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The Functional Characterization of Lung-Associated Natural Killer Activity and its Regulation by Alveolar Macrophages©

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This Thesis is submitted in fulfilment of the requirements
for the degree of PhD.

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to the love of my life Cheryl Mitchell
and to my parents

ABSTRACT

Natural killer (NK) activity plays an important role in host defense against tumors. It is likely that this defensive role is shaped by compartmental and local environmental factors. The purpose of this study was to characterize the natural killer activity of lymphocytes resident in the rat lung and explore its modulation. Lung lymphocytes (LL) were shown to possess potent NK activity against Yac-1 targets. The effector cells were found to be sensitive to complement-mediated lysis with anti-asialo GM-1 and to treatment with L-leucine methyl ester, which selectively kill NK cells.

Lung lymphocyte NK activity was compared to lymphocytes from the spleen and peripheral blood. LL were more potent NK effectors than peripheral blood (PBL) or spleen lymphocytes (SL), although the lung and spleen contained similar proportions of NK (3.2.3 positive) cells. Lung and peripheral blood NK activities were modulated to a similar extent by human recombinant interleukin-2 (IL-2) whereas spleen NK activity was significantly more responsive. Interferon-α/β (IFN-α/β) increased NK activity in all three compartments but to a lesser extent in PBL. By contrast, interferon-γ (IFN-γ) failed to augment lung or spleen NK activity but did significantly enhance NK activity in PBL. Interestingly, the regulatory effects of alveolar macrophages (AM) also varied depending upon the origin of

the lymphocytes. AM inhibited lung NK activity but had a stimulatory effect on spleen NK activity, despite the fact that both populations were equally sensitive to macrophage conditioned media.

A more thorough examination of the responsiveness of lung NK activity to cytokine treatment was undertaken once it had been established that LL behave differently than PBL or SL. Lung lymphocytes were treated *in vitro* for 18h with various cytokines. Human recombinant IL-2, purified rat IFN-α/β, or murine recombinant tumor necrosis factor-α (TNF-α) resulted in a dose-dependent increase in lung NK activity. The maximum stimulation by IL-2 and IFN-α/β was similar ($\simeq$55%) though much lower concentrations of IL-2 were required for the same effect. TNF-α stimulation was approximately half that seen for IL-2 and was only observed at a high concentration of the cytokine. Furthermore, doses of IFN-α/β and TNF-α that had little effect alone were able to synergize with suboptimal doses of IL-2. By contrast, lung NK activity was resistant to the influence of interleukin-1 (IL-1) and IFN-γ, alone or in combination with suboptimal doses of IL-2. The interaction of lung lymphocytes with alveolar macrophages may have a profound regulatory effect on NK activity. This interaction was explored using a two-chambered system which prohibited physical contact between the cells in the separate chambers but allowed diffusion of soluble factors. AM were found to

inhibit LL NK activity in a time-dependent and reversible manner. AM inhibition was shown to be sensitive to indomethacin which caused a decrease in prostaglandin E_2 (PGE_2) concentration. Quantitation of PGE_2 levels and treatment with exogenous PGE_2 indicated that it could not account for the entire inhibitory effect. It was subsequently found that exogenous transforming growth factor-β_1 (TGF-β_1) also inhibits lung NK activity and that treatment of inhibitory AM supernatant with a neutralizing antibody to TGF-β_1 absorbs up to 55% of the inhibitory activity. Moreover, the co-culture supernatant was found to contain 24 pg/mL TGF-β_1, enough to account for the remainder of the inhibition. By contrast, PDGF and nitric oxide were shown not to be involved in the inhibition.

The mechanism by which TGF-β_1 and thereby AM inhibit lung NK activity was investigated. It was found that AM inhibit lung NK at a post-binding step in the lytic pathway. An enriched population of lung NK cells (≈50% large granular lymphocytes) was used to further examine the mechanism. It was observed that treatment of LL with TGF-β_1, or co-culture with AM but not treatment with PGE_2 resulted in a decrease in BLT-esterase activity. A similar pattern of inhibition was seen in granzyme B mRNA by *in situ* hybridization. Taken together, these data indicate that AM inhibit lung NK activity, at least in part, through the production of TGF-β_1 which reduces the available granule protein mRNA.

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LIST OF ABBREVIATIONS

AM	alveolar macrophages
BLT	Nα-Cbz-lys-thiobenzylester
BRM	biological response modifier
BSA	bovine serum albumin
Ca^{++}	calcium ion
Cr^{51}	chromium-51
CTL	cytotoxic T lymphocyte
DAG	diacylglycerol
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
ELISA	enzyme linked immunosorbant assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage-colony stimulating factor
HEPES	N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]
%I	percent inhibition
ICAM	intercellular adhesion molecule
IFN	interferon
IgG	immunoglobulin Gamma
IL	interleukin
IP3	inositol triphosphate
LAK	lymphokine activated killer cell
LFA	lymphocyte functional antigen
LGL	large granular lymphocyte

LL	lung lymphocyte
L-LME	L-leucine methyl ester
L-NmmA	N^{α}-monomethyl-L-arginine
MHC	major histocompatibility complex
Mg^{++}	magnesium ion
MNC	mononuclear cells
NK	natural killer cells
NKCF	natural killer cytotoxic factor
NKR-P1	natural killer receptor protein 1
NO	nitric oxide
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PEM	Pipes, EGTA, and $MgCl_2$ buffer
PFP	pore forming protien
PGE_2	prostaglandin E_2
PLC	phospholipase C
PMA	phorbol ester myristate
poly I:C	polyinosinic-polycytidylic acid
SAL	small agranular lymphocytes
SI	stimulatory index
SL	spleen lymphocytes
TCI	tissue culture insert
TCM	tissue culture media
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor

INTRODUCTION

LITERATURE REVIEW

A. Peripheral and Tissue-Associated Natural Killer Cells

Natural killer (NK) cells are believed to play an important role in tumor surveillance (1), particularly in resistance to tumor metastasis (2-6). Tissue resident natural killer cells, particularly in the lungs and liver, are likely to play a key role in the resistance to metastasis (5,6). Some tissue-associated NK cells have been shown to respond differently than peripheral blood NK cells to *in vivo* stimulation (6-8) and this compartmentalization of activity appears to exist in clinical settings as well (9). This research project was undertaken to broaden the understanding of NK activity associated with lung lymphocytes through the characterization of the local regulation of these lymphocytes in the rat model.

Natural killer (NK) cells are lymphocytes that possess the ability to lyse certain tumor target cells in a major histocompatibility complex (MHC) unrestricted fashion without prior sensitization or stimulation. This definition attempts to delineate a cell type based on a function shared by phenotypically distinct cell populations (10,11). A morphological definition of NK cells was adopted when NK activity was found to be associated with large granular lymphocyte (LGL) morphology in the human (12) and the rat (13). However, this definition also fails to describe a unique population, in that cells of different lineages share LGL morphology (11) and small agranular lymphocytes bearing human NK cell surface markers possess lytic activity (14,15). NK cell surface markers have been developed to overcome these difficulties and provide an operational definition of NK cells. Natural killer cells share a wide range of surface molecules with other leukocyte populations including: CD2 (16), CD8 (17), CD11a/CD18 (18), CD16 (16), CD45 (19), CD53 (20), CD56 (21), and CD69 (22) among others. In the rat, asialo-GM1 which is a glycolipid on most NK cells (23) and NKR-P1, a triggering structure on rat NK cells (24) are the most common NK markers, although neither is unique to NK cells.

Natural killer cells have been described in peripheral blood (12,14), spleen (25), lymph nodes (26), liver (27,28), gut (29,30), and lungs (31,32). The vast majority of *in vitro* NK studies deal exclusively with the easily accessible compartments of the blood and the spleen. However, there is experimental evidence indicating that NK activity is regulated at the local level and may vary from tissue to tissue in the same animal (5-8). This limits the usefulness of generalizing data obtained from peripheral blood or spleen studies and makes it imperative that organ-specific NK activity be studied.

Very little was known about lung NK activity at the time this project was begun. Some early studies identified NK-like cells in the normal human lung but found that they were not lytically active (33). However, in this case, the lymphocytes were obtained from bronchoalveolar lavage fluid and thus were highly contaminated by alveolar macrophages, at least, upon initial isolation. Animal studies focused on the ability to augment lung NK activity through *in vivo* administration of various biological response modifiers, such as, polyinosinic-polycytidylic acid (poly I:C) (3,4), an inducer of interferon (IFN), or influenza virus infection (8). Additionally, it was shown that anti-asialo GM-1 administration *in vivo* increased experimental pulmonary metastases through depletion of lung NK cells (5,6). As this study began, there was a report comparing lung and spleen NK activity in the mouse (34). It was reported that there were similar numbers of asialo GM-1^+ cells in each compartment and that they possessed similar levels of NK activity. Furthermore, the lung was found to be more responsive to *in vivo* administration of poly I:C but less responsive to *in vitro* treatment with IFN (34). The scarcity of data relating directly to lung NK activity has obliged us to use the studies in other tissues as a guide in investigating lung NK activity. This has provided us with the opportunity to address various issues relating to NK activity from the pulmonary compartment.

B. Mechanisms of NK Activity

A great deal has been learned regarding the process by which natural killer activity operates in the past decade.

Several molecules have been discovered to play a more or less important role in NK cytotoxicity and many parallels have been found with cytotoxic T cell (CTL) activity. Natural killer activity can be divided into 4 relatively separate steps: i) conjugate formation and target cell recognition, ii) activation of lytic machinery, iii) secretion of lytic molecules, and iv) target cell destruction.

i) Target Cell Recognition.

Conjugate formation has long been thought of as the bridging of the target and effector cell through the interaction of multiple accessory structures such as CD2 with its ligand CD58 (LFA-3) and CD11a/CD18 (LFA-1) with CD54 (ICAM-1) or ICAM-2, in a Mg^{++} dependent manner (36-38). In fact, monoclonal antibodies (Mab) to LFA-1 and LFA-3 and the homotypic adhesion molecule CD56 have been shown to inhibit conjugate formation and lysis of some target cells (39). More recently, however, antibodies to LFA-1 and CD2 have been shown to inhibit cytotoxicity rather than conjugate formation, although they were found to significantly limit the area of contact between the target and effector cells (40). Furthermore, treatment of cloned NK cells with Mab CD2 has been shown to trigger granule exocytosis (41) and treatment with Mab to LFA-1 can trigger activation signals, such as, Ca^{++} influx and TNF-α production in lymphokine activated NK cells in conjunction with anti-CD16 (42) and on its own (43). Taken together, these findings cast doubt on the separate nature of the conjugation and recognition steps in the NK lytic cycle.

It has always been assumed that there was a natural killer target receptor through which NK cells recognize target cell structures. Recently, there have been reports characterizing candidate molecules in the mouse (44-46), the rat(47,48), and humans (49). The putative receptor molecules are type II integral membrane proteins possessing calcium-dependent lectin domains (50). It is not clear whether NK cells possess several distinct receptor molecules or whether there are several populations of monospecific NK cells. However, the human (48) and the mouse (50) appear to have NK specific gene complexes on chromosomes 12 and 6, respectively, containing several putative receptor genes. The NK receptor molecules belong to two distinct categories: NK cell activators and NK cell inhibitors.

The activator or 'classical' NK receptor has been characterized as a single entity, such as NKR-P1, which can cause redirected lysis of appropriate target cells and when blocked by specific antibodies results in inhibition of NK activity (47). However, there is evidence to indicate that the NK receptor is a multimeric complex much in the same way that the functional T cell receptor is a complex of several cell surface molecules. On the surface of NK cells CD2 has been found to be physically associated with CD53 (20). Furthermore, NKR-P1,CD2, and CD53 all require a functional

CD45 molecule with tyrosine phosphatase activity for signal transduction and NK cell activation (51). Although this evidence is from negative mutants of a rat NK cell line rather than freshly isolated lymphocytes, these cells were able to respond to pharmacological stimulation. It is interesting to postulate that several NK function related molecules interact during target recognition and that this composite signal activates NK cells for lysis.

Ly-49 is an example of the other subfamily of NK receptors which inhibits the action of NK cells against otherwise susceptible targets. Ly-49 is believed to interact with MHC-1 molecules and inhibit lysis of 'self' target cells (46). This structure explains the reported allospecificity of NK clones (52) and the findings that NK activity was often inversely proportional to MHC-1 expression (53,54). Recognition appears to take place in the presence of prohibitive MHC-1 molecules since Ca^{++} influx and phosphoinositide turnover occur despite the lack of a lytic event (55). The concept that this represents a recognition of 'self' and that NK activity occurs in the absence of self is strengthened by the findings that foreign peptides bound to MHC-1 molecules remove the protection from lysis (56,57).

ii) Activation of Lytic Machinery.

Activation of the lytic machinery follows recognition of a susceptible, non-self target. This process appears to be similar to cell activation during antibody dependent cell mediated cytotoxicity by NK cells (58) which is somewhat better understood. The transmembrane domain of CD16 is the low affinity IgG Fc receptor linked to integral membrane proteins $\zeta:\zeta$, $\zeta:\gamma$, or $\gamma:\gamma$, also found associated with the T cell receptor (TCR) (59). These disulfide linked dimers are required for cell surface expression of CD16 and its signal transduction (59). The dimers do not possess enzymatic activity but associate further with at least two non-receptor tyrosine kinases also found in T cells: ZAP-70 (60) and $p56^{lck}$ (61,62). Engagement of CD16 results in the rapid phosphorylation and activation of $p56^{lck}$ (61) which leads directly or indirectly to the tyrosine phosphorylation of phospholipase C (PLC)-γ1 and PLC-γ2 (63-64). There is evidence that tyrosine phosphorylation of PLC is also involved directly in NK activity against susceptible target cells (65). The activated PLC cleaves cell membrane phosphoinositol to inositol triphosphate (IP3) and diacylglycerol (DAG)(63). IP3 mobilizes calcium from intracellular stores and subsequent influx of extracellular Ca^{++} (66) while DAG stimulates protein kinase C (PKC) activity (67). Ca^{++} influx and PKC activation are associated with granule exocytosis in several systems though the mechanism is not entirely clear (68). Morphologically, the NK cell primed for lysis reorients its Golgi apparatus and microtubule organizing center towards the conjugated target cell and the cytoplasmic granules are released (69).

iii) Secretion of the Lytic Molecules.

The lethal hit has long been thought to come from granule exocytosis and the lytic potential of the granule components. Cytoplasmic granules of NK cells have been shown to contain pore-forming protein/cytolysin/perforin (PFP) and serine esterases (70) with various substrate specificities called granzymes and these granzymes are complexed to proteoglycans (71). Other granule components believed to be involved in NK activity include: Tumor Necrosis Factor (TNF)-like molecules, Natural Killer Cytotoxic Factor (NKCF) (72,73), leukalexin (74), calreticulin (75), and TIA-1 (T cell-restricted intracellular antigen-1), an RNA-binding protein (76). Together these components deliver the lytic potential of the NK cell.

However, the finding that small agranular $CD3^-CD56^+$ lymphocytes (SAL) are capable of potent NK activity has brought this into question (15). These cells contain identical pore-forming protein (PFP) to LGL but it is located in the cytoplasm rather than in azurophilic granules (15). When these SAL are stimulated with IL-2 the PFP become concentrated in granules identical morphologically to LGL granules (15). It is not clear whether the PFP in SAL is actually free in the cytoplasm or whether it is, in fact, present in very small granules or "pre-granules". SAL have also been shown to possess serine esterase activity equivalent to LGL. Additionally, LGL can be induced to express a lymphotoxin related protein on its cell surface which is capable of inducing lysis in susceptible targets (77). LGL are also known to express several non-granule proteolytic enzymes which may be involved in NK activity (78). Thus, the nature of the lethal hit is somewhat controversial despite the general belief that secretion of granule components, particularly PFP and granzymes are responsible for target cell death.

iv) Target Cell Death.

There is currently a debate regarding whether target cell death occurs by necrosis or apoptosis. Necrosis, the more established theory, hinges on the lytic potential of NK cell granule components, particularly PFP with its similarity to C9 of the complement system (79). The target cell membrane is essentially perforated and the cell bursts under the osmotic pressure. The apoptosis theory is much more recent and is based on the observation that NK targets often show DNA fragmentation (80) prior to target cell membrane destruction. The apoptosis theory contends that PFP, the major pore-forming-protein in the granules, makes holes in the target cell membrane but these perforations are not directly responsible for target cell death but rather allow the serine proteases, particularly granzymes A (81) and B (82) to enter the target cell and initiate a suicide program (75). The granzymes have not been found to possess endonuclease functions, in most cases, but are believed to be the effectors in the DNA fragmentation by activating either directly or through intermediates, the endonucleases responsible (75). It is likely that both effector mechanisms operate in the NK system but several questions remain, such as, whether they operate in tandem, whether one is preferentially utilized under various activation conditions, and whether each mechanism is equally susceptible to inhibition.

C. Cytokine Regulation of NK Activity

Several cytokines have been shown to increase NK activity *in vitro*. When this study was begun, IL-1 (83), IL-2 (84), IFN-α/β (85), IFN-γ (85), and TNF-α (86) were the chief stimulators of NK activity. Since then IL-6 (87), IL-7 (88), IL-12 (89) and platelet activating factor (PAF)(90) have been shown to have an augmenting effect.

The most potent single activator of natural killer cells is IL-2. Resting NK cells in the peripheral blood have been shown to constitutively express the intermediate affinity IL-2 receptor (p75) and are induced upon exposure to IL-2 to express CD25, together forming the high affinity IL-2 receptor (91). Short-term exposure of peripheral blood NK cells to IL-2 results in a substantial increase in cytotoxicity against NK-susceptible target cells that is independent of IFN production (92). IL-2 interaction with its receptor on NK cells results in phosphorylation and activation of the tyrosine kinase $p56^{lck}$ (93). It is interesting to note that $p56^{lck}$ is also involved in transduction of recognition signals from the natural killer cell receptor (NKR) complex, however, the alterations to $p56^{lck}$ seem to be more extensive in the IL-2R system. The significance of this has not been fully explored.

Long-term exposure (greater than 24h) of peripheral blood and spleen NK cells to high doses of IL-2 results in the acquisition of a broadened target spectrum and substantially increased cytotoxicity and proliferation (97). These effector cells are referred to as lymphokine activated killer (LAK) cells. The physiological relevance of LAK cells has not been adequately established, however, they provide a stable population with which to study IL-2 stimulation of NK cells and appear to be clinically relevant (98). The increased cytotoxicity of NK cells stimulated with IL-2 can, in part, be attributed to the enhanced expression of lytic mediators perforin (94), granzyme A (95), and granzyme B (96). This up-regulation occurs rapidly with the mRNA production peaking in as little as 3h (94). Another potential mechanism for the stimulation of NK activity is an increase in the ability of the effector cells to form stable conjugates eith target cells. LAK cells have been used to examine this potential mechanism. It has been shown that long-term IL-2 exposure results in a demonstrable increase in the expression of several adhesion molecules, particularly CD2 and LFA-1, in peripheral blood (99). Therefore, IL-2 strengthens the lytic potential of NK cells in the peripheral blood and spleen by increasing the number of adhesion molecules and increasing the amount of lytic factors available for secretion. A final area of influence of long-term IL-2 cultures of NK cells is in the induction of cytokine production. IL-2 stimulated LAK cells secrete significant levels of IFN-γ (88), TNF-α (100), and GM-CSF (88).

