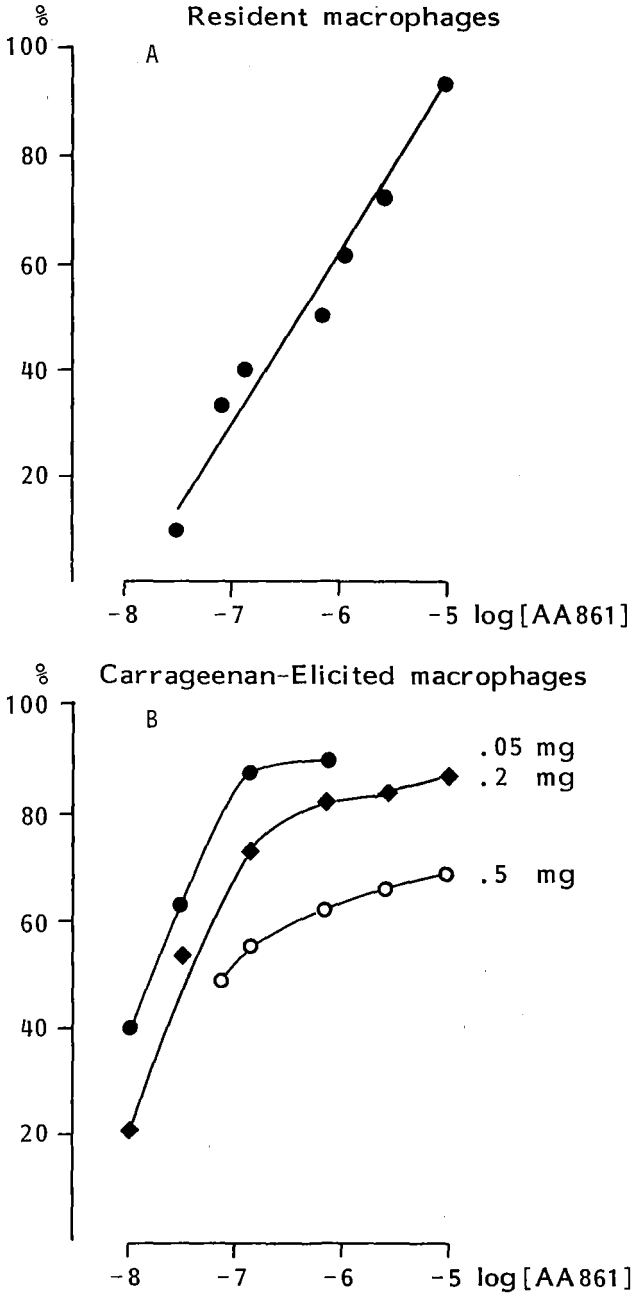
Carrageenan-elicited macrophages stimulated <u>in vitro</u> with .35 µM A23187 enhanced the release of eicosanoids during 60 minutes (.5 mg carrageenan/mouse), compared with nonstimulated cells:

.5 mg carrageenan: 22-->250 pg,
.2 mg carrageenan: 9 -> 150 pg and
.05 mg carrageenan: 1 -> 229 pg LTB_4 release/5 x 10^5 cells,
.5 mg carrageenan: 502-> 838 pg TXB_2 release/5 x 10^5 cells,
2.1->4.2 ng 6-keto $PGF_{1\alpha}$/5 x 10^5 cells.

The induced enhanced release of LTB_4 could be inhibited by 10^{-8}-10^{-5} M AA861, specifically (Figure 26):
IC_{50}(.5 mg)= .08 µM with max. inhibition of 70%
IC_{50}(.2 mg)= .04 µM with max. inhibition of 86%
IC_{50}(.05mg)= .02 uM with max. inhibition of 90% (Figure 6B.).

Figure 6.

% inhibition of A23187-induced LTB_4 release from resident & elicited macrophages



2.3 Separation of leukotrienes released from macrophages and P815 cells stimulated with A23187.

2.3.1 A23187-induced cysteinyl-leukotrienes release.

Cells from pooled samples I and II, stimulated with .35 µM A23187 in DMEM during 20 minutes, release LTD_4, predominantly. Also LTE_4 and an unidentified LT (T_R = 9 min.) were detected and separated by HPLC in these supernatants (Chapter 5). 400 pg LTC_4 was detected by RIA from the 6-7 min. (LTC_4) fraction after HPLC separation. This indicates that A23187-stimulated macrophages induce 5-lipoxygenase metabolites release and further metabolize LTC_4 into LTD_4, thus suggesting the presence of (active) GGT from A23187-stimulated macrophages.