It is important to note that LAK activity is a function of stimulated blood and spleen NK cells and has not been reproduced in lung lymphocytes (101). Human lung NK cells have recently been found to be stimulated by IL-2 in culture but were unable to express the LAK cell marker leu-19, up-regulate BLT-esterase activity (102)(granzyme A), or mediate LAK activity. This difference between the lung and the peripheral blood may reflect a difference in cell maturation wherein NK cells in the lung may have progressed to a differentiation state where proliferation is no longer possible. This concept is supported by the recent finding that lung NK cells possess a greater degree of basal BLT-esterase activity than PBL (102) indicating an *in situ* activation state, compared to PBL. Alternatively, the difference may reflect regulatory influences in the local lung environment.

Much less is known about the mechanism of regulation of NK cells by other cytokines probably because of the ease of studying LAK cells and the clinical promise of LAK cell activity.

Interferons have long been known to increase NK activity upon *in vivo* treatment with IFN or IFN inducers or *in vitro* treatment (103). However, the two major types of interferon, IFN-α/β and IFN-γ, have been shown to operate through separate receptors and function by different mechanisms (104). In fact, there have been some reports indicating the IFN-γ has little boosting activity (105). It is not clear whether this is a result of NK cell resistance to IFN-γ or previous exposure since NK cells are able to produce this cytokine. Both IFN-α/β and IFN-γ have been shown to interact with IL-2 to augment NK activity (106). IFN-α/β synergizes with IL-2 in stimulating NK activity but appears to inhibit the induction of LAK activity. IFN-γ, on the other hand, synergizes with the IL-2 effect on NK activity and enhances the cytotoxicity of LAK cells. This activity is of particular interest since IFN-γ is produced by IL-2 stimulated NK cells (107).

TNF-α has been shown to enhance peripheral blood NK activity *in vitro* (86) and *in vivo* (108), although the degree of stimulation appears to be less than that often associated with IL-2 stimulation. TNF-α may operate, in part, by inducing IL-2 receptor expression on NK cells. Consistent with this function, TNF-α is capable of synergizing with low doses of IL-2 to induce LAK activity in PBL (109). Peripheral blood LGL have been shown to produce TNF-α upon stimulation with IL-2 or by stimulation with sensitive target cells (100).

Human peripheral blood LGL can be induced to produce substantial amounts of IL-1 when stimulated with LPS (110).

The effects of IL-1 on NK activity are not clear; some report a direct stimulatory activity (83) while others find no evidence for direct stimulation but report synergistic augmentation with IFN or IL-2 (111). Like TNF-α, IL-1 may synergize with IL-2, at least in part, by inducing IL-2R (CD25) expression (112).

IL-6 has been shown to augment CD3 negative NK activity in PBL. The augmentation appears to operate at a post-conjugation step in the lytic cycle since conjugation was not affected. The effects of IL-6 on this population were largely a result of induction of IL-2 production in the PBL (87). When highly purified $CD3^{-}CD56^{+}$ human peripheral blood NK cells were used, IL-2 production was not induced, although a small increase in NK activity was reported (113). There is some debate over whether IL-6 can stimulate proliferation of this enriched NK cell population (114) or not (113) and whether IL-2 receptor expression is increased (114) or not (113). There is evidence to indicate that IL-6 synergizes with IL-2 in LAK induction and is capable on its own of inducing TNF production (114).

IL-7 is capable of inducing LAK activity in human peripheral blood NK cells, however, these activated cells have less proliferative activity than LAK cells derived from IL-2 stimulation. IL-7 induces the expression and secretion of lower concentrations of GM-CSF than LAK cells derived from stimulation of NK cells with IL-2, and only slightly induces

IFN-γ . Finally, IL-7 induces TNF receptor both on the surface and in a soluble form (88). Thus IL-7 may be important in cytokine cascades involving NK and other immunologically relevant cells.

IL-12 is a heterodimer glycoprotein that enhances the activity of freshly isolated NK cells. IL-12 appears to do this by promoting granulogenesis and stimulating the production of cytolytic granule components, perforin, granzyme A & B, and TIA-1 (115). Additionally, IL-12 is another cytokine capable of inducing LAK activity, in an IL-2 independent manner, in human peripheral blood NK cells, but is unable to promote significant proliferation (116). IL-12 induces the expression of high levels of IFN-γ and low levels of GM-CSF when compared to IL-7 (88). IL-12 acts in concert with IL-4 to induce moderate proliferation in LAK cells. IL-12 induces IL-4R expression while IL-4 in the presence of IL-12 induces IL-12R expression, and such interaction may account, in part, for the synergistic effect (117). These two cytokines also synergize in the production of TNF-α (117). The finding that IL-4 synergizes with IL-12 in the generation and proliferation of cells with LAK activity underscores the difference in the mechanism by which IL-2 and IL-12 operate, in that IL-2 induction of LAK activity is suppressed by IL-4 (118).

Platelet activating factor (PAF) has been reported to enhance lung NK activity (90), which is the reason for its inclusion in this list. PAF was reported to enhance NK activity through a Ca^{++} and PKC-dependent method, however, this was determined using chelators and other factors known to inhibit NK activity. Furthermore, PAF antagonists have been shown to inhibit basal NK activity (119) suggesting a role for this arachidonic acid metabolite in the NK lytic mechanism rather than as an externally-derived regulatory factor.

D. Modulation of NK Activity by Macrophages.

Cells of the monocyte/macrophage lineage are potent modulators of the immune response, in general, and of NK activity, in particular. Alveolar macrophages (AM) line the surface of the alveoli, the gas exchange surface of the lung, and are thus in close proximity to the interstitial lung lymphocytes of interest in this study. Therefore, a careful examination of the interaction of AM with lung lymphocytes was important to understanding the local regulation of these cells.

A role for monocytes in the regulation of the NK activity of peripheral blood lymphocytes has been established in several studies (120-124). The regulation can take the form of augmentation, as well as inhibition, depending on the source of the monocytes, the purity of NK cells, and the method of interaction. Macrophages, however, have been associated, predominately, with inhibition of NK activity (125).

Augmentation of NK activity by monocytes has been reported to take place when monocytes were added directly to the lytic assay or upon short-term co-culture and subsequent removal by carbonyl iron (120). This augmentation requires *de novo* protein synthesis, viable monocytes, and non-LGL accessory lymphocytes. Furthermore, the augmentation was sensitive to anti-IL-1 and anti-IL-2 but not to anti-IFN-γ, anti-IFN-α, or indomethacin (121). Thus, it appears that IL-1 and/or IL-2 may be produced in the co-culture with the help of accessory cells and that these cytokines are responsible for the observed augmentation of NK activity.

Augmentation has also been reported with monocytes differentiated *in vitro* (122). In this case, augmentation was dependent upon intact live macrophages being present in the co-culture and accessory cells were not required. Interestingly, the degree of maturation of the macrophages played a significant role in the modulation of NK activity in that monocytes cultured for 7 days augmented NK activity while monocytes cultured for 14 days inhibited NK activity. Additionally, alveolar macrophages have been found to be much more suppressive to NK activity than monocytes, a less differentiated cell type (123).

The vast majority of reports describe the inhibition of NK activity by monocytes/macrophages either through cell-to-cell contact mediated mechanisms or by production of inhibitory soluble factors. Monocytes (124) and macrophages (125) have been found to inhibit peripheral blood NK activity by means of cell-to-cell contact through the inhibition of Ca^{++} influx (125). A wide range of cytokines, growth factors, and other immunoregulatory molecules are produced by macrophages. Several of these have been found to inhibit, or potentially inhibit, natural killer activity, including: transforming growth factor (TGF)-β (126), platelet-derived growth factor (PDGF) (127), prostaglandins (PG) particularly PGE_2 (128), lipoxins A and B (129), and reactive forms of oxygen (130) or nitrogen (131).

Prostaglandins are cyclooxygenase metabolites of arachidonic acid. Several prostaglandins of the E series have been shown to inhibit NK activity with PGE_2 being the most prominent (128). PGE_2 inhibition of NK activity has been studied extensively with the use of indomethacin, a cyclooxygenase inhibitor, and with addition of exogenous PGE_2 into cytotoxicity assays. PGE_2 inhibits freshly isolated spleen NK activity and IFN-stimulated NK activity (128) and may be involved in AM mediated inhibition of LAK activation (132). The mechanism of action of PGE_2 is not well understood but it involves an inhibition of conjugate formation and an increase in cAMP, which may suppress granule secretion (94). Recently, the effects of PGE_2 have come into question when it was reported that pretreatment of target cells but not effector cells with PGE_2 resulted in suppression of NK activity in the murine system (133). The experimental protocols used in previous studies were not designed to discount this possibility so the effects of exogenous PGE_2 may, in part, reflect its action on the target cell. However, it has been reported that *in vivo* treatment with indomethacin increases murine peritoneal NK activity (148). This clearly suggests an effector cell component in the inhibition, given that the target cells were not exposed to the indomethacin or the inhibitory microenvironment of the peritoneum.

Lipoxins are other arachidonic acid metabolites produced by macrophages and capable of inhibiting peripheral blood NK activity. The mode of action of lipoxins is substantially different than that of prostaglandins (129). Lipoxins do not alter conjugate formation or cAMP but appear to disrupt signals involved in the orientation of the Golgi apparatus toward the target cell (134). Lipoxins have not been studied as extensively as other potential inhibitory factors and their physiological relevance to monocyte/macrophage inhibition of NK activity has not been shown.

Reactive oxygen species, such as hydrogen peroxide, superoxide anion, hydroxyl radical and singlet oxygen are known to be produced by the oxidative burst of monocyte/macrophage. They may act through effector cell damage or through unexplored reversible means (130). Nitric oxide, a reactive nitrogen molecule, may behave in a similar fashion. Nitric oxide is produced by macrophages and is known to inhibit T cell activation and proliferation (131), however, the effect on NK activity is not known.

The last group of inhibitory factors to be examined is that of the cytokines, TGF-β and PDGF. PDGF is produced by monocytes stimulated by a variety of means (135). NK inhibition takes place after relatively short incubation times (2h) and results from an inhibition of conjugate formation (127). This inhibition is resistant to reversal by IL-2 or IFN-α, but PDGF is unable to inhibit NK cells stimulated with these cytokines (136).

In contrast, TGF-β is a potent inhibitor of freshly isolated NK cells (126) and lymphokine activated NK cells (137). It appears that this effect on LAK induction is a result of an inhibition of TNF-α production because exogenous TNF-α added at the initiation of culture can reverse the effects of TGF-β (137). In addition to TNF-α suppression, TGF-β inhibits the production of IFN-γ, TNF-β, and the expression of IL-2Rα (CD25) in LAK cells (138). TGF-β inhibits cytotoxicity by down-regulating the expression of cytolytic granule components perforin and granzyme A, at least in NK cells stimulated with IL-2 (94). The fact that TGF-β is produced by alveolar macrophages (132) and its potent inhibitory capacity makes it an important candidate in AM-mediated suppression of NK activity.

OBJECTIVES

Having explored the relevant literature regarding the regulation of natural killer activity, it is apparent that tissue-associated regulation is an important and neglected area of study. This is particularly true of lung-associated NK activity given the situation of the lung in contact with the external and internal environment, as well as its importance in metastasis. We have undertaken the examination of the lung-associated NK cell population and its regulation, to fill the void in this area of research.

A major aim of our study was to characterize lung-associated natural killer activity in the rat in terms of accepted NK parameters. The initial experiments were designed to establish that a population of "standard NK cells" were present in the lung. The lung NK cell activity was then compared to spleen and peripheral blood NK activity in the rat to determine the extent of similarity and/or difference between these more commonly studied populations of NK cells. Having established these parameters, our study focussed on the local regulation of lung NK activity. First, the responsiveness of lung NK activity to various cytokines and combinations of cytokines was examined. Second and most importantly, the local regulation of lung NK activity by alveolar macrophages and its mechanism of action was investigated.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 225 to 300g were purchased from Charles River Canada, Inc. (St-Constant, Quebec). These animals were derived from a pathogen-free colony, shipped behind filter barriers and housed in isolated temperature controlled quarters in an animal isolator unit (Upjohn Scientific, Toronto, Ontario). The animals were used within 2 weeks of arrival.

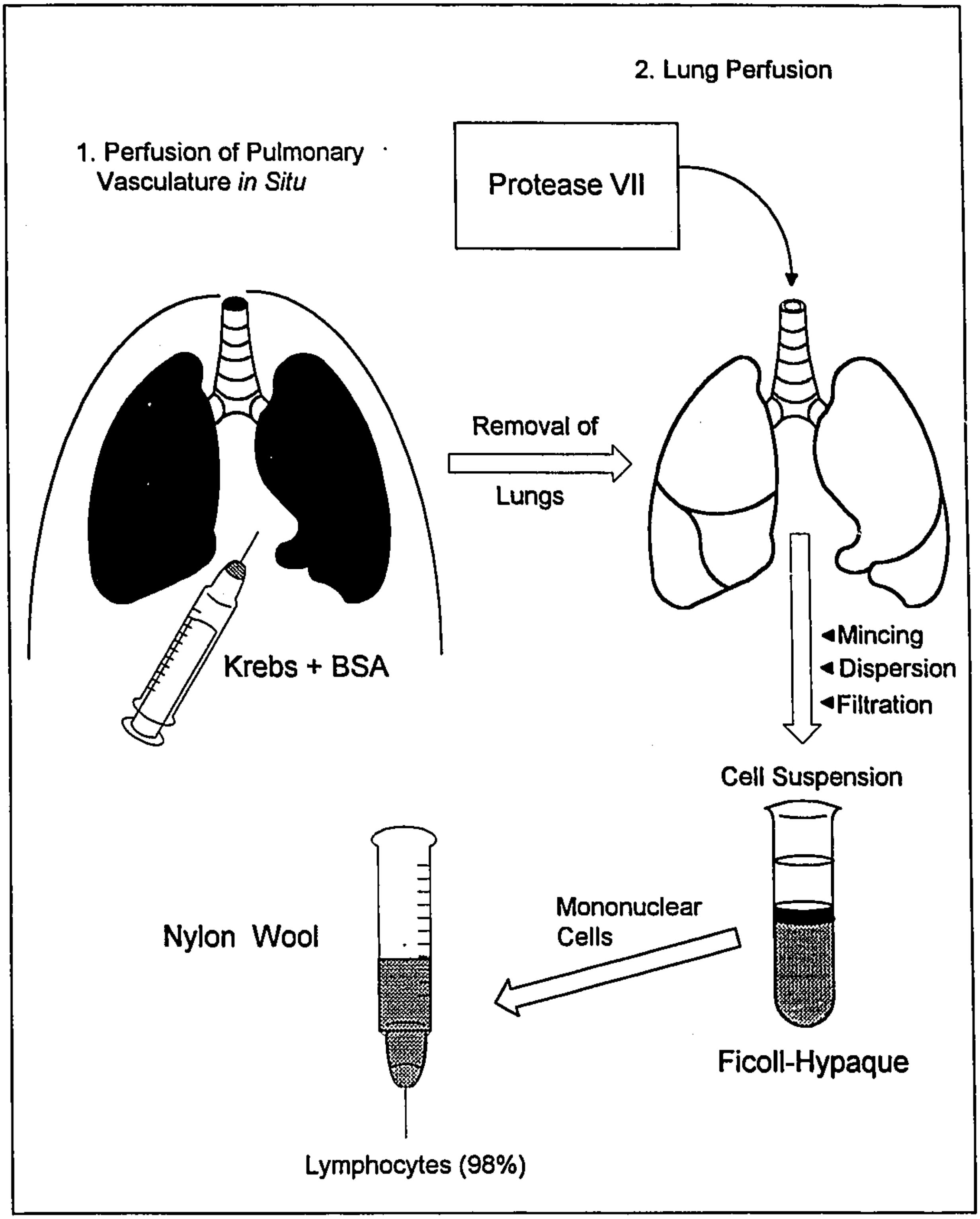
Cell Preparations

A. Lung Lymphocytes (LL)

Rat lung interstitial lymphocytes (LL) were prepared according to Figure 1, as previously described (139). The anesthetized rat was given a lethal dose (0.5 mL) of sodium pentobarbital, Somnotol, (MTC Pharmaceuticals, Cambridge, ONT) via the renal vein. The rat was injected with 3000 U of heparin (Organon Teknika, Toronto, ONT) Immediately after sacrifice, the lung was inflated and perfused with 120 mL Krebs buffer containing 2.5% bovine serum albumin (BSA) (Sigma, St. Louis, Mo), 5 mM glucose, and 1000 U of heparin (37°C, pH 7.4) via the pulmonary artery to remove all blood elements from the pulmonary vasculature. The lung and trachea were removed from the animal and attached to a peristaltic pump. The lung was perfused via the trachea for 10 min with Ca^{++}, Mg^{++}-free Krebs buffer containing 0.5-1.0 mg/mL protease type VII (Sigma, St. Louis, Mo) and 1 mM

FIGURE 1. Isolation of Lung Lymphocytes.

The rat was anesthetized, then perfused via the pulmonary artery with warmed buffer. The lungs were removed from the rat and attached to a peristaltic pump by the trachea. A protease solution was pumped into the lungs. The digested lungs were minced, passed through a 60 cc syringe, and filtered. The single cell suspension was layered on to a Ficoll-Hypaque gradient. The mononuclear cells were removed from the interface between the Ficoll-Hypaque cushion and the buffer layer and passed through a nylon wool column. The resultant population was used in most of the experiments described in this study. The cell population contained approximately 98% lymphocytes and 2% macrophages.


2. Lung Perfusion
1. Perfusion of Pulmonary Vasculature *in Situ*
Protease VII
Removal of Lungs
Krebs + BSA
◄Mincing
◄Dispersion
◄Filtration
Cell Suspension
Mononuclear Cells
Nylon Wool
Ficoll-Hypaque
Lymphocytes (98%)

Ethylenediamine tetraacetate (EDTA) (150 mL, 37°C, pH 7.4). The lung tissue was minced and dispersed by repeated passage through a 60 mL syringe and filtered twice through Swiss Nytex nylon monofilament (110 μm)(B & SH Thompson, Mont-Royal, PQ). The cell suspension was washed twice in PBS and filtered after each wash. Mononuclear cells (MNC) were obtained by centrifugation on a Ficoll-Hypaque (Pharmacia, Dorval, PQ) gradient (specific density, 1.077) at 1800 rpm for 45 minutes at room temperature. The cells at the interface between the Ficoll-Hypaque and the phosphate buffered saline (PBS) layer were carefully harvested and washed twice in PBS and once in RPMI-1640 medium containing 0.1% Gentamicin, 10% fetal bovine serum (FBS) and 0.8% HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), which will henceforth be referred to as tissue culture media (TCM). The resulting cell preparation contained approximately 30×10^6 cells per rat. Except where noted, MNC were incubated for 30 minutes in a pre-washed nylon wool column (Wako Chemicals, Dallas, Texas) to permit the adherence of macrophages and B cells. The non-adherent population was washed out of the column and collected for further study. The final population routinely consisted of an average of 98% lymphocytes and 2% macrophages by light microscopy and Wright-Giemsa staining.