P815 tumor cells release in supernatants (DMEM) a small amount of LTD_4, detected by HPLC (Chapter 5).

2.3.2 Effect of serum containing-TGM on cysteinyl-leukotrienes formation.

In TGM alone and in supernatants of macrophages from pooled samples I and II in TGM incubated for 20 minutes at 37° C no detectable LTs by HPLC was observed. Exogenous LTC_4 and ^3H-LTC_4 incubated in serum containing TGM, convert partially into LTE_4 and ^3H-LTE_4, respectively (Chapter 5). This suggests the presence of GGT and cysteinyl-glycinase in serum.

Macrophages from pooled samples I and II incubated with .35 µM A23187 release LTE_4 and an unidentified LT (T_R = 9 min.) separated by HPLC (Chapter 5 and 6). This indicates that the 5-lipoxygenase metabolite LTC_4 from A23187-stimulated macrophages is an intermediate metabolite in the formation of LTE_4, by the presence of GGT (in serum and from A23187 - activated macrophages) and cysteinyl-glycinase (in serum).

P815 tumor cells incubated with .35 µM A23187 release LTD_4 in TGM (Chapter 5).

2.3.3 Effect of AA861 and L-serine on cysteinyl-leukotrienes formation.

Macrophages from pooled sample I and II, preincubated with 2.5 µM AA861 and stimulated additionally with A23187 during 20 min. release no LTs in supernatant as detected by HPLC separation (Chapter 5).

L-serine (200 mM)- pretreated macrophages from sample I and II stimulated with A23187 (and 100 mM serine) during 20 min. release LTC_4 in the supernatant, as detected by HPLC separation (Chapter 6). Macrophages from samples I and II incubated with 100 mM L-serine in TGM during 24 hrs. release a large amount of LTC_4 and a small amount of LTE_4, whereas macrophages from samples I and II incubated in TGM alone during 24 hrs. release small amounts of LTC_4 and LTE_4 (Chapter 6). These results indicate that L-serine induces accumulation of macrophage LTC_4 by inhibition of conversion of LTC_4 into LTD_4.

3. Cytostatic activity of macrophages under cocultured conditions with P815 tumor cells.

3.1 Macrophage/P815 cell ratio dependency

Non-stimulated macrophage-enriched samples I and II, cocultured for 40 hrs. with 6 x 10^3 P815 tumor cells in effector cell (macrophage): tumor cell ratios (R) > 2:1 express cytostatic activity (Figure 7A), whereas cells from samples III and IV (with ratios > 2) do not express antitumor cytostatic activity under these conditions in vitro. This indicates that resident macrophages have small endogenous cytostatic activity by theirselves under these in vitro conditions.

3.2 Increased macrophage antitumor cytostatic activity by A23187

Concentrations A23187 > .1 µM and < .25 µM promote P815 tumor cell growth, as measured by increased ^{3}H-thymidine uptake, whereas [A23187] >.35 µM is toxic for P815 cells (Figure 7B). Within the narrow concentration range between .25 and .35 µM, A23187 induces antitumor cytostatic activity towards P815 cells cocultured with the macrophage- enriched samples I and II in a non-cytostatic ratio 2:1 (Figure 7B). Cells from samples I and II exposed for 24 hrs. to .35 µM A23187, express 53-49% (n=6 experiments) inhibition of P815 tumor cell growth (Chapter 4, 5 and 6) whereas cells from samples III and IV do not express significant antitumor cytostatic activity under these circumstances (Chapter 4). Preliminary experiments demonstrated that macrophage express cytostasis towards the cocultured P815 cells without A23187 (in 100 µl TGM), when they were preincubated with .35 µM A23187 during at least 1 hr.. Supernatants from macrophages incubated more than 1 hr. with .35 µM A23187 were not cytostatic towards P815 cells in vitro. This indicates a requirement of at least 1 hr exposure of macrophages to .35 µM A23187 for the induction of macrophage antitumor cytostasis.

These results indicate that stimulation of macrophage eicosanoids release by A23187 could play an important role in A23187-induced macrophage antitumor cytostasis. Preliminary studies indicate that macrophage-tumor cell contact is required in A23187-induced macrophage antitumor cytostasis.