This protocol was altered in the experiments performed to determine whether enzymatic treatment significantly influenced NK activity. In this set of experiments, three non-enzymatic treatments were tested. The lung was handled as above until removal. The lung was then either, a) minced in Hank's balanced salt solution, homogenized and fractionated on Ficoll-Hypaque, b) minced and pushed through a rigid nylon mesh, or c) flushed through a 10 mL syringe 10 times. In each case the resulting cell suspension was analyzed by differential staining of cytospin samples and assayed for NK activity.

B. Spleen Lymphocytes (SL)

The rat was anesthetized as above and the spleen was excised and placed in PBS on ice. The connective tissue was removed and the spleen was transferred twice to fresh PBS on ice. The spleen was gently pressed through a Collector Tissue Sieve (E-C Apparatus). The cell suspension was collected in 10 mL PBS and transferred to a 15 mL glass tube. This mixture was allowed to sediment at room temperature for 5 min and the sediment was discarded. After centrifugation at 1000 rpm for 10 min, the supernatant was discarded and the pellet resuspended in PBS. This cell suspension was filtered through Swiss Nytex, as above, then fractionated on a Ficoll-Hypaque gradient. Spleen cells were then passed through a nylon wool fibre column in the same manner as the LL described above. This procedure yielded approximately $55x10^6$ cells per spleen. The resulting population was shown to have less than 1% macrophages contamination by differential analysis.

C. Peripheral Blood Lymphocytes (PBL)

Blood was collected by cardiac puncture of anesthetized rats into 10 mL syringes containing 100 U of heparin. The blood was diluted 2-fold with PBS and layered onto a Ficoll-Hypaque gradient. On the average, 9.75×10^6 cells were obtained from the interface of the PBS and Ficoll-Hypaque layers of the gradient. These cells were treated as described above (Fig. 1) for the lung resulting in a nylon wool non-adherent population which contained greater than 99% lymphocytes.

D. Alveolar Macrophages (AM)

Alveolar Macrophages (AM) were obtained by bronchoalveolar lavage. The rat was injected with a lethal dose of somnotol as was the case with the other procedures. The diaphragm was punctured and the abdominal aorta was severed to exsanguinate the rat. The trachea was cannulated and a volume of 48 mL warm PBS was used to lavage the lung in aliquots of 8 mL. The lung surface was massaged gently while the lavage fluid was in place. The first three aliquots were kept in the lung for four minutes while the final three were for two minutes each. Each aliquot was placed on ice after removal from the lung. Lavage recovery was at least 90% of the volume infused. The total lavage fluid was centrifuged at 1000 rpm for 10 min at 4° C and the pellet was resuspended in tissue culture media. Differential cellular analysis has shown these cells to be greater than 99% of macrophage morphology.

Differential Cellular Analysis

Differential counts of LL, SL, PBL and AM populations were made from cytocentrifuged smears prepared with 4×10^4 cells/mL in tissue culture media containing 20% FBS. The smears were stained with Wright-Giemsa and examined under a light microscope.

Cytotoxicity Assay

NK activity was measured through a chromium-51 release assay where target cells, Yac-1 (murine lymphoma) in the case of rat or mouse NK, were labelled with ^{51}Cr then incubated with the effector cells. The radioactivity released from the target cell was directly proportional to the lytic activity of the effector cell. To obtain meaningful information, several target to effector cell ratios were employed. Using this procedure, lymphocytes were added to 96-well microtiter plates in 100 μL of TCM at various concentrations, typically 2×10^6/mL, 10^6/mL, 5×10^5/mL, and 2.5×10^5/mL. Yac-1 target cells were labelled for 90 min with ^{51}Cr (Amersham, Oakville, Ontario or New England Nuclear, Bedford, Ma) (0.1 mCi). Labelled target cells were washed three times in warm TCM and added into 96-well plates at a concentration of 10^4 cells/well in 0.1 mL of media to give effector:target (E:T) ratios of 20:1 to 2.5:1. The plates were incubated for 4h, after which, 0.1 mL of the supernatant was removed and ^{51}Cr content was measured in a γ-counter (Raytest). Cytotoxicity was expressed for each ratio as the percent specific ^{51}Cr release using the following formula:

$$\% \text{ specific } ^{51}\text{Cr release} = \frac{\text{ER} - \text{SR}}{\text{TR} - \text{SR}} \times 100\%$$

where ER represents cpm in the presence of lymphocytes; SR, cpm due to spontaneous release and TR, cpm due to the total incorporated ^{51}Cr in 10^4 target cells.

Analyzing differences across a range of ratios is cumbersome and the results are often highly dependent upon the particular ratios used. The use of lytic units to give a single value representing the potential of all of the ratios combined allows for more reliable comparisons to be made. Therefore, the ^{51}Cr release data were, except where noted, further processed into lytic units to give a single value of the lytic potential from the assorted experimental ratios. The lytic units were derived from an exponential fit equation using a computer program generously provided by Dr. H. Pross (Queen's University) (140). One lytic unit was defined as the number of effector cells required to cause 20% lysis of 10^6 target cells. The data were often expressed as stimulatory index (SI) or as percent inhibition (%I), which was calculated as follows:

$$\text{S.I.} = \frac{\text{LU}_{20} \text{ from experimental}}{\text{LU}_{20} \text{ from control}} \times 100\%$$

$$\%\text{I} = \left(1 - \frac{\text{LU}_{20} \text{ from experimental}}{\text{LU}_{20} \text{ from control}}\right) \times 100\%$$

Treatment with Anti-Asialo GM-1 and Complement

Lung lymphocytes were suspended in cytotoxicity media (CM: RPMI + 0.3% BSA + 25 mM HEPES) (Cedarlane Laboratories, Hornby, Ontario) at a concentration of $5x10^6$ cells/mL. Asialo GM-1 rabbit antisera (Wako Chemicals, Richmond, Virginia) was added at 1/25 and 1/50 dilution and incubated on ice for 45 min with intermittent agitation. The cells were then washed three times in CM. The labelled cells were then incubated at 37° C with intermittent agitation for 45 min with a 1/10 dilution of Low-Tox M rabbit complement (Cedarlane). The cells were washed three times in CM and the cytotoxicity was determined. As a control, cells were treated with complement without prior exposure to the specific antisera. The average recovery of cells treated with complement alone was 80%, while 65% were recovered following treatment with anti-asialo GM-1 and complement.

Treatment with L-Leucine Methyl Ester

The method of Shau and Golub (141) was used to deplete the lung lymphocyte population of NK cells. L-leucine methyl ester (L-LME)(Sigma) was prepared fresh in RPMI-1640 medium and the pH was adjusted to 7.4 with 1N NaOH. Lung lymphocytes were suspended at 10^7 cells/mL in TCM containing 20 mM or 40 mM of L-LME. They were incubated for 30 min at 37° C, then washed three times in TCM and tested for cytotoxicity against Yac-1 target cells. The average recovery of L-LME treated lung lymphocytes was 85%.

Immunofluorescent Staining

Lung and spleen lymphocyte preparations were examined for reactivity with the rat NK specific monoclonal antibody 3.2.3 (142). Cells were incubated for 30 min on ice with mAb 3.2.3, a mouse IgG_1 (generously provided by Dr. J. Hiserodt, University of Pittsburgh) at a dilution of 1:500. Labelled cells were washed twice in PBS and incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (Fab_2 fragment) (Sigma) at a dilution of 1:128. As a control, cells were also incubated with the secondary antibody in the absence of the primary antibody.

Cells were washed twice and layered onto poly-L-lysine coated cover slips and allowed to settle for 10 min at room temperature. The lymphocytes were washed once in PEM buffer (80 mM Pipes, 5 mM EGTA, 1 mM $MgCl_2$, pH 6.8) then fixed for 10 min with 3.7% formaldehyde in PEM buffer. The cover slips were mounted on slides with 7 μL of mounting medium (p-phenylene diamine in 50% glycerol-PBS, pH 7.8). The percentage of positively staining lymphocytes was determined by counting greater than 200 cells per slide under the fluorescent microscope. Positive cells were never seen in the cells treated only with the secondary antibody.

Co-Culture of Lymphocytes with AM

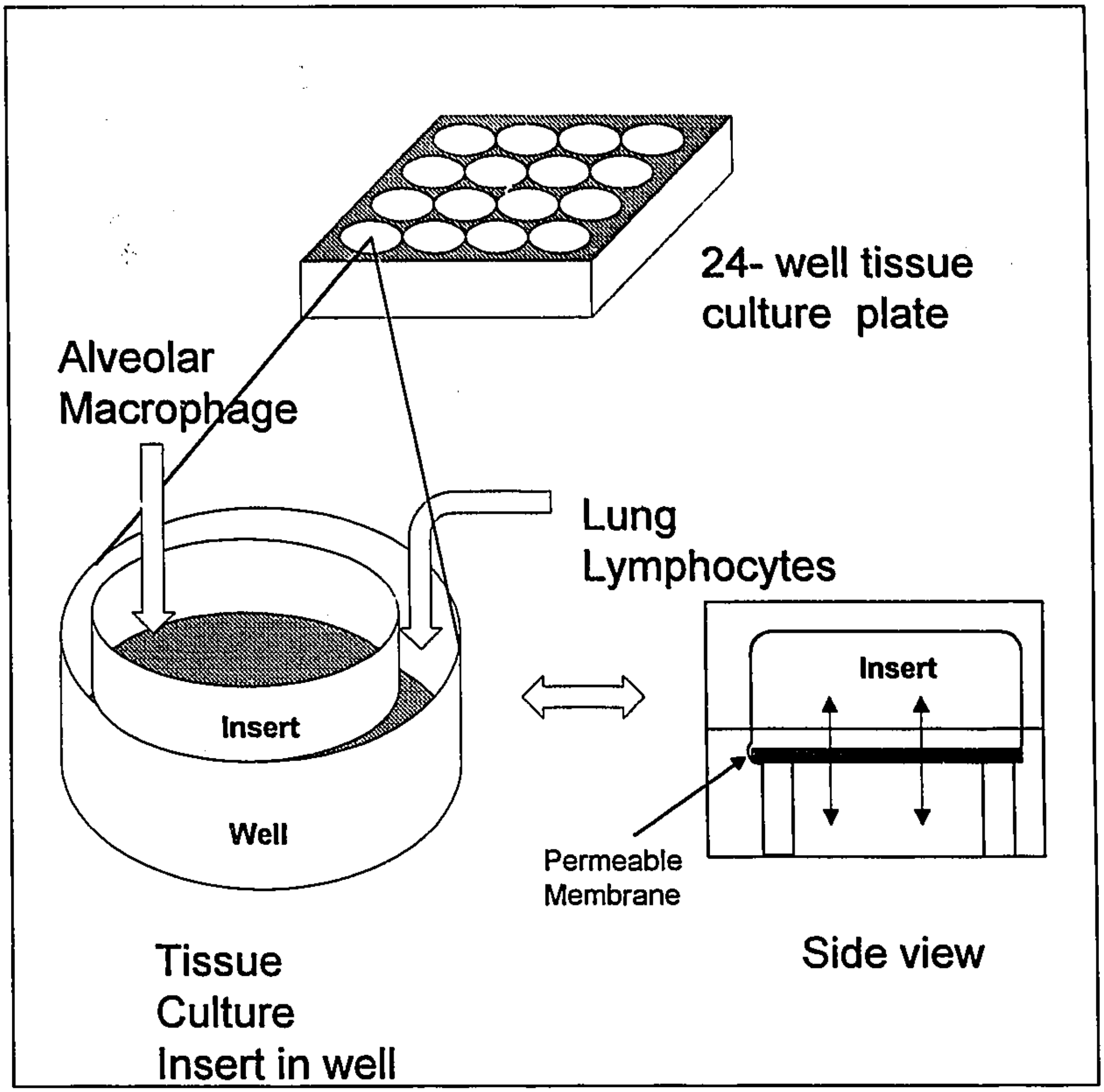
Lymphocytes and alveolar macrophages were co-cultured in a two-chamber system separated by a permeable barrier, as shown in Figure 2. Lymphocytes were placed into 24-well plates at a concentration of 2 x 10^6 cells/mL in 0.5 mL of TCM. Millicell-CM Culture Plate Inserts (TCI) (Millipore, Bedford, Ma) were placed into the wells in such a way that no air bubbles were trapped under the TCI and the TCI membrane was uniformly moistened by the underlying media. Freshly isolated AM were added into the TCI at a concentration of 2 x 10^6 cells/mL in 0.5 mL, or 0.5 mL of media alone was added. The plates were incubated at 37°C for 18h. After the incubation time, the TCI, still containing the AM, was removed and discarded. The lymphocytes were collected from the plate by repeated pipetting, washed, and counted. They were then placed in a 96-well plate and a cytotoxicity assay was performed.

Supernatant Preparation

Alveolar macrophages obtained through bronchoalveolar lavage were plated into 24-well plates in 1 mL TCM at concentrations of 1 x 10^6 AM/mL, 2 x 10^6 AM/mL, 3 x 10^6 AM/mL, and 4 x 10^6 AM/mL. The plates were incubated at 37°C for 18h. The supernatant was then removed from the plates and like supernatants pooled. The pooled supernatant was centrifuged at 1800 rpm for 15 min to remove cells and was stored at -80°C until needed.

FIGURE 2. Co-Culture of Lung Lymphocytes with Alveolar Macrophages.

The lung lymphocytes were placed into a 24-well microtiter plate at a concentration of $2x10^6$ cells/mL in 0.5 mL of tissue culture media. Tissue culture inserts were placed in the well in such a way that the entire membrane was moistened by the media. Alveolar macrophages obtained by bronchoalveolar lavage were added to the tissue culture insert also at a concentration of $2x10^6$ cells/mL in 0.5 mL of tissue culture media. The plates were incubated for 18h at 37°C, then the insert containing the alveolar macrophages was removed. The treated lung lymphocytes were harvested, washed, and used as lymphocytes co-cultured with AM.


24- well tissue culture plate
Alveolar Macrophage
Lung Lymphocytes
Insert
Well
Insert
Permeable Membrane
Tissue Culture Insert in well
Side view

Biological Response Modifiers (BRM)

Human recombinant interleukin 2 (rIL-2) with specific activity of 5 x 10^6 U/mg of protein was kindly provided by Cetus Corp., Emeryville, CA. or purchased from Gibco/BRL, Burlington, Ont. with a specific activity of 3.1 x 10^6 U/mg of protein. Both preparations were used in the experiments reported here, with equal effectiveness. Murine recombinant interleukin 1 (rIL-1) was purchased from Genzyme, Boston, MA, with a specific activity of 1 x10^8 U/mg of protein. Rat recombinant interferon gamma (rIFN-γ) with specific activity of 4 x 10^6 U/mg of protein was purchased from Amgen, Thousand Oaks, CA. Purified rat interferon alpha/beta (IFN-α/β) with specific activity of 3.5 x 10^6 U/mg of protein was purchased from Lee Biomolecular, San Diego, CA. Murine recombinant tumor necrosis factor alpha (rTNF-α) with a specific activity of 4 x 10^7 U/mg of protein was purchased from Genzyme, Boston, MA. Prostaglandin E_2 (PGE_2) with a purity of greater than 99% as determined by thin layer chromatography was purchased from Sigma. Ultrapure human transforming growth factor-β_1 (TGF-β_1) with a specific activity of 10^6 U/mg was purchased from Genzyme.

Incubation with BRM

Nylon wool non-adherent lymphocytes were placed into 96-well microtiter plates at 2-fold dilutions (2.0-0.25x10^5 cells/well) in triplicate in 0.1 mL of TCM. The desired concentration of BRM(s) was then added to the wells in 0.1 mL of TCM. The plates were incubated for 18h at 37°C. The plates were centrifuged and 0.1 mL of the supernatant was discarded from each well. ^{51}Cr labelled Yac-1 target cells were added to the wells and the cytotoxicity assay was performed as described above.

Kinetics of AM Inhibition of Lung NK Activity

Lung lymphocytes were co-cultured with alveolar macrophages in the two-chamber system described above. At 2h, 4h, 6h, 12h, and 18h after the beginning of the co-culture period, lymphocytes that had been co-cultured and parallel lymphocytes that had been incubated alone were harvested. These lymphocytes were placed in 96-well plates and cytotoxicity assays were performed with freshly labelled Yac-1 target cells.

In a different set of experiments, lung lymphocytes were co-cultured with alveolar macrophages, as above, for 18h. The TCI containing the AM were then removed and the lymphocytes harvested. These cells were washed three times in TCM and plated in 96-well plates. The plates were incubated at 37° C for 2h, 4h, or 6h before the addition of labelled Yac-1 cells for the beginning of a cytotoxicity assay. Lung lymphocytes that had been incubated alone were treated in parallel to the co-cultured cells and were used as controls at each time point.

Co-culture of AM with LL conjugated to Yac-1

Cell-to-cell contact co-cultures were performed in 96-well plates to examine the effects of AM on binding versus post-binding events. Lymphocytes were aliquoted at 2×10^{6}/mL, 10^{6}/mL, 5×10^{5}/mL, and 2.5×10^{5}/mL in 100 µL TCM. Labelled Yac-1 cells were added and the plates were incubated for 30 min at 4°C. AM were added to the wells to give a final ratio of 1:1 and 1:5 AM:LL and the plates were incubated at 37°C for 1h, 3h, or 4h. After the indicated time, 100 µL of the supernatant were counted for radioactivity and the lytic activity determined as above. To control for non-specific cold target inhibition due to the presence of a non-effector / non-target cell type in the assay, the 'NK resistant' P815 (murine mastocytoma) cell line was added in place of AM as a filler cell in control wells.