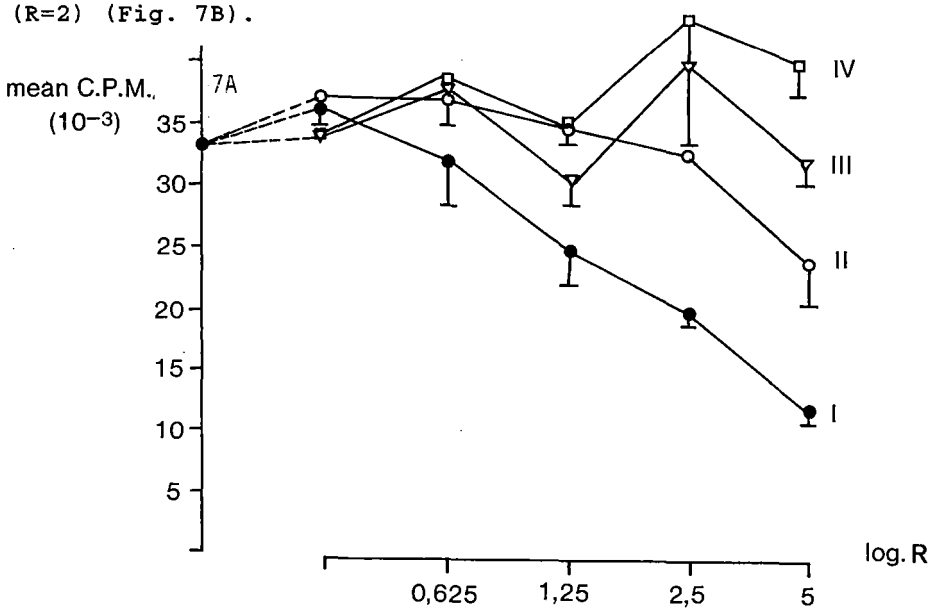
3.3 Effect of 5-lipoxygenase inhibitors on macrophage cytostatic activity.

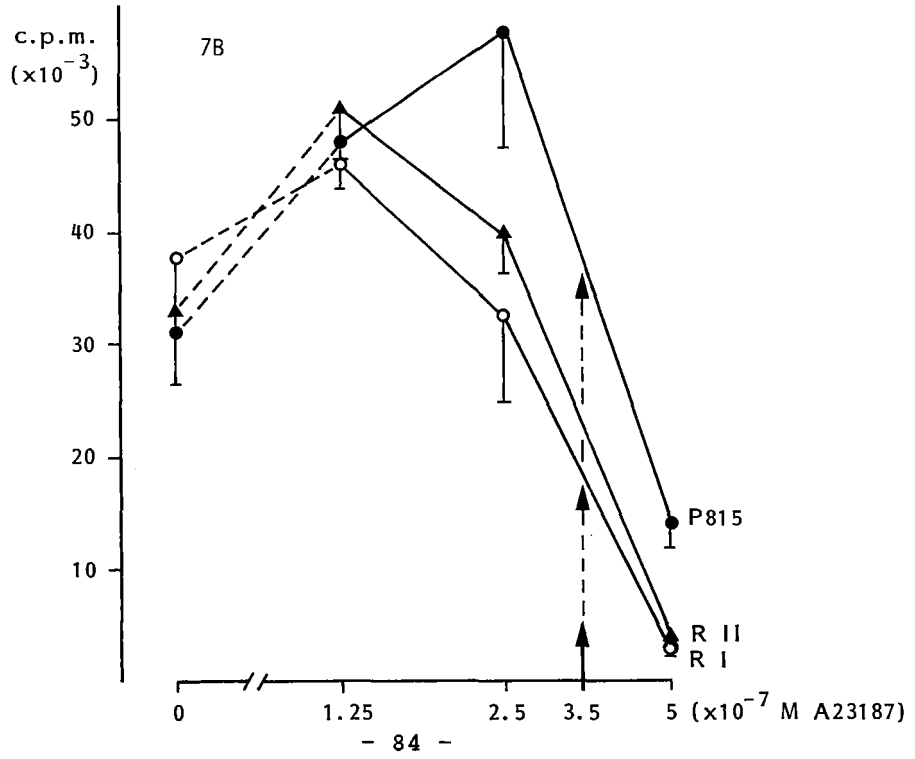
Preincubation of the specific 5-lipoxygenase inhibitor AA861 (.6 - 5 µM) with cells from macrophage-enriched sample I and II could inhibit A23187-induced macrophage antitumor cytostasis (Chapter 4 and 5), whereas NDGA, did not affect A23187-induced macrophages anti-tumor cytostasis (Chapter 4).