Indomethacin Treatment

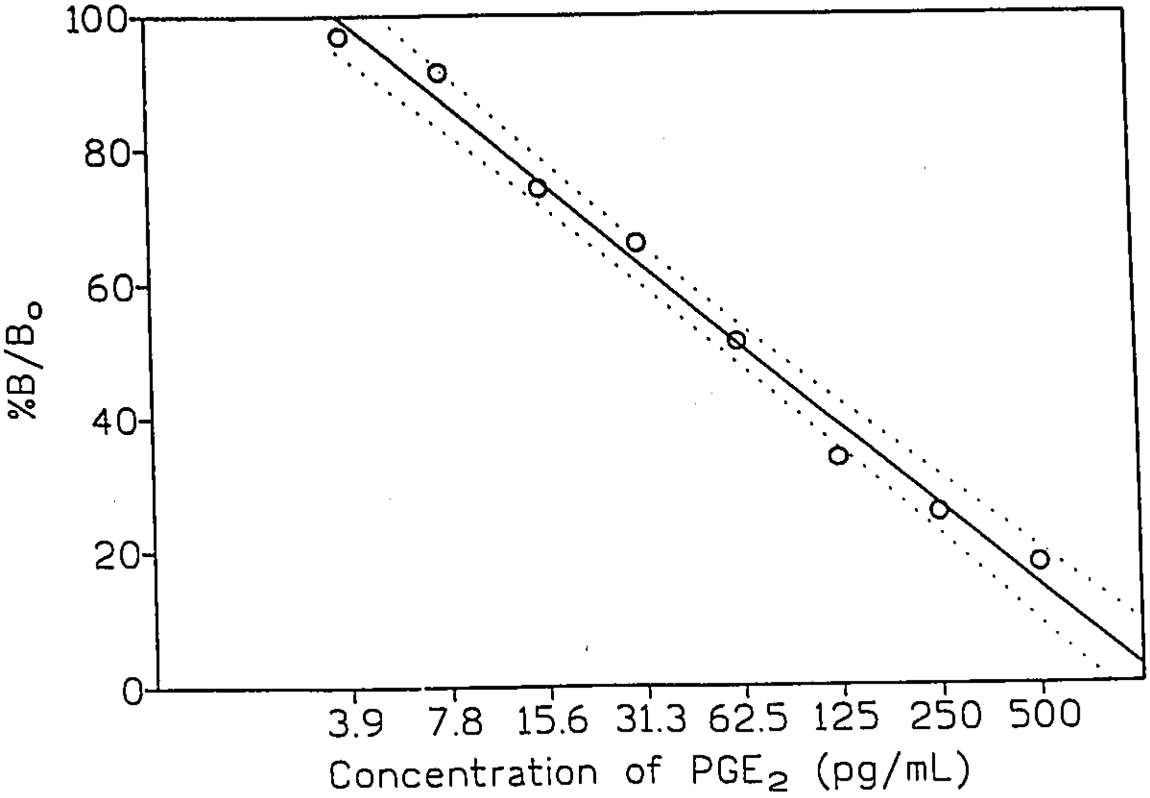
Indomethacin was used to inhibit the production of prostaglandins. Indomethacin (Sigma, St. Louis, Mo) was added to both lymphocytes alone and to lymphocyte/macrophage co-culture wells at the beginning of the incubation period in concentrations ranging from 0.5 µM to 10 µM. The lung lymphocytes were harvested at the end of the 18h incubation and the cytotoxicity was assessed in a ^{51}Cr release assay. There was no effect of indomethacin on lung NK activity in the absence of AM except at the highest concentrations (10 µM).

Prostaglandin E_2 Determination

Prostaglandin E_2 (PGE_2) was detected in cell-free supernatants using an ELISA kit (Cayman Chemical, Ann Arbor, MI). The ELISA was a competitive acetylcholinesterase linked immunoassay. It was performed according to the manufacturer's instruction. Nunc certified maxisorp microtiter plates were coated for 24h at room temperature with mouse monoclonal anti-rabbit IgG then blocked with BSA at 4°C for at least 18h. The plates were kept in the dark throughout all incubations. Standard concentrations of PGE_2, 2-fold dilutions from 500 pg/mL to 3.9 pg/mL or appropriate dilutions of cell-free supernatants were added to the wells with a fixed concentration of acetylcholinesterase-linked PGE_2 and rabbit PGE_2 antiserum. The plates were incubated at room temperature for 18h then developed with fresh Ellman's Reagent for 90 min. The absorbance was read at 450 nm in an optical microplate reader (Biotek). The values were corrected for non-specific binding. The standard curve was plotted as % standard bound/maximum bound versus log PGE_2 concentration. The concentrations in the samples were derived from the linear regression of the straight line portion of the standard curve. A representative standard curve appears in Figure 3.

FIGURE 3. Representative Standard Curve for PGE_2 ELISA.

PGE_2 was measured by a competitive acetylcholinesterase-linked immunoassay. The standard curve was derived by plotting the absorbance for a particular concentration of PGE_2 (2-fold dilutions from 500 pg/mL to 3.9 pg/mL) as a percentage of the maximum absorbance for the assay against the log of the concentration of PGE_2 used. The line of best fit was determined by linear regression (GraphPad) and the test values were obtained from the equation of the standard curve. The solid line represents the linear regression and the dotted lines are the 95% confidence interval.


100
80
60
40
20
0
%B/B$_o$
3.9
7.8
15.6
31.3
62.5
125
250
500
Concentration of PGE$_2$ (pg/mL)

Neutralization Experiments

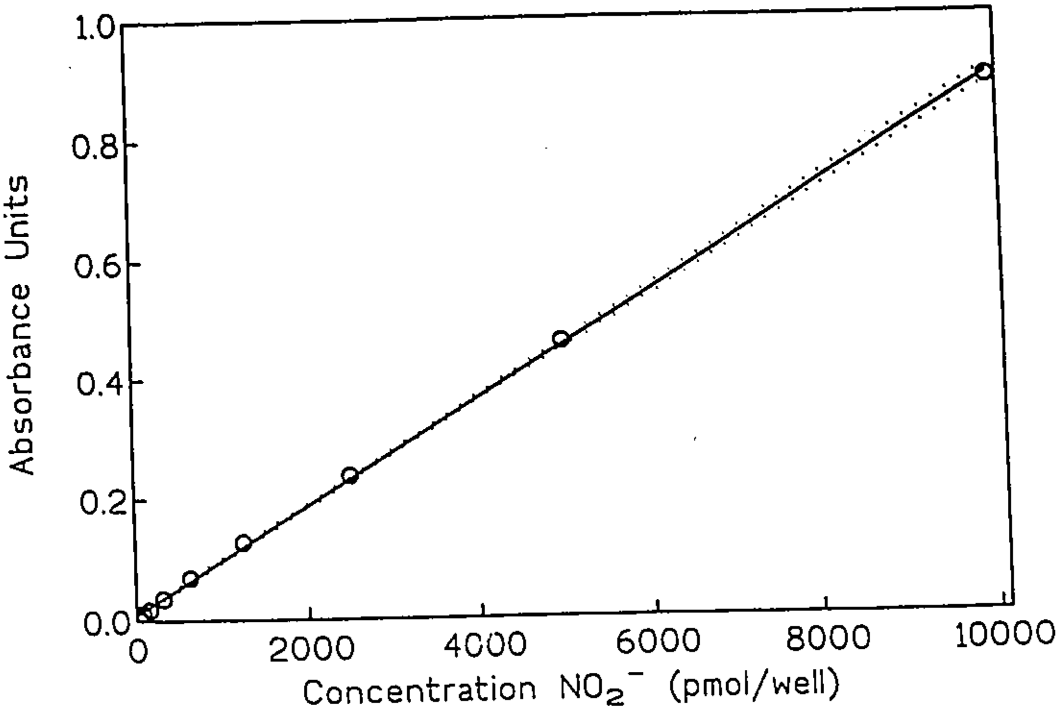
Supernatants from 18h incubations of 4 x 10^6 AM/mL were reacted for 30 min either with a purified turkey IgG antibody to human Transforming Growth Factor-β_1 (anti-TGF-β_1) with neutralizing activity against rat TGF-β_1 (Collaborative Research, Bedford, MA), a goat IgG antibody against human Platelet Derived Growth Factor (anti-PDGF) with neutralizing activity against rat PDGF (Collaborative Research, Bedford, Ma), or media at 37°C. The treated supernatant was added to the lymphocytes at the start of the cytotoxicity assay. In control experiments it was determined that turkey serum had no effect on the inhibitory potential of these supernatants.

Nitrite Determination

Nitric oxide production was estimated by measuring the nitrite concentration produced in the culture media. Nitrite concentration was measured by the method of Ding et al. (143). Concentrations of sodium nitrite ($NaNO_2$), 2-fold dilutions from 10^{-8} mol/well to 3.9 x 10^{-11} mol/well, were added to 96-well plates in 100 μL of phenol-free TCM or 100 μL of appropriately diluted cell-free supernatant produced in phenol-free TCM was added. Griess reagent (100 μL) was added to the wells and the plates were incubated in the dark at room temperature for 10 min. The absorbance was read at 550 nm in a microplate reader (Biotek). The absorbance was plotted against the sodium nitrite concentration and the nitrite concentrations

FIGURE 4. Typical Standard Curve for Nitrite Determination.

Two-fold dilutions of sodium nitrite were added to the assay from 10^{-8} mol/well to 3.9×10^{-11} mol/well. The absorbance from the colorimetric reaction with Griess reagent recorded by an optical plate reader at 550 nm was plotted against the concentration. The solid line represents the linear regression of the data points while the dotted line represents the 95% confidence interval. The concentrations of nitrite in the test wells were determined from the absorbance values applied to the standard curve. The concentration was then converted to molarity from the 100 μL volume.


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance Units
0
2000
4000
6000
8000
10000
Concentration NO$_2^-$ (pmol/well)

of the samples were estimated from a linear regression of the standard curve. An example of the standard curve appears in Figure 4, where the linear regression is the solid line and the 95% confidence interval is the dotted line. Control supernatants were prepared with 100 μM of N^{α}-monomethyl-L-arginine (L-NmmA)(Calbiochem, LaJolla, CA), an inhibitor of nitric oxide synthetase. Nitric oxide was assumed to be the L-NmmA inhibitable portion of the observed nitrite concentration. Griess reagent was prepared on ice immediately prior to use by combining one part 0.1% naphthylethylenediamine dihydrochloride in distilled water and one part 1% sulfanilamide in 2.5% phosphoric acid (H_3PO_4). These reagents were purchased from BDH (Toronto, Ont).

TGF-β Assay

The CCL-64 mink lung epithelial cell line (ATCC, Rockville, MD) was used in a growth inhibition assay to measure TGF-β_1 by the method of Danielpour et al. (144). CCL-64 cells were harvested from the tissue culture flask prior to becoming confluent, approximately 24h after initiation of the culture. They were seeded at 2.5x10^4 per well in 96-well microtiter plates in TCM and incubated for 4h at 37°C. Standard concentrations of TGF-β_1 (Genzyme) between 10 ng/mL and 0.1 pg/mL were added to the wells. Cell-free supernatants were pre-incubated for 45 min at room temperature with either neutralizing chicken anti-human TGF-β_1 (R&D Systems), naive chicken yolk immunoglobulin (IgY) (R&D Systems) or TCM, then added to the seeded cells. The plates were incubated at 37°C for an additional 18h. The cells were pulsed for 6h with [^{3}H]thymidine (1.5 μCi/well, NEN), washed with PBS and treated with 0.5% trypsin-0.03% EDTA for 40 min at room temperature and finally collected with a Skatron cell harvester. The filters were dried and counted in a Beckman liquid scintillation counter. The counts per minute were plotted against the log of the TGF-β_1 concentration and the TGF-β_1 content of the samples was estimated from the non-linear regression of the standard curve. An example of the standard curve for TGF-β_1 appears in Figure 5. TGF-β_1 concentrations reported were the amounts found in supernatants treated with IgY minus the amounts found in supernatants treated with the specific neutralizing antibody.

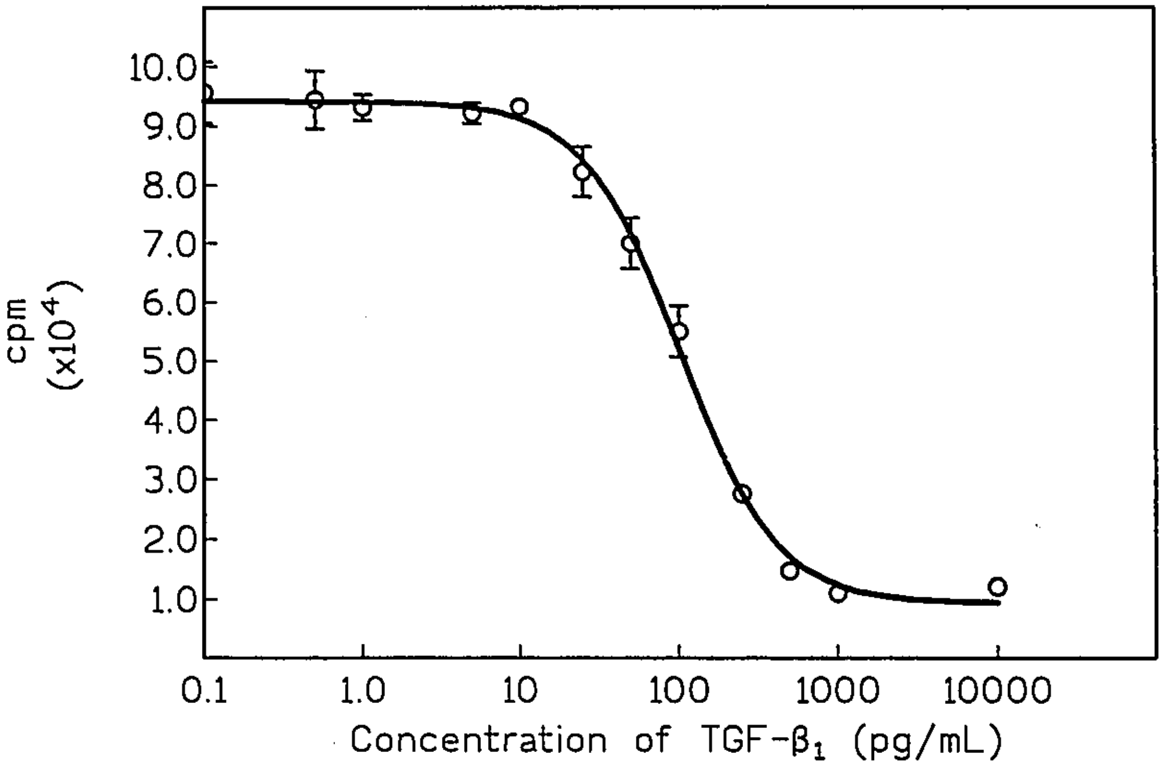
Purification of NK Cells

A. Percoll Fractionation of Lung Lymphocytes

The nylon wool non-adherent lymphocytes were further separated on a self-generating Percoll gradient by a modification of the method of Ravnik et al. (145). Lymphocytes were resuspended in 0.5 mL of Percoll (Pharmacia) solution diluted in Hank's balanced salt solution (HBSS) at the osmolality of rat serum (307 mosm/L) and a starting density of 1.077 (54% Percoll). This cell suspension was gently layered onto 7.5 mL of the same Percoll solution.

FIGURE 5. Representative Standard Curve for TGF-β_1 Assay.

The TGF-β_1 assay was a growth inhibition assay measured by ^{3}H-thymidine incorporation. Ultrapure TGF-β_1 was added to CCL-64 indicator cells at concentrations of 10^5, 10^3, 500, 250, 100, 50, 25, 10, 5, 1, and 0.5 pg/mL. The radioactivity (cpm) was plotted against the log of the concentration. The line of best fit was determined by non-linear regression (GraphPad). The TGF-β_1 concentration of the test wells was calculated from the equation of the curve. The concentration of TGF-β_1 in the conditioned media was determined by subtracting the concentration obtained when a neutralizing antibody to TGF-β was present from the concentration obtained when normal chicken immunoglobulin was present.



cpm
(x10⁴)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.1
1.0
10
100
1000
10000
Concentration of TGF-β₁ (pg/mL)

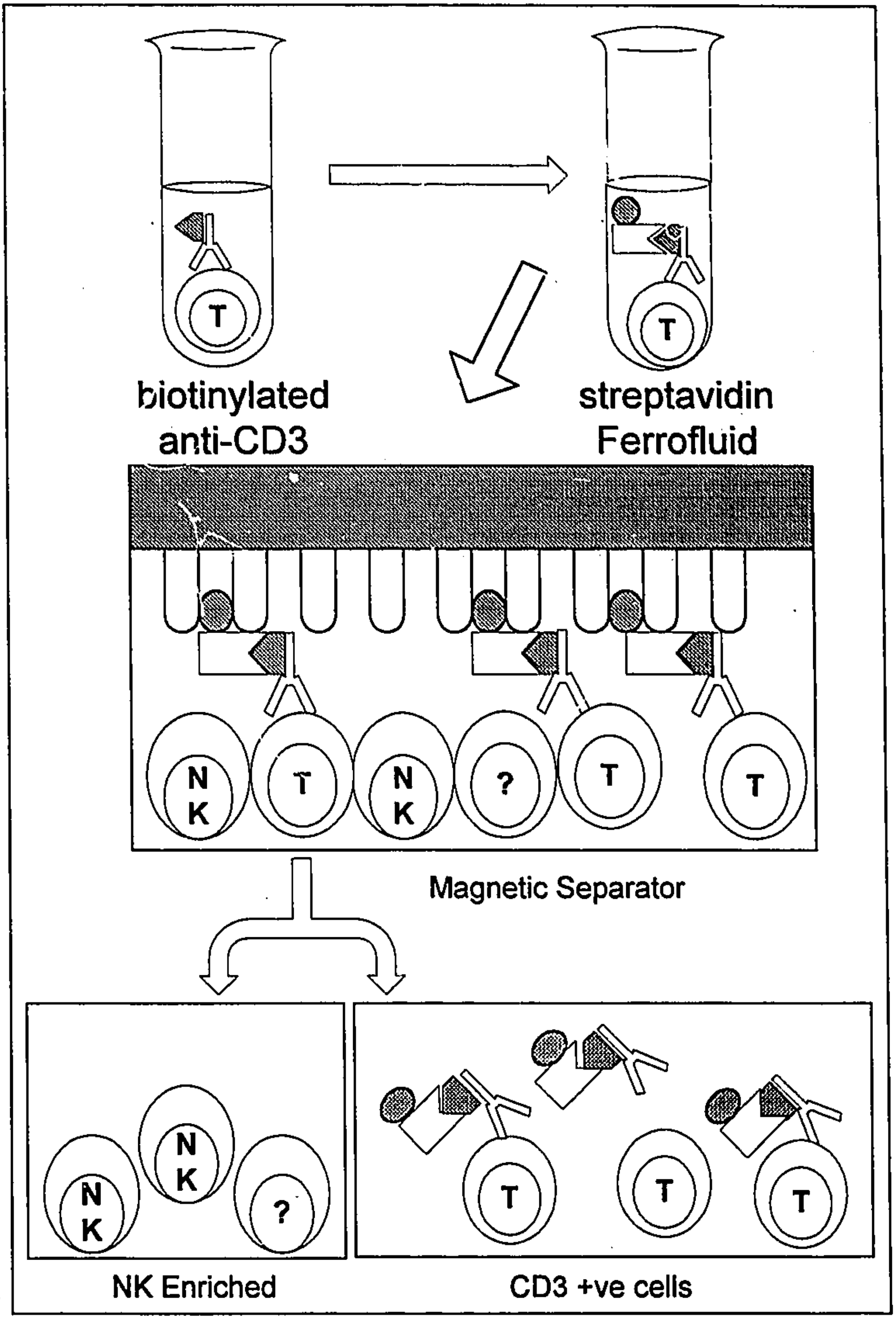
The gradient was formed by centrifugation at 16000 rpm for 2.5 min at 4°C in a Beckman J21-M2 high speed centrifuge using a JA-20 fixed angle rotor with the brake off. A second tube of Percoll solution containing density marker beads (Pharmacia) was spun at the same time to indicate the density zones in the generated gradient. The fractions were collected with a siliconized pasteur pipette as follows: fraction 1, d=1.016-1.047; fraction 2, d=1.064; fraction 3, d=1.077, fraction 4, d=1.087, fraction 5, d=1.098-1.142. Each fraction was washed three times in TCM, analyzed by differential staining and the cytotoxicity was determined.