These results indicate that a 5-lipoxygenase metabolite is required in the expression of A23187-induced macrophages cytostasis and that the ratio of 5-lipoxygenase-/cyclooxygenase metabolites release is important in regulation of this macrophage cytostasis.

Figure 7.

Uptake of ^{3}H-thymidine by 6 x 10^{3} P815 tumor cells cocultured with separated samples I,II,III and IV with increasing ratios (R) macrophages/P815 cells (Fig. 7A) and stimulated with A23187 (R=2) (Fig. 7B).





3.4 Effect of exogenous leukotrienes on macrophage cytostatic activity.

Inhibition of A23187-induced macrophage cytostasis by 5 µM AA861 from cells of sample I could be restored by 10^{-8}M exogenous LTC_4 (38% inhibition of tumor cell growth) (TABLE). However, exogenous [LTC_4] < 10^{-7}M could not induce antitumor cytostasis in macrophages cocultured with P815 cells (without A23187 and AA861) (Chapter 5). Other exogenous LTs (LTB_4 and LTE_4) neither restore (with A23187 and AA861) nor induce (without A23187 and AA861) P815 cell cytostasis by macrophages (Chapter 5 and unpublished observations).

These results indicate that LTC_4 is the essential 5-lipoxygenase metabolite in A23187-induced macrophage antitumor cytostasis. However, increased cytosolic [Ca^{2+}] appeared also to be required in the mechanism by which A23187 induces macrophage antitumor cytostasis.

3.5 Effect of L-serine on macrophage cytostatic activity.

Macrophages from sample I and II preincubated with L-Serine (>100 mM) and exposed subsequently to concentrations of L-serine >50 mM expressed antitumor cytostasis against P815 cells (62-78%). Addition of similar amounts of L-serine, without macrophages, promoted tumor cell growth of P815 cells (Chapter 6). Serine (200 mM)- pretreated macrophages subsequently exposed to .35 µM A23187 (53-49% inhibition of P815 cell growth) showed an additive (91-95%) antitumor cytostatic activity. This additive cytostatic activity of macrophages was related to accumulation of LTC_4 formed in serine-treated macrophages (with and without A23187) (Chapter 6).

% CYTOSTATIC ACTIVITY

TABLE

SAMPLE	A23187	A23187+AA861	A23187+AA861 +LTC_4 10^{-8}
I	53 ± 5	19 ± 6*	38 ± 2**
II	49 ± 10	17 ±12*	19 ± 14

**ε* :$p < 0.01$

Chapter 9.

DISCUSSION

Non-stimulated resident peritoneal macrophages (from macrophage-enriched samples I and II) expressed small eicosanoid (LTB_4-, 6-keto $PGF_{1\alpha}$- and TXB_2) synthesis activity under serum-free-medium cultured conditions in vitro (Chapter 4 and 7) and slight LTC_4 synthesis in serum-containing-medium (TGM) (Chapter 6). Endogenous leukotrienes could augment macrophage/monocyte activity (Rola-Pleszczynski and Lemaire, 1985a; and Gagnon et al., 1988) and eicosanoid release in favor of cyclooxygenase metabolites release might be involved in down regulation (Bonta and Parnham, 1982; Taffet and Russell, 1981a, b and 1982; Adams and Hamilton, 1988; and Elliott et al., 1989) of macrophage activity in steady state under in vitro circumstances. Cytostatic activity against P815 tumor cells was observed with non-stimulated macrophages cocultured in TGM in an effector cell:tumor cell ratio > 2:1 (Chapter 8; Figure 7A). The antitumor cytostatic activity by non-stimulated macrophages might be related to release of small amounts of prostanoids and leukotrienes.

Stimulated by .35 µM A23187, macrophages released increased amounts of eicosanoids during 60 minutes (Chapter 4,5,6 and 7). Although the amounts of 6-keto $PGF_{1\alpha}$ and TXB_2 released were much higher than the amounts of LTB_4 released during the 40 minutes incubation period, the change in macrophage steady state of eicosanoid release induced by A23187, was in the first 5 minutes of incubation in favor of 5-lipoxygenase metabolites release. This initial increase of 5-lipoxygenase/cyclooxygenase metabolite release of A23187-stimulated macrophages, could be involved in the mechanism by which endogenous leukotrienes could overcome the deactivating effect of PGE_2 and PGI_2 (Cantarow et al., 1978; Bonney et al., 1978; Leung and Mihich, 1980; Snyder et al., 1982; and Renz et al., 1988) on macrophage inflammatory - and of PGE_2 on macrophage antitumor activity (Schultz et al., 1978 and 1979; and Taffet, 1982). The differential effect of A23187 on increase of 5-lipoxygenase- and cyclooxygenase metabolites release might be explained by the Ca^{2+}-dependent 5-lipoxygenase activity and the stimulation of LTC_4 on cyclooxygenase metabolites release (Feuerstein et al., 1981); first (after 5 minutes incubation) in favor of the Ca^{2+}-dependent 5-lipoxygenase activation, which could enhance cyclooxygenase metabolites release, subsequently. The cyclooxygenase metabolites might inhibit 5-lipoxygenase metabolites release (Elliott et al., 1989) and therefore establish a second effect (in the additional 35 minutes of incubation) in favor of cyclooxygenase metabolites release, because the stimulated LTB_4 release was inhibited and the cyclooxygenase metabolites release increased further (Chapter 7).

The quantity of 0.35 µM A23187, which is not cytostatic by itself, induced macrophage antitumor cytostasis

(53-49% inhibition of thymidine incorporation by P815 tumor cells) in serum-containing TGM when cells from macrophage-enriched samples were cocultured with P815 cells in an effector cell:tumor cell ratio 2:1. Enhanced release of cyclooxygenase - and 5-lipoxygenase metabolites from resident macrophages by A23187 were both inhibited by nordihydroguaiaretic acid (NDGA). AA861 inhibited only A23187-induced 5-lipoxygenase metabolite release of macrophages. The inhibition of A23187-induced macrophage antitumor cytostatic activity by AA861, but not by NDGA, indicated the requirement for antitumor cytostatic activity of an endogenous 5-lipoxygenase metabolite(s) and supported the importance of 5-lipoxygenase-/cyclooxygenase metabolites release ratio for expression of macrophage antitumor cytostasis (Chapter 4).

The increase in LTB_4 release induced by A23187 was in straight relation with increase in relative percentage of MAC-2^+ peritoneal cells. This straight relation supports the assumption that A23187-stimulated macrophages are the major source of released LTB_4 and that contamination of other cell types does not affect this stimulated release from macrophages (Chapter 7).

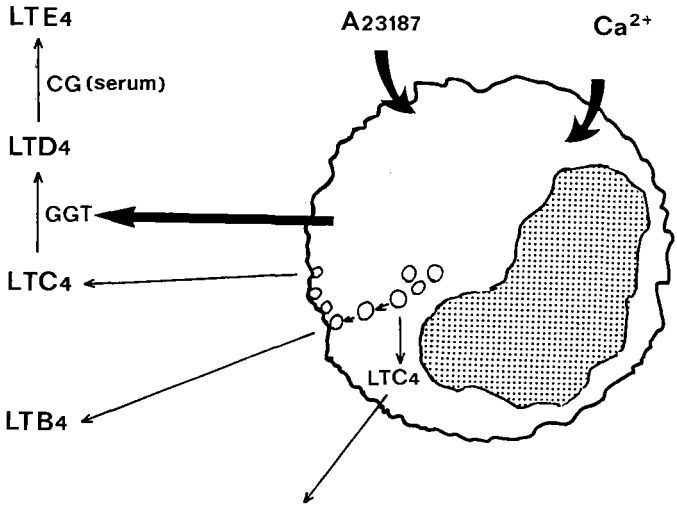
Carrageenan-elicited peritoneal mononuclear phagocytes stimulated with .35 µM A23187 release increased amounts of LTB_4. The release of LTB_4 was inhibited by AA861. The IC_{50} of AA861 of A23187-induced LTB_4 release from carrageenan-elicited macrophages was 10 x more than the IC_{50} of resident macrophages treated with AA861 and A23187. The shift of the inhibition of LTB_4 release by AA861 indicated difference in site (decompartmentalization; Balter et al., 1989) of A23187-induced membrane-translocated (Rouzer and Kargman, 1988b) 5-lipoxygenase between resident and elicited macrophages in vivo. Additionally, we showed that high doses of AA861 failed to inhibit more than 60% of the A23187-induced LTB_4 release from peritoneal macrophages elicited with .5 mg carrageenan. These results might be explained by a disturbance of decompartmentalization of translocated 5-lipoxygenase in elicited- (by toxic carragenan) macrophages in which 5-lipoxygenase activation by increased cytosolic Ca^{2+} was not inhibited by high [AA861] 's.