B. Magnetic Removal of CD3$^+$ Cells

The lung lymphocyte population was enriched for NK cells by selective removal of CD3$^+$ cells using a magnetic antibody system, represented schematically in Figure 6. Nylon wool non-adherent lung lymphocytes were incubated on ice for 45 min in 0.1% sodium azide in PBS with a 1/50 dilution of biotinylated mouse monoclonal anti-rat CD3 (Cedarlane) with periodic agitation. The cells were washed twice in 0.1% sodium azide and resuspended in 5 mL. An identical amount of streptavidin conjugated Ferrofluid (Immunicon, Huntingdon Valley, Pa), which had been diluted 1/25 in proprietary dilution buffer (Immunicon) 4h previously and stored on ice, was added. The mixture was incubated on ice for 45 min with occasional shaking. The cells were gently placed into the separation vessel with a siliconized pasteur pipette; care

FIGURE 6. Enrichment of NK Cells in the Lung Lymphocyte Population.

Lung lymphocytes from six rats were isolated and pooled. The cells were incubated with biotinylated anti-CD3 on ice, washed, then incubated with streptavidin ferrofluid, a magnetic colloidal solution. The cells were placed in the separation vessel and raised into contact with the magnetic field coils. The vessel was lowered out of the magnetic field and the cells were counted. These cells were used as the NK enriched population of lung lymphocytes.


T
T
biotinylated anti-CD3
streptavidin Ferrofluid
N K
T
N K
?
T
T
Magnetic Separator
N K
N K
?
T
T
T
NK Enriched
CD3 +ve cells

was taken to avoid bubbles. The separation vessel was placed inside the Magnetic Cell Separator (Immunicon) and the magnetic collector was set in place. The separation vessel platform was elevated, submerging the magnetized coils of the collector. The magnetic separation was allowed to proceed at room temperature for 5 min. The cells remaining in the separation vessel were washed twice in TCM and were ready for further analysis. The cells attached to the coils were obtained by submerging the detached collector in TCM in a clean separation vessel, which was then agitated repeatedly. These cells were then washed twice in TCM as were untreated lung lymphocytes incubated throughout the procedure in 0.1% sodium azide at 4°C. Cytocentrifuge slides were made of each of the preparations and used to determine the percentage of LGL. These results were periodically confirmed by flow cytometry.

Flow Cytometry

In some cases, microscopic determination of the efficacy of the separation was verified by FACS analysis. The lymphocytes were incubated at a concentration of 10^6 cells in PBS containing 0.1% sodium azide at 4°C with mouse monoclonal anti-rat CD5 (IgG_1)(Cedarlane), a pan-T marker or mouse monoclonal anti-rat NKR-P1 (Cedarlane), identical to clone 3.2.3 described above, for 45 min. The cells were washed three times in cold 0.1% sodium azide and incubated with phycoerythrin-conjugated goat anti-mouse IgG for 45 min at

4°C. After washing three times in PBS the cells were analyzed on the flow cytometer gated on all live cells. The autofluorescence gate was set with cells incubated with the secondary antibody alone.

BLT-Esterase Assay

Natural killer cell enriched lung lymphocytes were treated with 1 µg/mL of PGE_2 (Sigma), 1 ng/mL TGF-β_1, co-cultured with AM, or incubated with TCM for 18h. The cells were resuspended in RPMI supplemented with 10% heat inactivated FBS (inactivated at 70°C for 1h) at a concentration of 10^7 cells/mL and 50 µL were placed in 96-well plates in triplicate. The cells were stimulated with 25 µL of 200 µg/mL phorbol ester myristate (PMA) and 25 µL of 1 µg/mL calcium ionophore, A23187, for 4h at 37°C. Controls of media alone (blank), Triton X100 (total release) at the begining and the end of the 4h incubation, and calcium ionophore without PMA (control) were prepared from cells pre-treated with media alone. The plate was centrifuged for 10 min at 1500 rpm and 50 µL of supernatant were harvested and stored at -80°C until needed. The BLT-esterase activity was determined by a colorimetric assay. A solution of 3.38 mg of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 3.16 mg of Nα-Cbz-Lys-thiobenzylester (BLT) in 10 mL of 0.1 M Tris buffer was made immediately prior to use and 50 µL were added to each supernatant. The optical density was read at 30 min and 1h at 405 nm in a microplate reader (Biotek). The background absorbance was subtracted from each result and the data were expressed as percentage inhibition compared to the cells pre-treated with media alone.

In Situ Hybridization

A. Preparation of Probes

The cDNA probes C11, granzyme B, and B10, granzyme D, were kindly provided by Dr. R.C. Bleackley (University of Alberta, Edmonton) (146). C11 was provided in a pGEM 3Z cloning vector. The full coding sequence was contained in a 800 bp EcoR1-BamH1 fragment. B10 was provided in a Bluescript cloning vector and BamH1-HindIII digestion resulted in the 900 bp fragment containing the full coding sequence. After digestion, the probes were run on a 0.8% agarose gel. The appropriate bands were extracted from the gel using the Qiaex Gel Extraction Kit (Qiagen) The probes were labelled with [α-^{35}S]dATP (NEN) by random priming using the Megaprime DNA Labelling Kit (Amersham). The labelled probes were then purified on a Nuctrap probe purification column (Stratagene).

B. Hybridization

The influence of AM, TGF-β, and PGE_2 on mRNA levels of the NK proteins granzyme B, involved in the lytic process, and granzyme D, which is not, were determined by *in situ* hybridization. Treated NK enriched lung lymphocytes were cytocentrifuged onto RNAse-free microscope slides. The slides were air dried then dried by graded ethanol washes and stored at -70°C until needed. Slides were fixed for 10 min in acetone at -20°C and washed twice in PBS. Negative controls were treated with 100 μL of RNAse A (100 μg/mL) for 1h at 37°C and, subsequently, processed separately from the other slides. The slides were acetylated then pre-hybridized for 1h at room temperature with the hybridization cocktail lacking the specific probe to decrease non-specific background. The indicated probe, which had been labelled with [α-^{35}S]dATP at 37°C for 1h by random priming using a MegaPrime kit (Amersham), was added to the hybridization cocktail. The cocktail was applied to the slides at 75°C and the slides were covered by snapping a baked coverslip into place, avoiding air pockets. The covered slides were placed into humidity chambers and incubated overnight at 44°C. The coverslips were removed and the slides were extensively washed and allowed to air dry overnight. The hybridized slides were dipped in Kodak Nuclear Track Type 2 photographic emulsion (Interscience, Markham, ONT), dried in the dark and placed in light-tight boxes. The boxes were stored at 4°C to permit exposure of the emulsion to the labelled probe. After exposure, the slides were developed in D-19 developer (Henry's Photographic), fixed in Kodak Rapid Fix (Henry's Photographic) and dried overnight. The slides were analyzed by dark field microscopy using the NIH Image 1.47 software (NIH, Bethesda, Md). At least 200 cells were counted per slide. A cell was considered positive if it was at least 3 times brighter, under dark field microscopy, than background.

Statistical Analysis

Results were expressed as mean values ± SEM of at least three independent experiments. In many cases, the cells used for the various assays are pooled from more than a single animal, eg. isolation of $CD3^-$ lung lymphocytes requires the sacrifice of 6 rats per experiment while co-culture experiment with AM typically require the lung lymphoctyes of 2 rats.

Statistical significance was determined using Student's t test ($p<0.05$) (Instat, GraphPad, San Diego, CA). Linear regression, non-linear regression, and 95% confidence limits were determined using the computer program Inplot (GraphPad).

RESULTS

Establishing the Presence of NK Activity in Lung Lymphocytes.

A. Differential Analysis of Effector Cell Preparations.

The lymphocyte populations from the lung, spleen, and peripheral blood were counted in a hemocytometer with trypan blue staining. It can be seen in Table 1 that each of the populations retained greater than 90% viability. The cells were further examined by Wright-Giemsa staining of cytocentrifuge preparations. The differential analysis of the populations from the various anatomical compartments described in Table 1, show that each contains a vast majority of lymphocytes: 97% for LL, and 99% for SL and PBL with only minor proportions of monocytes/macrophages and negligible amounts of neutrophils.

B. Cytotoxicity of Lung Interstitial Lymphocytes.

Lung lymphocytes were tested for NK activity against the mouse lymphoma target, Yac-1, prior to nylon wool passage. The lung lymphocytes possessed NK activity at each of the effector-to-target ratios tested (Table 2). As the lung lymphocytes were obtained after exposure to protease, it was of interest to prepare lung lymphocytes without this treatment to verify that the cytotoxicity was a function of the cells and not a spurious effect of the enzyme treatment. Lung lymphocytes were prepared by three separate methods of mechanical dissociation. As shown in Table 2, each method

TABLE 1. Differential Analysis of Cell Preparations

	LL	PBL	SL
Total Cell Yield ($\times10^6$)[a]	27.2 ± 1.0	9.8 ± 2.4	53.6 ± 4.8
Viability (%)	93.7 ± 0.6	99.2 ± 0.2	97.5 ± 0.4
Macrophages[b]	2.3 ± 0.2	0.8 ± 0.1	0.4 ± 0.1
Lymphocytes[b]	97.4 ± 0.4	99.3 ± 0.1	99.2 ± 0.2
Neutrophils[b]	0.1 ± 0.1	0 ± 0	0.5 ± 0.2

Lymphocytes were obtained from the lung, spleen and peripheral blood as outlined in **Materials and Methods.** The viability was determined by trypan blue exclusion, while the cell types were determined by light microscopy.
LL=lung lymphocytes, PBL=peripheral blood lymphocytes, and SL=spleen lymphocytes

[a] Data represent the mean ± SEM of at least 4 experiments.
[b] Differential counts (% of total cells)

TABLE 2. Cytotoxicity of Lung Lymphocytes Prepared by Enzymatic Digestion or Mechanical Dissociation.

	Differential[a] Staining		Specific Cytotoxicity (E:T) (%)		
Treatment	LYMP (%)	AM (%)	50:1	25:1	12.5:1
Protease 37°C 10 min.	94	6	21.6 ± 1.6	17.2 ± 1.7	8.5 ± 1.6
Mechanical Dissociation[b]	71	29	14.8 ± 2.3	11.4 ± 1	7.1 ± 1.2
Mechanical Dissociation[c]	66	34	11.8 ± 1	8.5 ± 0.9	6.7 ± 0.9
Mechanical Dissociation[d]	27	73	2 ± 1	1.4 ± 0.4	0.9 ± 0.3

[a] Wright-Giemsa.

[b] Cell suspensions were prepared by mincing in Hank's balanced salt solution followed by gentle homogenization and fractionation on Ficoll, as described in **Materials and Methods**.

[c] Cell suspensions were prepared by mincing the lungs and pushing the mince through a rigid nylon mesh.

[d] Lung mince was flushed through 10 mL syringe 10 times.

yielded cells lytically active against Yac-1. The level of cytotoxicity in each case was substantially lower than that found with enzymatic treatment, however, the levels correlated with the proportion of lymphocytes obtained. In addition, the primary contaminant in these preparations was macrophages which may suggest a role for adjacent macrophages in the negative modulation of lung NK activity.

C. Effects of NK Reactive Agents in LL Cytotoxicity.

Having shown that the NK activity was not a result of enzymatic treatment, the identity of the cell type responsible for NK activity in LL was examined by two different methods shown to eliminate NK cells in other systems. The first technique employed anti-asialo GM-1 which reacts with ganglio-N-tetraosylceramide expressed on NK but not cytotoxic T cells (23). Treatment of LL with anti-asialo GM-1 plus complement inhibited their ability to lyse Yac-1 target cells (Table 3). The cytotoxicity was completely abrogated at both antisera dilutions tested, whereas LL treated with complement alone retained 95% of their lytic activity. The second method involved the treatment of LL with L-leucine methyl ester (L-LME), a lysosomotropic agent which has been reported to kill NK cells (141). Treatment of LL with toxic concentrations of L-LME (20-40 mM) almost completely abolished the NK activity (Table 3).

TABLE 3. Effects of NK Reactive Agents on LL Cytotoxicity.

Treatment		Specific Lysis 50:1	 25:1
None[a]		23.1 ± 2.1	17.1 ± 1.7
Anti-asialo GM_1	1/25	0	0
Anti-asialo GM_1	1/50	0.1 ± 0.1	0.04 ± 0.03
None		21.6 ± 2.6	14.2 ± 2.1
L-LME	20 mM	6.9 ± 1	3.9 ± 1.1
L-LME	40 mM	2.4 ± 0.8	0.8 ± 0.4

Lung lymphocytes were isolated as described in **Materials and Methods.** The cells were then incubated with anti-asialo GM-1 followed by rabbit complement or complement alone. The cells were washed and the cytotoxicity assay was performed.
In the case of the NK depletions with L-LME, the indicated concentration of L-LME or media was added to the cells and they were incubated for 30 min then washed and the cytotoxicity was determined.

[a] Data represents the mean ± SEM of 3 experiments.

Comparison of Lung NK Activity and NK from Other Compartments.

A. Basal Activity.

Once it was established that lung NK cells possess the minimal criteria of NK cells in other systems, the question became, how does lung NK activity compare to the NK activity in established tissue models, the spleen and peripheral blood. The underlying hypothesis was that lung lymphocytes, by virtue of their anatomical location, may possess unique characteristics with regard to regulation. The NK activity of lung, spleen, and PBL was compared in fresh and 24h cultures of lymphocytes. Freshly obtained lung lymphocytes (LL) were found to possess a significantly greater basal activity (5.7 LU ± 0.5) than SL (3.1 LU ± 0.5) or PBL (2.5 LU ± 0.2)(Figure 7). Additionally, incubation of the lymphocyte populations at 37°C for 24h, to allow the cells to recover from the isolation procedure and the removal of cytophilic antibodies, did not have a significant effect on NK activity.

B. NK Cell Content.

The mouse monoclonal antibody 3.2.3, directed against the rat NK cell triggering structure NKR-P1, reacts against 95% of blood and spleen large granular lymphocytes (LGL) (142). Since LL displayed a higher cytotoxicity than SL, this mAb was used to determine the proportion of NK cells present in the LL and SL populations by indirect immunofluorescence. LL and SL were shown to possess similar numbers of 3.2.3 positive cells, 12.6% and 13.6% respectively (Table 4), despite a significant

FIGURE 7. Comparison of the Cytotoxicity of LL, SL and PBL.

Effector cell populations, freshly obtained or incubated at 37°C for 24h were tested for their cytotoxicity using four different E:T ratios (40:1 to 2.5:1). Data are expressed as lytic units (LU_{20}) and values represent the mean ± SEM of at least 4 experiments.
**Significantly different from SL and PBL ($p \langle 0.05$).
*Significantly different from PBL ($p \langle 0.05$).

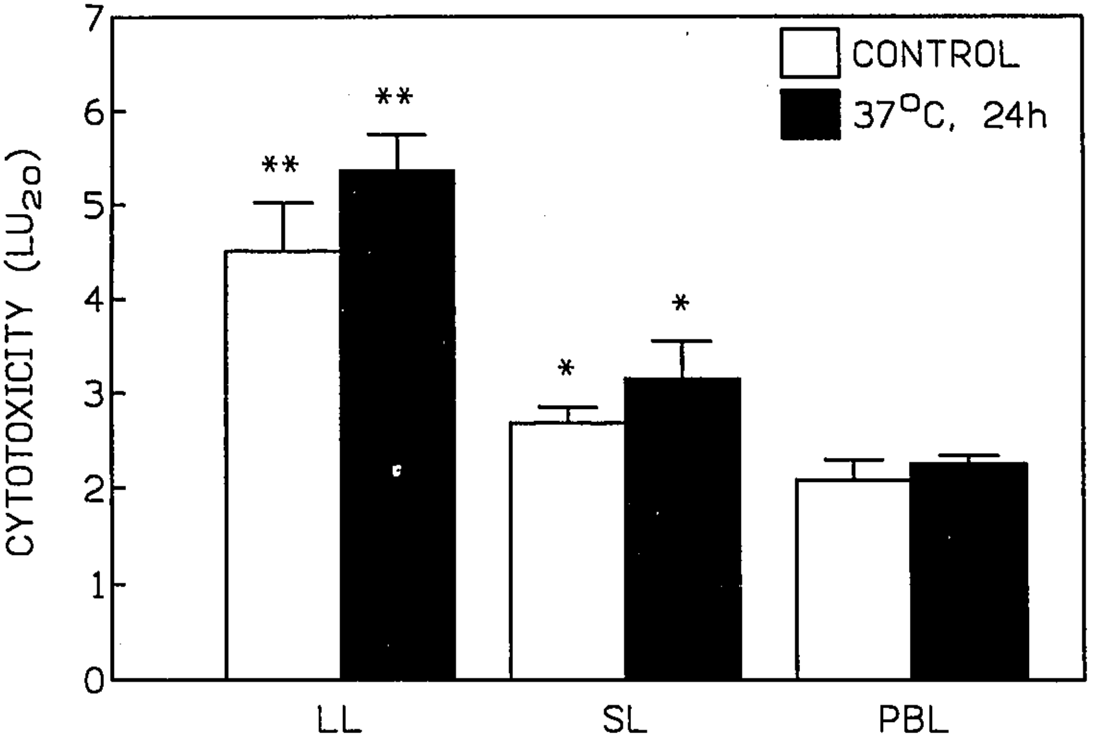

CYTOTOXICITY (LU$_{20}$)
0
1
2
3
4
5
6
7
CONTROL
37°C, 24h
**
**
*
*
LL
SL
PBL

TABLE 4. Frequency of 3.2.3 Reactive Cells in LL and SL.

Cell Preparations	Percentage of 3.2.3 Positive NK Cells[a]
LL	12.6 ± 0.2
SL	13.6 ± 0.4

[a] Immunofluorescent staining with mAb 3.2.3 and rabbit anti-mouse IgG (Fab_2 fragment) was performed as described in **Materials and Methods.** Random fields were examined by microscopy and the percentage of lymphocytes positively stained was determined. The microscopic preparations were scored blind and the values represent the mean ± SEM of at least 4 experiments.

difference in basal NK activity (Figure 7).

C. Response to Various BRM.

The responsiveness of LL, SL, and PBL to 18h exposure to some of the BRM which have been reported to enhance NK activity in various systems was determined. There were significant differences found in the reactivity of the different effector cell populations (Figure 8). IL-2 enhanced the NK activity of all three compartments but SL were significantly more responsive than the other two effectors. Similarly, IFN-α/β augmented NK activity in each compartment but LL were significantly more responsive than PBL. By contrast, neither LL nor SL responded to treatment with IFN-γ, although treatment did result in a small but significant enhancement of PBL cytotoxicity.

D. Macrophage Modulation.

Alveolar macrophages (AM) line the alveolar walls of the lung, thus, they are located in an ajacent but physically separate compartment from interstitial lymphocytes. We have compared the effects of AM and their conditioned media on LL and SL natural killer activity. 10^6 cells from each lymphocyte population was co-cultured with eqal numbers of AM in a two-chamber system, whereby the cells were able to interact chemically but not physically. As illustrated in

FIGURE 8. Comparative Effects of Various BRM on LL, SL, and PBL Cytotoxicity.

LL, SL, and PBL were incubated in the absence or presence of either rIL-2 (100 U/mL), rIFN-γ (1000 U/mL), or IFN-α/β (500 U/mL) for 18h. Cytotoxicity was determined and calculated as lytic units using four different E:T ratios. The data are expressed as percent stimulation and values represent the mean ± SEM of at least 3 experiments.
*Significantly different from LL and PBL ($p < 0.05$).
**Significantly different from LL and SL ($p < 0.05$).
***Significantly different from PBL ($p < 0.05$).

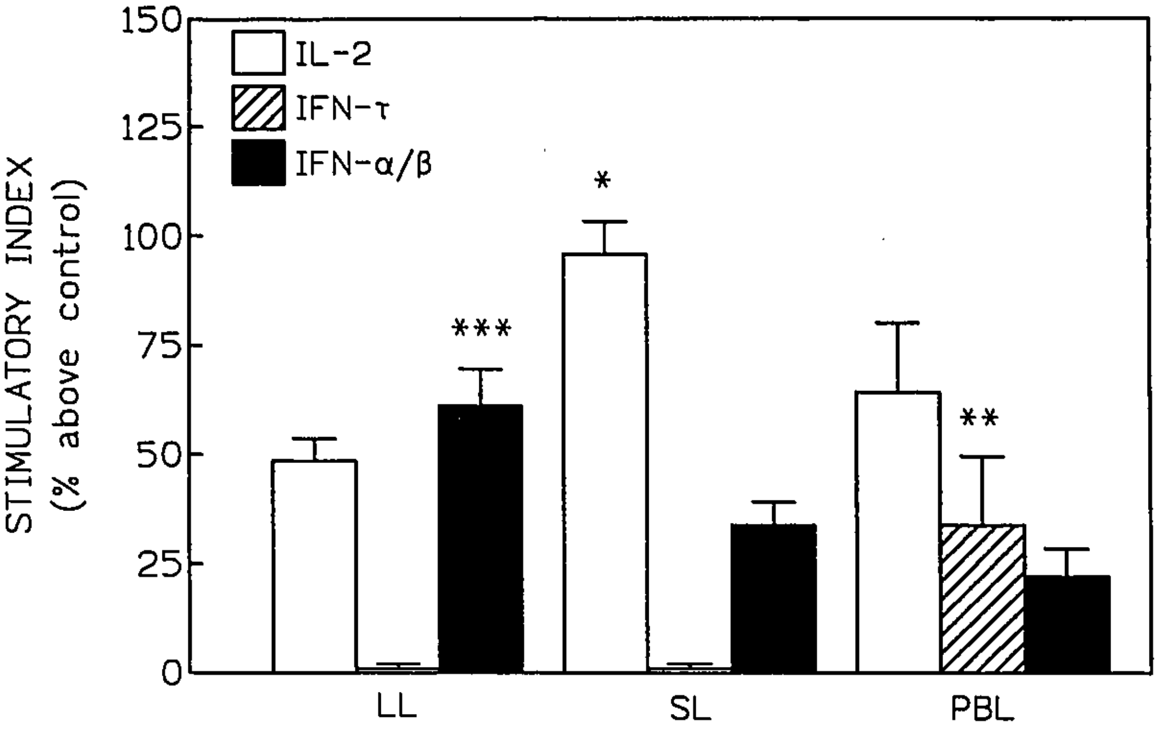

150
125
100
75
50
25
0
STIMULATORY INDEX
(% above control)
IL-2
IFN-τ
IFN-α/β
*
***
**
LL
SL
PBL

Figure 9, LL NK activity was significantly inhibited by 36.8 ± 3.4% after 18h co-culture with AM while SL NK activity was significantly enhanced by 28.1 ± 5.2%. When the AM were replaced with 18h AM conditioned media, this dichotomy of effect disappeared and LL and SL behaved similarly in response to AM supernatant. As the number of AM used in producing the supernatant was increased, both SL and LL were similarly inhibited to a greater degree. The highest concentration of AM used to produce supernatant was 4×10^6 cells/mL which resulted in the significant inhibition of LL (by 37.7%) and SL (by 40.2%) at levels equivalent to those found with the co-culture of AM and LL (by 36.8%). This suggests an active role for LL in the production of inhibitory factor(s) in the co-culture system.

Lung Lymphocyte Response to Exogenous BRM

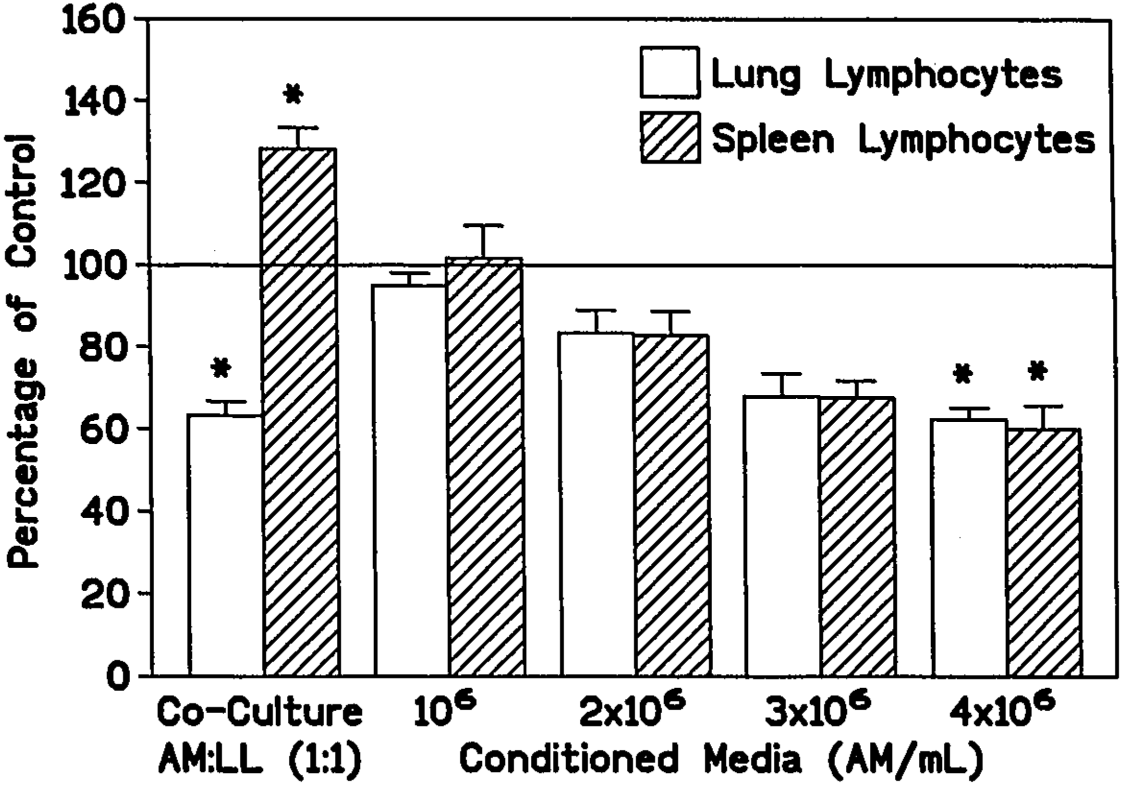
The tissue-specific response to biologic response modifiers (BRM) suggested a need for a closer examination of the reactivity of lung NK activity to cytokines shown to enhance the NK activity of other NK cell populations.

A. Effects of BRM on LL NK Activity.

We investigated the response of lung interstitial lymphocytes to some of the BRM which have been reported to enhance peripheral blood and/or spleen NK activity, at the time of our studies. The effects of recombinant interleukin-2 (rIL-2), interferon-alpha/beta (IFN-α/β), and interferon-gamma (IFN-γ) on lung NK activity were studied in

FIGURE 9. Effects of AM Co-Culture and Supernatant of NK Activity.

LL and SL were co-cultured with AM for 18h or incubated for 18h in tissue culture media, or the 18h conditioned media from 10^6 AM/mL, 2×10^6 AM/mL, 3×10^6 AM/mL, or 4×10^6 AM/mL was added directly to the cytotoxicity assay. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. The data are expressed as a percentage of control, cytotoxicity of TCM incubated lymphocytes, and values represent the mean ± SEM of at least 3 experiments. *Significantly different from control values ($p < 0.05$).



160
140
120
100
80
60
40
20
0
Percentage of Control
Lung Lymphocytes
Spleen Lymphocytes
*
*
*
*
Co-Culture
AM:LL (1:1)
10⁶
2x10⁶
3x10⁶
4x10⁶
Conditioned Media (AM/mL)

greater detail and the modulatory effects of recombinant interleukin-1 (rIL-1), and tumor necrosis factor-alpha (TNF-α) were also examined. It was determined that 18h treatment of LL with rIL-2, IFN-α/β, or TNF-α prior to commencement of the cytotoxicity assay augmented LL NK activity (Figure 10), whereas rIFN-γ (Figure 11) and rIL-1 (Figure 12) treatment was ineffective. The concentration of BRM required to elicit a response varies in each case (Fig. 10). LL were responsive to rIL-2 concentrations as low as 25 U/mL and reached a stable maximum enhancement of about 55% above the basal activity at a concentration of 50 U/mL. The maximum enhancement of LL NK activity by IFN-α/β was similar to rIL-2 but a much higher concentration (500 U/mL) of IFN-α/β was required to obtain maximal stimulation. The dose response curve for TNF-α was found to be similar to that found for IFN-α/β, in that relatively little effect was seen at concentrations below 250 U/mL. However, the maximum stimulation by rTNF-α, requiring 500 U/mL, was less than half of that found for either IFN-α/β or rIL-2.

B. Combined Effects of BRM with IL-2 on LL Cytotoxicity.

The responsiveness of LL to 18h treatment with rIL-1, IFN-α/β, IFN-γ, and TNF-α when combined with a suboptimal dose (10 U/mL) of rIL-2 was examined. It was found that low doses of IFN-α/β, up to 100 U/mL, interact synergistically with rIL-2 to enhance LL NK activity (Figure 13). However, the NK

FIGURE 10. Comparative Effects of Various BRM on Lung NK Activity.

Lung lymphocytes were incubated with the indicated concentrations of rIL-2, IFN-α/β, or TNF-α for 18h. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent stimulation and values represent the mean ± SEM of at least 4 experiments.

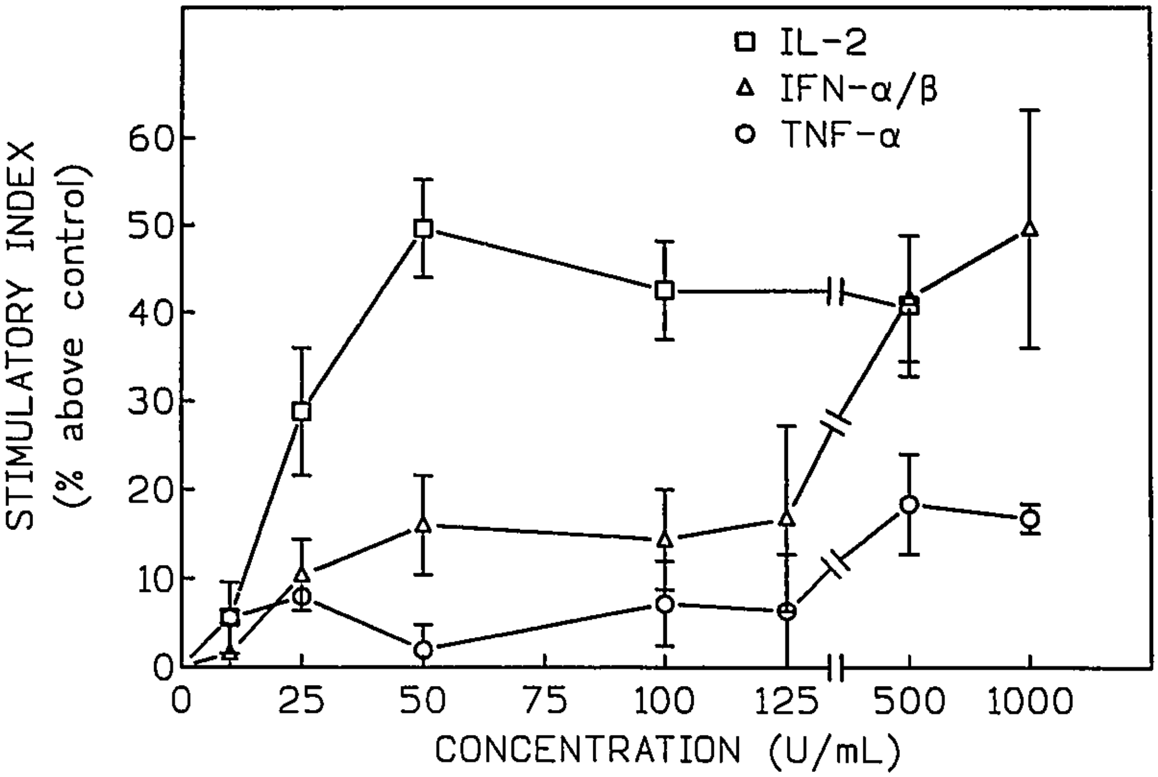
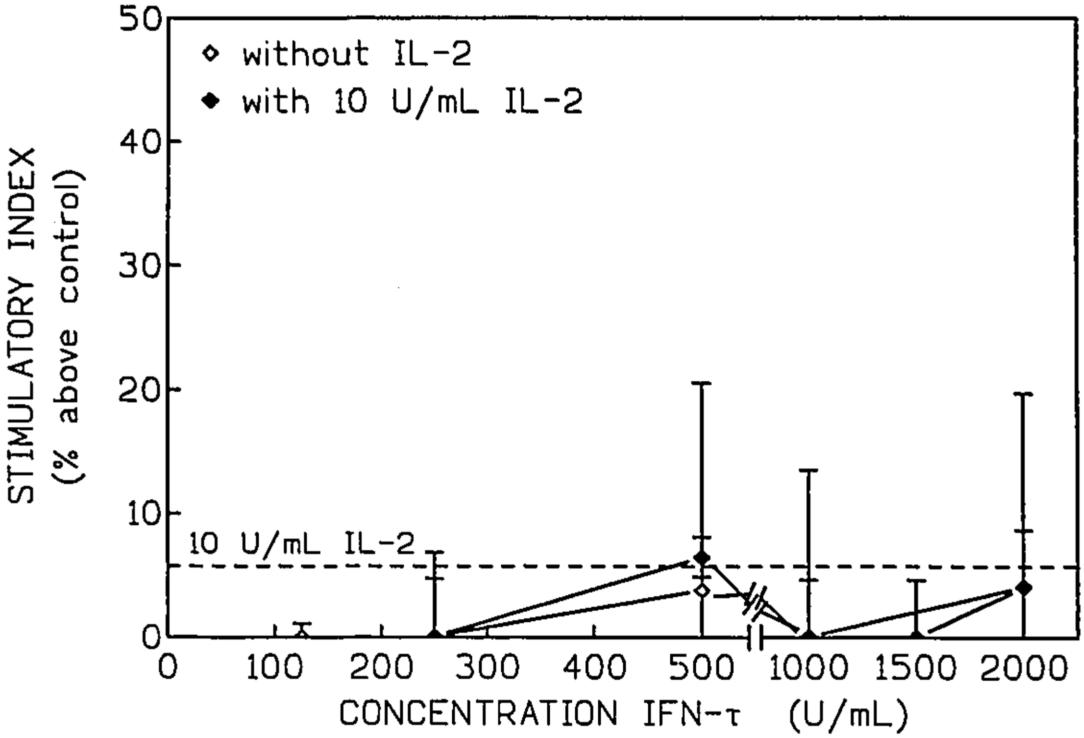

□ IL-2
△ IFN-α/β
○ TNF-α
STIMULATORY INDEX
(% above control)
0
10
20
30
40
50
60
0
25
50
75
100
125
500
1000
CONCENTRATION (U/mL)

FIGURE 11. The Effects of IFN-γ on Lung NK Activity.

Lung lymphocytes were treated with indicated concentrations of rIFN-γ or with a combination of rIFN-γ and 10 U/mL rIL-2. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent stimulation and values represent the mean ± SEM of at least 4 experiments.



◇ without IL-2
◆ with 10 U/mL IL-2
STIMULATORY INDEX (% above control)
50
40
30
20
10
0
10 U/mL IL-2
0
100
200
300
400
500
1000
1500
2000
CONCENTRATION IFN-τ (U/mL)

FIGURE 12. The Effects of rIL-1 on Lung NK Activity.

Lung lymphocytes were treated with indicated concentrations of rIL-1 or rIL-1 and 10 U/mL rIL-2. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent stimulation and values represent the mean ± SEM of at least 3 experiments.