A23187-induced 5-lipoxygenase metabolites release was in favor of LTD_4, but also LTE_4 was identified by HPLC separation in supernatants of resident macrophages stimulated by A23187 in serum free medium. This implicated activation of macrophage- τ-glutamyl transpeptidase (GGT) and - cysteinyl glycinase (CG) by A23187, a finding which was also observed with granulocytes (Raulf et al., 1985) (Figure 8).

Exogenous LTC_4 in serum-containing TGM converted into LTE_4, which indicated the presence of GGT and CG in serum. TGM itself and supernatants of non-stimulated macrophages incubated in TGM during 20 minutes revealed no detectable LTs by HPLC separation. A23187-stimulated macrophages in serum-containing TGM released large amounts of LTE_4 and an unidentified LT.

Figure 8.

A23187-induced macrophage leukotriene formation and -release, including intracellular translocation of 5-lipoxygenase



Abbreviations in Figure 8.

A23187 = Calcium ionophore A23187
Ca^{2+} = Calcium ion
CG = Cysteinyl glycinase
GGT = τ-glutamyl transpeptidase
LT = Leukotriene

This release was inhibited by incubation of macrophages with 2.5 µM AA861. These results indicated that A23187-induced macrophage 5-lipoxygenase activity results ultimately in LTE_4 (and an unidentified LT). However, LTC_4, but not LTE_4 reversed AA861-inhibition of macrophage antitumor cytostasis, thus indicating that endogenous LTC_4 is an essential metabolite in the mechanism of induction of macrophage cytostatic activity against P815 tumor cells by A23187 (Chapter 5).

The calcium ionophore can activate many macrophage functions, which might be initiated by increased cytosolic Ca^{2+}. Ca^{2+}-influx may be involved in the activation of cascades of metabolites which play a role in the pathway of signals involved in enhancement, initiation or inhibition of certain macrophage functions (Chapter 1; Figure 4). Increased cytosolic Ca^{2+} and LTC_4 release from macrophages were found in macrophage responses to certain inflammatory stimuli (Rouzer et al., 1980; Young et al., 1984; and Aderem et al., 1986a). Endogenous LTC_4 was reported to be also essential in other macrophage activities (Miller et al., 1986; and Ziboh et al., 1986). Our study demonstrates the importance of these increases in macrophage antitumor cytostasis. However, exogenous LTC_4 did not induce macrophage antitumor cytostasis when macrophages were cocultured with P815 cells alone. This result indicates that Ca^{2+} influx is required for macrophage antitumor cytostasis. The failure of exogenous LTC_4 to bypass the Ca^{2+} signal may indicate that A23187 has to establish an very high concentration of endogenous LTC_4 (in the initial 5 minutes) before decrease in endogenous [LTC_4] by breakdown to LTE_4 and/or to an unidentified LT.

Our study indicates also that the rate of conversion of LTC_4 into the non active LTE_4 (by GGT and CG in serum and in macrophages) might be a limiting factor in expression of macrophage antitumor cytostasis. In order to investigate this assumption, the effect of L-serine on macrophage antitumor cytostasis was determined. L-serine binds to the active τ-glutamyl site of GGT (Thomson and Meister, 1977) and inhibits the conversion of LTC_4 into LTE_4. Indeed, L-serine - (200mM) pretreatment of macrophages and subsequent incubation (by dilution) with 100 mM serine, with and without 0.35 µM A23187 revealed release of large amounts of LTC_4 only. Moreover, macrophages pretreated with 200 mM L-serine and exposed subsequently to 100 mM serine induced macrophage antitumor cytostasis (62-78%). Additive macrophage antitumor cytostasis (91-95%) was observed when serine-pretreated macrophages were exposed also to A23187, subsequently. These results support the assumption that a high [LTC_4] is important for macrophage antitumor cytostasis. An accelerated accumulation of endogenous LTC_4 in A23187+serine-treated macrophages could cause the additive macrophage antitumor cytostatic effect (Chapter 6).