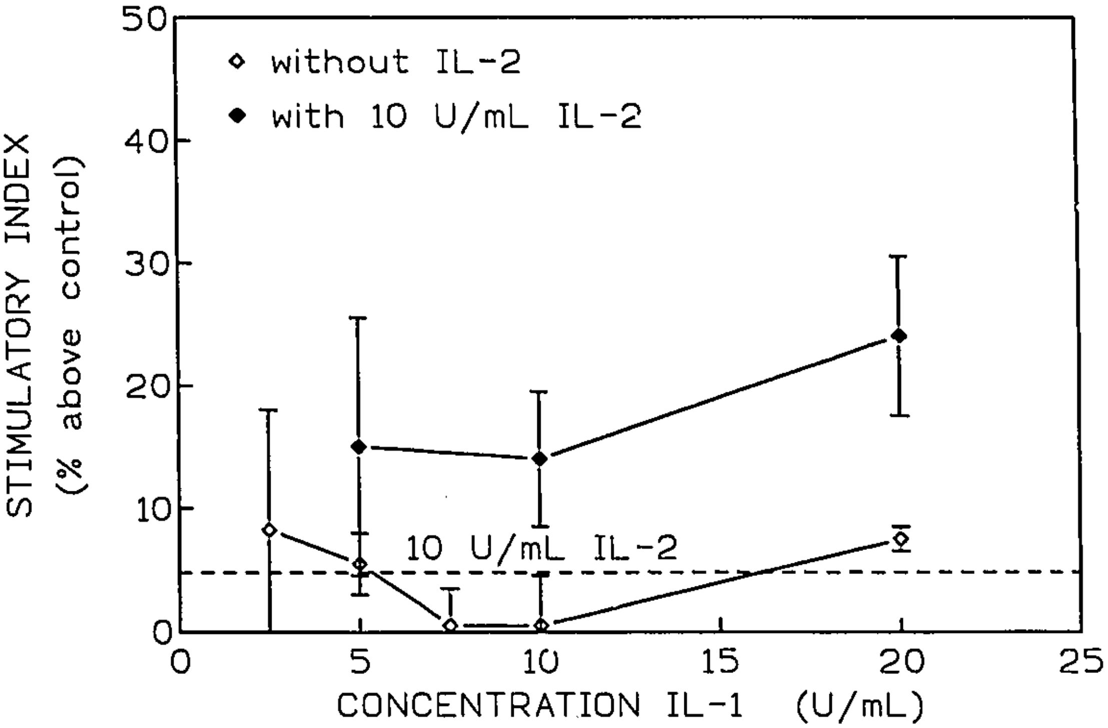
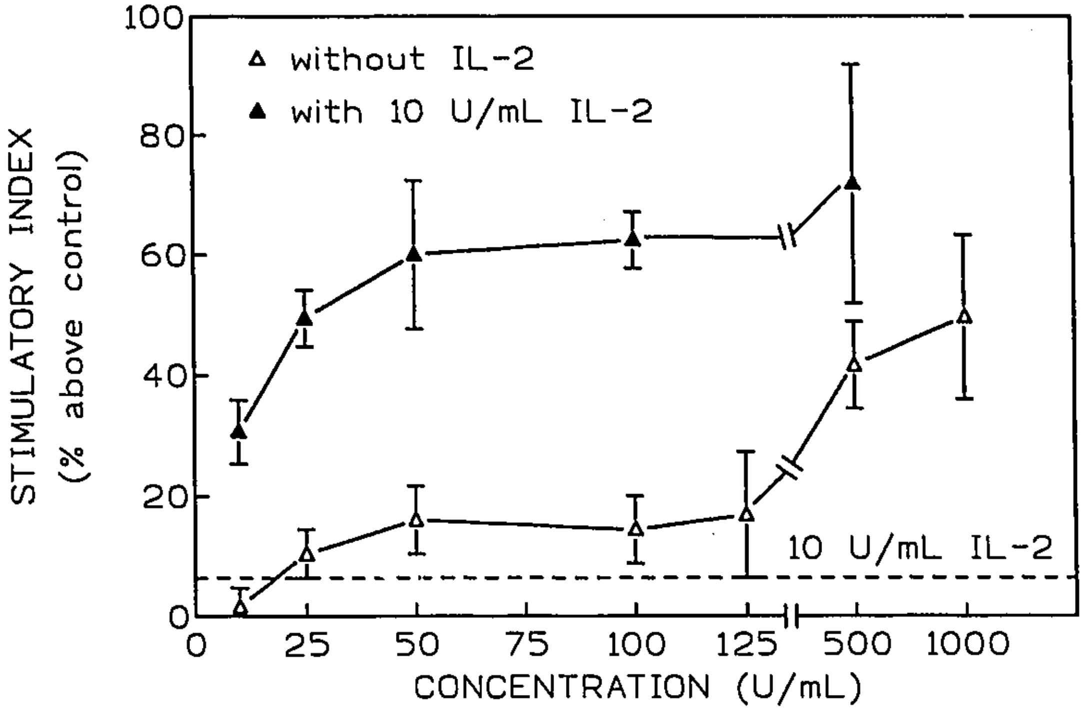

◇ without IL-2
◆ with 10 U/mL IL-2
10 U/mL IL-2
STIMULATORY INDEX
(% above control)
0
10
20
30
40
50
0
5
10
15
20
25
CONCENTRATION IL-1 (U/mL)

Figure 13. Effects of rIL-2 on IFN-α/β Enhancement of Lung NK Activity.

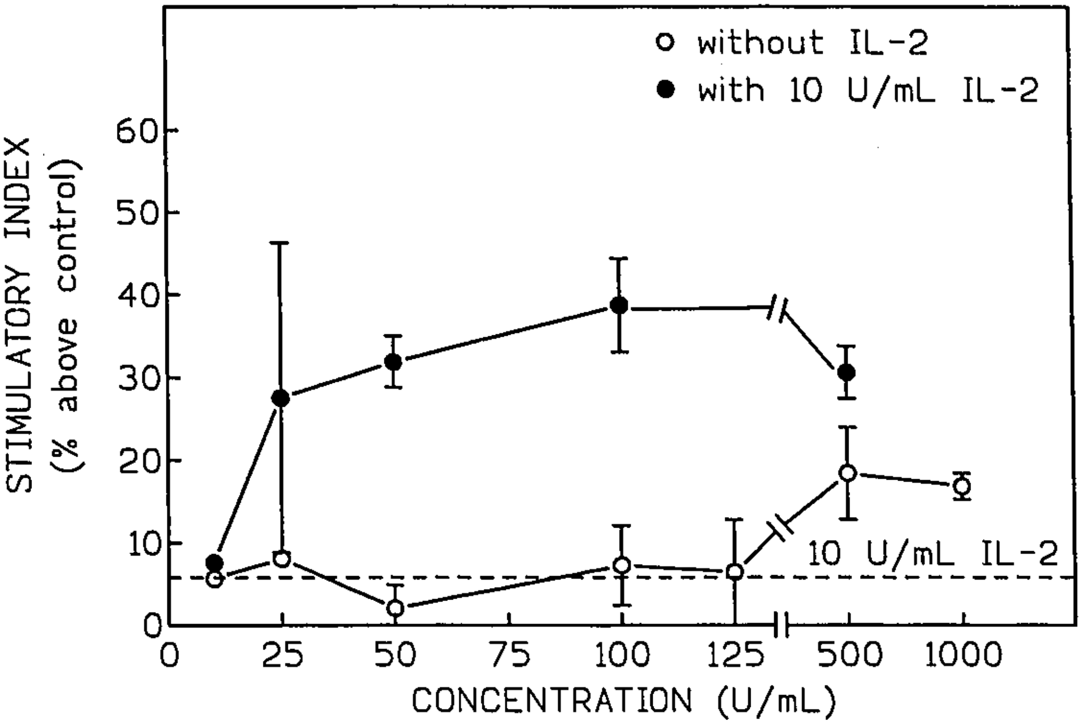
Lung lymphocytes were incubated with various concentrations of IFN-α/β alone or in the presence of 10 U/mL of rIL-2 for 18h. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent stimulation and values represent the mean ± SEM of at least 5 experiments.



△ without IL-2
▲ with 10 U/mL IL-2
STIMULATORY INDEX
(% above control)
100
80
60
40
20
0
10 U/mL IL-2
0
25
50
75
100
125
500
1000
CONCENTRATION (U/mL)

Figure 14. Effects of rIL-2 on TNF-α Enhancement of Lung NK Activity.

Lung lymphocytes were incubated with the indicated concentrations of rTNF-α alone or in the presence of 10 U/mL rIL-2 for 18h. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent stimulation and values represent the mean ± SEM of at least 3 experiments.


○ without IL-2
● with 10 U/mL IL-2
STIMULATORY INDEX
(% above control)
60
50
40
30
20
10
0
10 U/mL IL-2
0
25
50
75
100
125
500
1000
CONCENTRATION (U/mL)

activity rapidly reached the maximum level of stimulation of about 55% at a concentration of 50 U/mL of IFN-α/β and 10 U/mL rIL-2. Higher concentrations of IFN-α/β in conjunction with rIL-2 were ineffective in further boosting LL NK activity. A similar pattern was found for rTNF-α, as shown in Figure 14. The synergism, in this case, was even more pronounced at concentrations of rTNF-α that were ineffective in enhancing LL NK activity in the absence of rIL-2. However, the presence of rIL-2 became less of a factor at concentrations of rTNF-α shown to be an effective modulator of LL NK activity. Neither rIFN-γ (Figure 11) nor rIL-1 (Figure 12) significantly affected the enhancement of lung NK activity by low concentrations of rIL-2.

Local Regulation of Lung NK Activity by Alveolar Macrophages

An important question to be addressed in the characterization of lung lymphocyte regulation was, how do NK cells interact with alveolar macrophages, the chief airway immunoregulator? An attempt was made to experimentally mirror the physical proximity of the alveolar macrophages and the interstitial lymphocytes and yet maintain the compartmentalization of the populations within the lung.

A. Kinetics of Alveolar Macrophage Inhibition of Lung-Associated NK Activity.

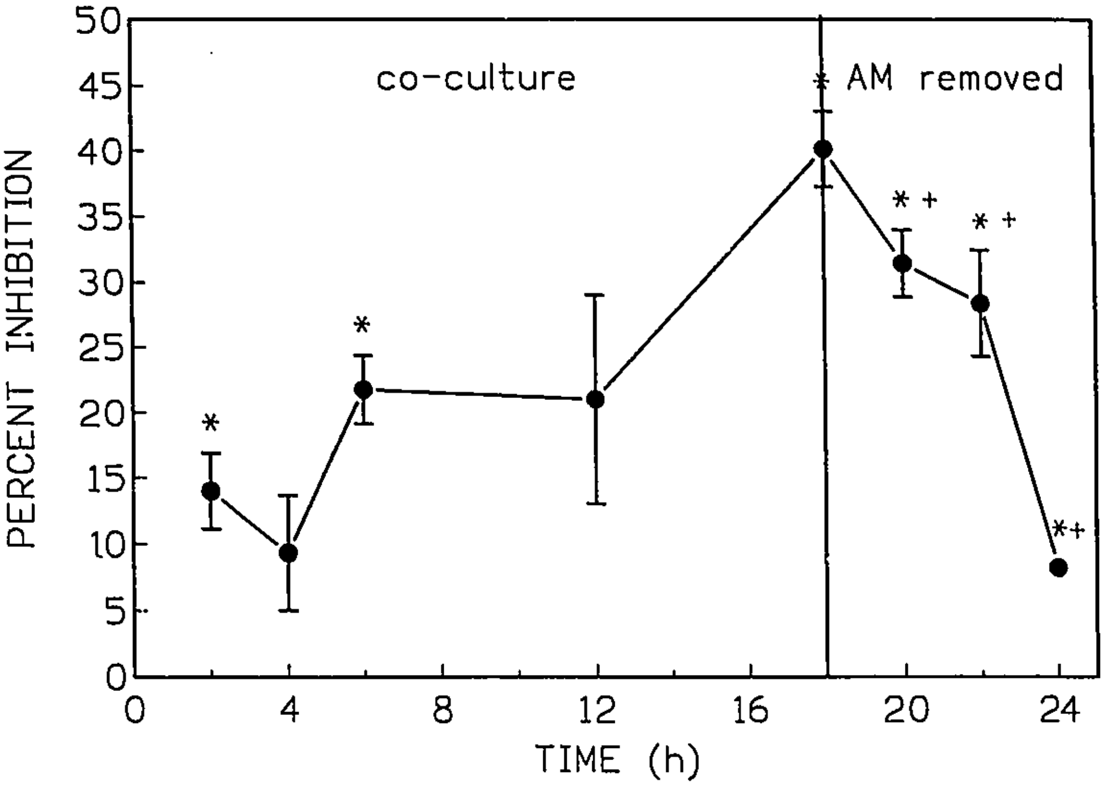
Lung lymphocytes were co-cultured with alveolar macrophages using permeable tissue culture inserts, to give a rough approximation of the *in situ* circumstances and to permit separation of the cell populations at the end of the incubation time. The detection of inhibition in this system demonstrated that soluble factor(s) are involved because physical cell-to-cell contact was avoided. AM inhibited LL NK activity in a 2-step, time-dependent fashion (Figure 15). The first plateau of activity (22% inhibition) was achieved after 6h of co-culture. This was followed by a maximum inhibition (~43%) after 18h co-culture. Inhibition in this system required the continuous presence of AM. When the AM were removed after 18h co-culture and the LL washed, the inhibition was reduced in a time-dependent manner which resulted in a return to greater than 90% of the basal activity by 6h after removal of the AM (Figure 15). Thus, suppression of LL NK activity was not related to permanent damage of the effector cells but rather resulted from a down-regulation of NK activity.

B. Role of PGE_2 in AM Inhibition of LL NK Activity.

The next question to be addressed was: what were the soluble factor(s) involved in the inhibition? The first candidate to be examined was prostaglandin E_2 which has been reported to inhibit blood and spleen NK activity and is known to be produced by AM. This question was investigated through the treatment of the LL-AM co-culture with indomethacin, an inhibitor of the cyclooxygenase pathway of arachidonic acid metabolism and PGE_2 synthesis. An inverse relationship was found to exist between the concentration of indomethacin

FIGURE 15. Effects of AM on Lung NK Activity over Time.

LL were placed in a 24-well plate at 10^6 cells/mL, AM were placed in tissue culture inserts in the wells at the same concentration and they were co-incubated for the times shown. AM were removed and LL were washed and a standard Cr^{51} assay was performed. The LL were further incubated for the indicated times before they were washed and the Cr^{51} release assay was performed. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent inhibition and values represent the mean ± SEM of at least 3 experiments.
*Significantly different ($p \leq 0.05$) from lymphocytes incubated in the absence of AM.
*+Significantly different from the degree of inhibition seen at 18h in the presence of AM.



PERCENT INHIBITION
0
5
10
15
20
25
30
35
40
45
50
TIME (h)
0
4
8
12
16
20
24
co-culture
AM removed
*
*
*
* +
* +
*+

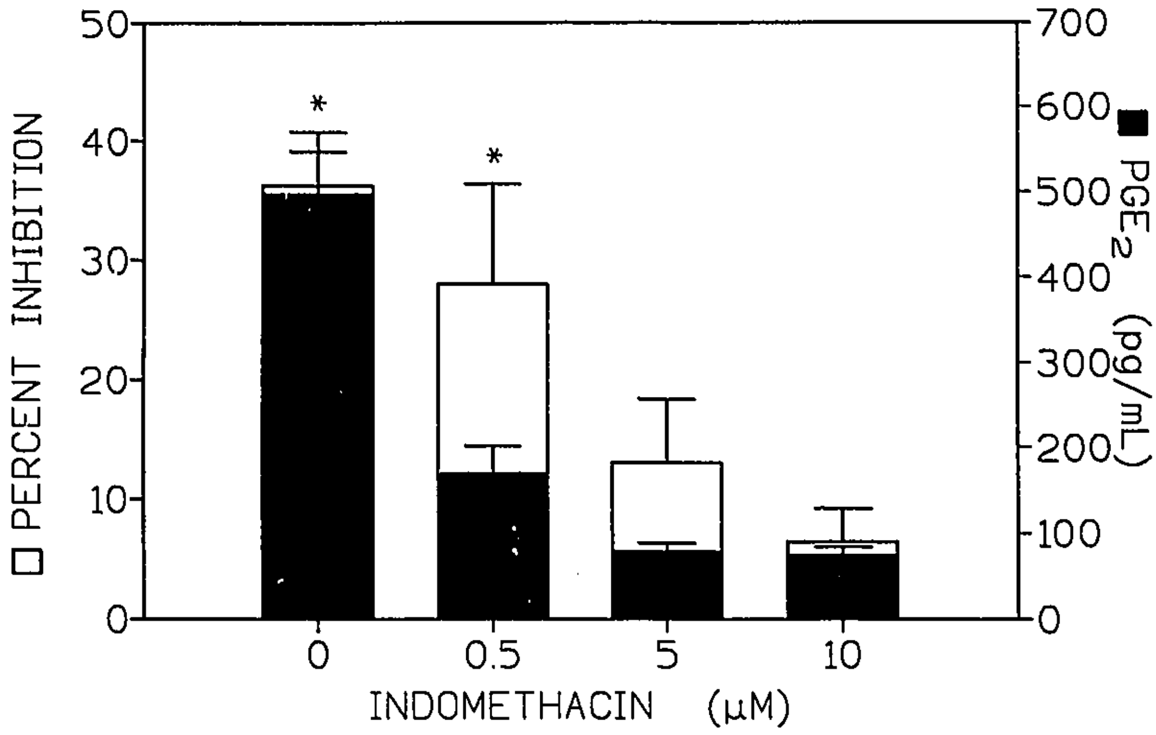
and percent inhibition (Figure 16), suggesting the involvement of PGE_2.

Given the suggested involvement of PGE_2 in the inhibition and the dependence of inhibition by AM supernatant on AM concentration, it was of interest to determine if PGE_2 was responsible for the supernatant effect. The production of PGE_2 was measured by ELISA in co-culture supernatant of 10^6 AM/mL (which is the amount used in the co-culture system), and in the supernatant of 4×10^6 AM/mL (which was found to significantly inhibit NK cells). The level of PGE_2 in the co-culture supernatant was found to be 720.6 pg/mL which is significantly more than was found for 10^6 AM/mL supernatant (144.3 pg/mL, Table 5). This difference indicates that LL and AM interact in the co-culture system to increase the production of inhibitory factors, such as PGE_2.

The effects of exogenous PGE_2 on LL NK activity was then examined in an attempt to determine whether PGE_2 could account for the entire inhibition in the co-culture. PGE_2 was shown to have a dose-dependent inhibitory effect on LL NK activity when added directly to the cytotoxicity assay (Figure 17). Exogenous PGE_2 at 1200 pg/mL resulted in an approximately 31% inhibition of LL NK activity when added to the assay. This was much greater than the concentration of PGE_2 found in the co-culture supernatant (~720 pg/mL) which was shown to cause 38% inhibition (Table 5). This indicated that the concentration of PGE_2 found in the co-culture supernatant was

FIGURE 16. Effects of Indomethacin on the Inhibition of Lung NK Activity and the Production of PGE_2.

AM were co-cultured with LL using the tissue culture insert system for 18h with the appropriate concentration of indomethacin. The AM were removed and the LL were washed and a standard Cr^{51} release assay was performed (□). Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent inhibition and values represent the mean ± SEM of at least 3 experiments. An ELISA for PGE_2 was performed on the co-culture supernatant (■). The values represent the mean ± SEM of at least 3 experiments.
* Significantly different from control ($p \leq 0.05$).



50
40
30
20
10
0
□ PERCENT INHIBITION
700
600
500
400
300
200
100
0
■ PGE2 (pg/mL)
*
*
0
0.5
5
10
INDOMETHACIN (μM)

TABLE 5. Inhibitory Effect and PGE_2 Levels of Conditioned Media.