P815 mastocytoma tumor cells release LTC_4 when exposed to A23187 (Murphy et al., 1979). Our study indicates that P815 cells incubated with A23187 in serum free medium release a small amount of LTD_4. This release is enhanced when

P815 cells are cultured in TGM in addition to A23187 (Chapter 5). Compared with leukotriene release from stimulated macrophages, the putative effect of leukotrienes released from P815 cells on macrophage cytostatic activity is negligible.

The mechanism by which A23187-stimulated macrophages inhibit growth of P815 cells is not fully clear yet. Cytostasis of P815 cells by release of IL-1β from A23187-stimulated macrophage (Matsushima and Oppenheim, 1985) seems not to be involved, because P815 cell growth appears to be resistant to the cytostatic effects of IL-1β (Lovett et al., 1986). Grand-Perret and coauthors (1986) observed also macrophage cytostasis against P815 cells, when macrophages were preincubated (2 hrs.) with A23187. Results from our study indicated a cell-to-cell contact requirement in the mechanism by which A23187-stimulated macrophages express antitumor cytostasis, because supernatants from A23187-treated macrophages had no cytostatic effect on P815 cells. It was also described that growth of P815 cells could be affected by PGs (Balazsovits et al., 1988) and TNFα (Carswell et al., 1975). It is not known whether A23187 induced TNFα release from these macrophages, but it is unlikely that PGs express antitumor cytostasis directly in this assay of macrophage mediated antitumor cytostasis, because supernatants from A23187-stimulated macrophages failed to express cytostasis towards P815 cells.

In conclusion, our results indicate that LTC_4 is an essential 5-lipoxygenase intermediate in A23187-induced macrophage antitumor cytostasis. LTC_4 converted into LTE_4 by the presence of τ-glutamyl transpeptidase (GGT) and CG in serum, present in TGM and this process was accelerated by increased activity of these enzymes due to exposure of macrophages to A23187. The conversion of LTC_4 into LTE_4 can limit A23187-induced macrophage antitumor cytostasis. Based on our results on the time course of 5-lipoxygenase-/cyclooxygenase metabolites release ratio, the activating role of LTC_4 might be exerted in the initial 5 minutes of stimulation of macrophages by A23187. Ca^{2+}-influx-mediated macrophage 5-lipoxygenase activation by A23187 could establish the required signal of high LTC_4 production in the initial 5 minutes incubation in TGM. Serine inhibits the conversion of LTC_4 into LTD_4 by binding GGT. The straightforward relation between induction of accumulation of LTC_4 in macrophages and induction of macrophage antitumor cytostasis by L-serine, indicats the importance of [LTC_4] for macrophage antitumor cytostasis in vitro. The additive antitumor cytostatic activity by combined L-serine and A23187 treatment of macrophages may be explained by acceleration of high [LTC_4] in macrophages.

Modulation of macrophage LTC_4 biosynthesis by L-serine and A23187 provides an insight into the role of endogenous LTC_4 in expression of macrophage antitumor cytostasis in vitro. Further research is required for the use of such combined treatment for cancer immunotherapy.

SUMMARY

The main aim of the present work was to study the possible interrelationship between induction of antitumor cytostasis and of production of prostanoids and leukotrienes by murine peritoneal macrophages stimulated with the calcium ionophore A23187. A method of cell separation by velocity sedimentation was adapted for obtaining macrophage-enriched preparations from the resident peritoneal macrophage population.

The following results were obtained:

1. Macrophage antitumor cytostatic activity was induced by A23187 (53-49% inhibition of thymidine incorporation by P815 tumor cells) in an in vitro coculture system of macrophages and tumor cells in serum containing medium (TGM).
2. Induction of increased release of macrophage eicosanoids (leukotrienes B_4, C_4, D_4, E_4 and the prostanoids; 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_4) by A23187, including demonstration of increased net A23187-induced 5-lipoxygenase/cyclooxygenase metabolites release ratio in the initial 5 min., in respect of the ratio from non-stimulated macrophages in 'steady state' conditions in vitro.
3. Conversion of LTC_4 into LTE_4 incubated at 37° C in TGM.
4. Inhibition by AA861 of macrophage cytostatic activity was related to inhibition of 5-lipoxygenase metabolites release, whereas the failure of inhibition of macrophage cytostatic activity by N.D.G.A. was related to inhibition of both 5-lipoxygenase- and cyclooxygenase metabolites release.
5. Exogenous leukotriene C_4 (10^{-8} M), but neither leukotriene B_4 nor leukotriene E_4, reversed AA861-inhibited macrophage antitumor cytostasis.
6. Effect of L-serine, which binds to the active site of τ-glutamyl transpeptidase used for the conversion of leukotriene C_4 to leukotriene D_4, on macrophage antitumor cytostatic activity had been studied. Increased leukotriene C_4 release was related to increased antitumor cytostatic activity (62-78%) by serine-(100 mM) treated macrophages.
7. Additive antitumor cytostatic effect (91-95%) by serine-(200 mM) pretreated - and subsequently exposure to A23187-(and by dilution 100 mM serine) treated macrophages was demonstrated. This additive effect was related to accelerated release of (accumulated) LTC_4 and inhibition of LTE_4 formation in macrophage supernatants.

The main conclusion of the present work is that induction of antitumor cytostatic activity in peritoneal macrophages treated by A23187 and/or L-serine is related to induction of production of leukotrienes, especially of leukotriene C_4.

SAMENVATTING

Het voornaamste doel van dit werk was het onderzoeken van de mogelijke relatie tussen het op gang brengen van antitumor cytostatische activiteit en de productie van prostanoiden en leukotrienen van muis peritoneaal macrofagen gestimuleerd d.m.v. het calcium ionofoor A23187. Hiertoe werd allereerst een methode geoptimaliseerd voor het verkrijgen van een zo zuiver mogelijke peritoneaal macrofaag fractie d.m.v. cel scheiding op basis van het verschil in bezinkingssnelheid van cellen in een oplopende gradient van FBS (foetale serum van het rund). In dit onderzoek werden de volgende resultaten verkregen:

1. 53-49% remming van inbouw van thymidine in P815 tumor cellen (antitumor cytostatische activiteit) tesamen gekweekt met macrofagen en het calcium ionofoor A23187 in serum-verrijkt medium.
2. Toeneming van productie- en vrijmaking van eicosanoiden (leukotrienen B_4, C_4, D_4, E_4 en de prostanoiden; 6-keto prostaglandine $F_{1\alpha}$ en thromboxaan B_2) van macrofagen o.i.v. A23187, inclusief een toeneming van de netto verhouding 5-lipoxygenase / cyclooxygenase metabolieten productie o.i.v. A23187 binnen 5 minuten, t.o.v. de verhouding van deze vrijgemaakte metabolieten van niet gestimuleerde macrofagen, gekweekt in vitro.
3. Een snelle omzetting van LTC_4 in LTE_4 werd gevonden in serum-verrijkt medium bij 37° C.
4. A23187 - geinduceerde macrofaag antitumor cytostatische activiteit werd geremd o.i.v. AA861 en werd in verband gebracht met remming van de 5-lipoxygenase metabolieten productie van macrofagen, terwijl o.i.v. NDGA, het ontbreken van remming van antitumor cytostatische activiteit in verband werd gebracht met remming van zowel 5-lipoxygenase-, als cyclooxygenase metabolieten productie van macrofagen.
5. Noch LTB_4, noch LTE_4, doch wel LTC_4 kon de door AA861 geremde macrofaag antitumor cytostatische activiteit weer tot stand brengen.
6. Bepaling van antitumor cytostatische activiteit van macrofagen o.i.v. L-serine, welke zich hecht aan de actieve bindingsplaats van τ-glutamyl transpeptidase (dit enzym is noodzakelijk voor de omzetting van leukotriene C_4 in leukotriene D_4). Verhoogde macrofaag antitumor cytostatische activiteit (62-78%) werd gevonden o.i.v. serine (100 mM) en werd in verband gebracht met een toename in leukotriene C_4 geproduceerd door serine-behandelde macrofagen.
7. Een additieve antitumor cytostatische activiteit (91-95%) werd gevonden met macrofagen voorbehandeld met serine (200 mM) en daarna A23187 (0.35μM) met een verdunning van serine (100 mM). Deze toeneming van activatie van macrofagen werd in verband gebracht met een snelle stapeling van leukotriene C_4 formatie en remming van leukotriene E_4 vorming in medium van gekweekte macrofagen.

De voornaamste conclusie van dit werk is dat stimulatie van antitumor cytostatische activiteit van peritoneaal macrofagen o.i.v. A23187 en/of serine in verband gebracht kan worden met toename in productie van 5-lipoxygenase metabolieten in het algemeen, en wel leukotriene C_4 in het bijzonder.

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