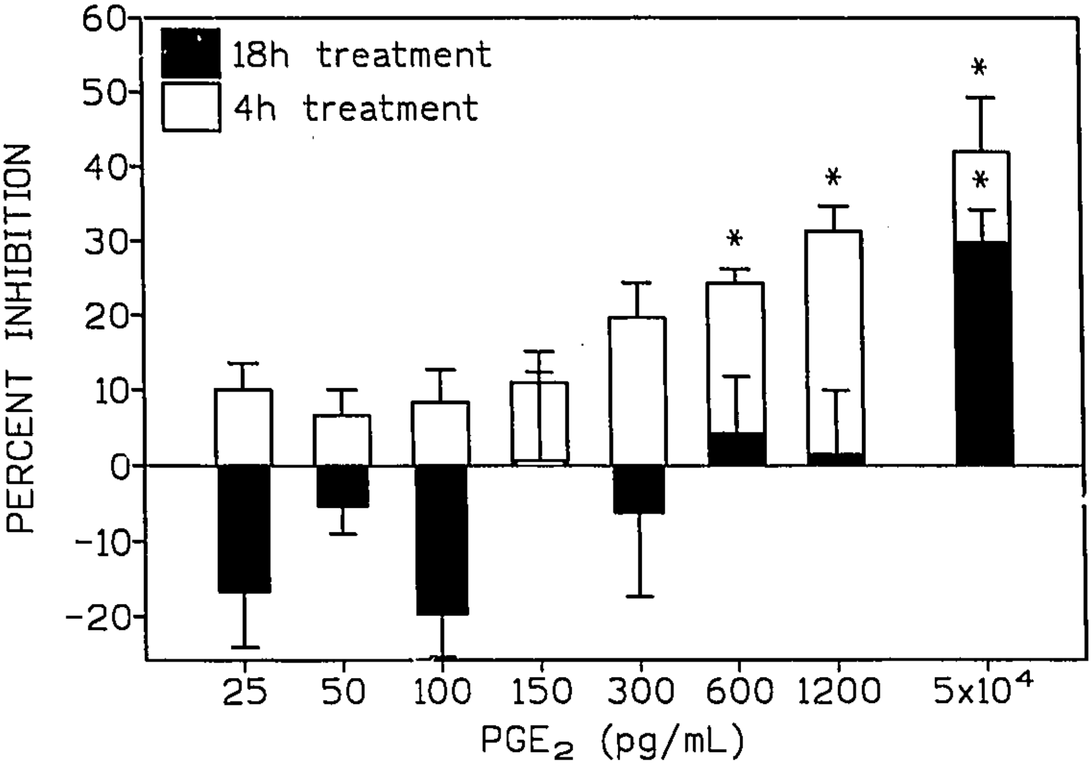
	NK activity (% Inhibition)	PGE_2 (pg/mL)
Co-culture LL + AM	$37.8 \pm 4.5^{a*}$	720.6 ± 79.0^{a}‡
10^6 AM/mL Supernatant	4.7 ± 2.4	144.3 ± 54.5
$4x10^6$ AM/mL Supernatant	$33.8 \pm 3.3^{*}$	411.1 ± 81.7‡

Lung lymphocytes were co-cultured with AM or incubated with conditioned media for 18h and a cytotoxicity assay was performed. The concentration of PGE_2 was determined by ELISA in 18h conditioned media from co-culture, 10^6 AM/mL, and 4 x 10^6 AM/mL.

[a] Values represent the mean ± SEM of at least 3 experiments.
[*] Significantly different ($p<0.05$) inhibition compared to lymphocytes incubated alone.
‡ Significantly different ($p<0.05$) PGE_2 concentration compared to 10^6 AM/mL supernatant.

FIGURE 17. Effects of PGE_2 on lung NK activity.

Lung lymphocytes were treated with the indicated concentrations of PGE_2 18h prior to (■) or at the beginning of (□) the Cr^{51} release assay. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent inhibition and values represent the mean ± SEM of at least 3 experiments. *Significantly different from control ($p \leq 0.05$).


18h treatment
4h treatment
PERCENT INHIBITION
60
50
40
30
20
10
0
-10
-20
*
*
*
*
25
50
100
150
300
600
1200
5x10^4
PGE2 (pg/mL)

insufficient to cause the degree of inhibition observed in the presence of AM (38-43%). Specifically, a 60-fold increase (up to 5×10^4 pg/mL) in PGE_2 concentration would be required to produce the observed inhibition (Figure 17).

Based on the kinetics of AM inhibition of LL NK activity, we further compared the effects of PGE_2 during 4h and 18h treatments. As shown in Figure 17, physiologically relevant concentrations of PGE_2 (600-700 pg/mL) were inhibitory ($\approx$25%) during short-term treatment (4h) but had no significant effect during long-term treatment (18h). Therefore, PGE_2 may be responsible for an early inhibitory effect, whereas the more pronounced inhibition at later time points may require additional factor(s).

C. Role of Nitric Oxide.

Nitric oxide (NO) has been shown to be an important effector molecule in activated macrophage suppression in other systems (131,147,149). Furthermore, NK cells have been found to stimulate peritoneal macrophages to produce NO (150). The potential role of nitric oxide in AM inhibition of LL NK activity was investigated. The production of NO was assessed by quantitating the concentration of nitrites in the various cell culture supernatants and determining whether the nitrite content could be inhibited by N^G-monomethyl-L-arginine (L-NmmA), an inhibitor of NO synthase. AM stimulated with LPS produced significant amounts of nitrite (93.2 μM) that were inhibited to 14.6 μM in the presence of L-NmmA (Table 6). By contrast, LL, AM, and LL + AM co-cultures produced only low or additive levels of nitrite that were unaffected by L-NmmA. This suggests that nitric oxide is not involved in AM mediated suppression of LL NK activity.

D. Involvement of TGF-β_1 but not PDGF

Previous studies have shown that transforming growth factor-β (TGF-β) (94,126) and platelet-derived growth factor (PDGF) (127,136) are capable of inhibiting NK activity. We examined the relevance of these cytokines to the inhibition of LL NK activity using neutralizing antibodies. The neutralizing antibodies were incubated with the supernatant of $4\text{x}10^6$ AM/mL, which has been shown to inhibit NK activity to a similar extent as co-culture. The adsorbed supernatant was then added to the cytotoxicity assay. Anti-TGF-β_1 suppressed the inhibitory effect of AM supernatant by up to approximately 55% while anti-PDGF had no significant effect at any concentration used (Figure 18).

E. Support for TGF-β_1 Involvement.

The ability of neutralizing anti-TGF-β_1 to block AM-mediated suppression was a strong but indirect indication that this cytokine was involved. To verify and extend this observation, the effects of exogenous TGF-β_1 (0.25-20 ng/mL) on LL, as well as the amount of active TGF-β_1 present in the co-culture supernatant were determined. TGF-β_1 was able to inhibit LL NK activity to a maximum of approximately 10%

TABLE 6. Nitric Oxide Production by AM, LL, and AM-LL Co-Culture.

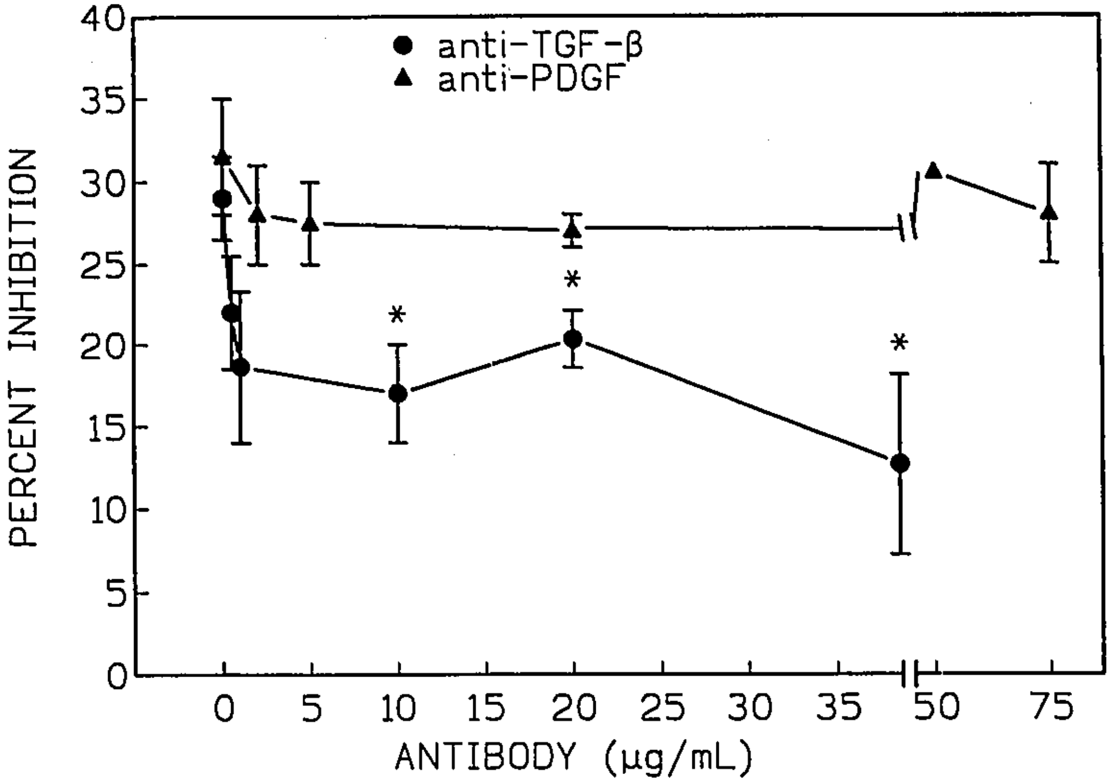
	Media Control $[NO_2^-]$ (μM)	L-NmmA Treated $[NO_2^-]$ (μM)
Lung Lymphocytes	10.0 ± 3.2[a]	9.5 ± 1.8
Alveolar macrophages	17.6 ± 4.1	15.1 ± 3.9
Co-culture (LL + AM)	36.1 ± 7.3	22.8 ± 0.49
Alveolar macrophages stimulated with LPS[b]	93.2 ± 10.5[*]‡	14.6 ± 14.3

Cells were incubated for 18h in the presence and absence of nitric oxide synthase inhibitor (L-NmmA). Supernatants were obtained from each cell culture described above and equal volumes were mixed with Griess reagent and the color change was monitored by an optical plate reader at 550 nm.

[a] Values represent mean ± SEM of at least 3 experiments.
[b] 1 μg/mL of LPS was used to stimulate AM.
[*] Significantly different ($p<0.05$) compared to AM alone.
‡ Significantly different ($p<0.05$) when compared to L-NmmA treatment.

FIGURE 18. Effects of Neutralizing Antibody to TGF-β or PDGF on the Inhibition by 18h Supernatant of $4x10^6$ AM/mL on Lung NK Activity.

The appropriate concentrations of anti-TGF-β or anti-PDGF was incubated with the supernatant for 30 min. This was then added to a Cr^{51} release assay. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent inhibition and values represent the mean ± SEM of at least 3 experiments.
*Significantly different from control ($p \leq 0.05$).



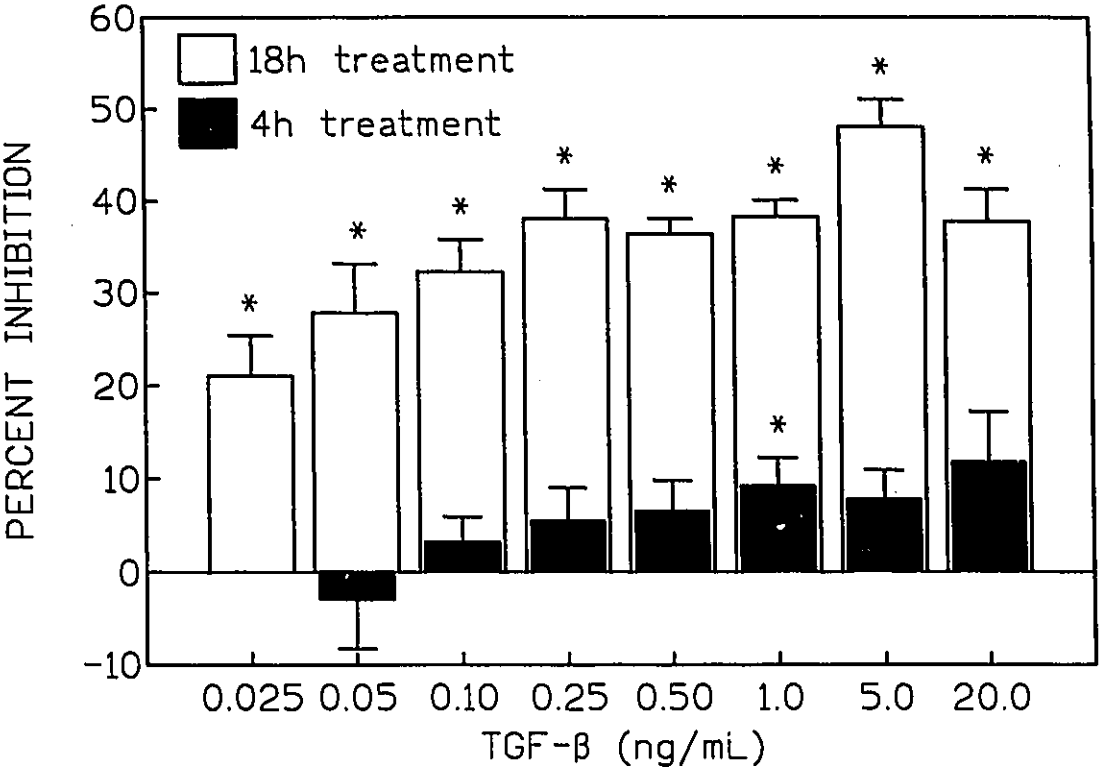
● anti-TGF-β
▲ anti-PDGF
PERCENT INHIBITION
0
5
10
15
20
25
30
35
40
*
*
*
0
5
10
15
20
25
30
35
50
75
ANTIBODY (μg/mL)

when added directly in the cytotoxicity assay (4h)(Figure 19). Thus, it may account for the inhibition found in 4x10^6 AM/mL supernatant that could not be ascribed to PGE_2. Moreover, pretreatment of LL for 18h with exogenous TGF-β_1 (0.25-5 ng/mL) resulted in a significant inhibition of up to 47% (Figure 19). This suggests that TGF-β_1 may be mainly responsible for the late phase inhibition seen in Figure 15.

To further substantiate the role of TGF-β_1 in mediating AM suppressive effect on NK activity, the levels of TGF-β_1 in the supernatant of 10^6 LL/mL, 10^6 and 4x10^6 AM/mL, and LL + AM were measured using the standard CCL-64 bioassay. As shown in Table 7, supernatant from 10^6 AM/mL and 10^6 LL/mL contained less than 5 pg/mL of TGF-β_1. By contrast, co-culture supernatant contained 24 pg/mL of active TGF-β_1 and 4x10^6 AM/mL supernatant contained 48 pg/mL, these concentrations were found to be sufficient to significantly inhibit LL NK activity (Figure 19). The values found in Table 7 are representative of 3 independent experiments. This experiment was chosen for Table 7 because the standard curve was derived from the same TGF-β_1 lot as used to determine the exogenous effects in Figure 19. The other two experiments employed a less potent batch of TGF-β_1 which consequently yielded a depressed standard curve and inflated experimental values. The trends were similar in each experiment. Thus, the amount of TGF-β_1 found under these conditions correlated well with observed NK inhibition.

FIGURE 19. Effects of Exogenous TGF-β on Lung NK Activity.

LL were treated with the indicated concentration of TGF-β, added 18h prior to (□) or at the initiation of (■) the cytotoxicity assay. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent inhibition and values represent the mean ± SEM of at least 3 experiments. *Significantly different from control ($p \leq 0.05$).



18h treatment
4h treatment
PERCENT INHIBITION
60
50
40
30
20
10
0
-10
*
0.025
0.05
0.10
0.25
0.50
1.0
5.0
20.0
TGF-β (ng/mL)

TABLE 7. TGF-β_1 production by LL, AM, and AM-LL co-culture.

	TGF-β_1 (pg/mL)[a]
Lung Lymphocytes	<5
Alveolar macrophages	<5
Co-culture (LL + AM)	24.0
4x alveolar macrophages conditioned media	48.0

Values were derived from a standard curve of TGF-β_1. The concentrations shown represent the quantity of TGF-β in the supernatants neutralized by chicken anti-human TGF-β_1 as opposed to the background value obtained with normal chicken IgG.

[a] Values are representive of the results of 3 independent experiments.

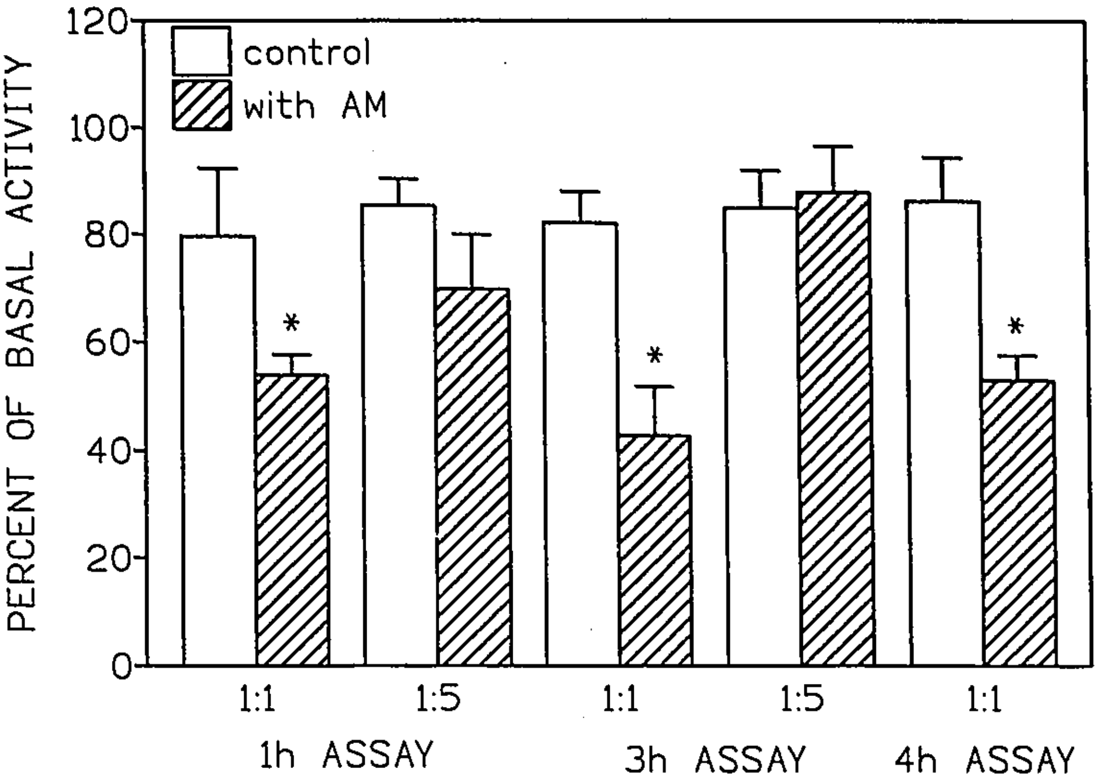
Mechanism of NK Inhibition by AM

A. Conjugate Formation versus Post-Binding Step.

The first step in investigating the mechanism of action of AM was to determine whether the inhibition was at the level of conjugate formation or at a post-conjugation step in the lytic pathway. This was examined by allowing the lymphocytes to form conjugates with labeled target cells at 4°C, where conjugation takes place but activation of lytic machinery cannot, prior to the introduction of AM. In this experiment, the AM were introduced directly into the tissue culture wells because tissue culture inserts were not available for the 96-well plates used in the cytotoxicity assay and the assay could not be practically up-scaled to the 24-well plates. Filler cells were used to eliminate cold target inhibition due to the presence of a non-effector/non-target cell type in the assay. Thus, LL were permitted to interact with ^{51}Cr labeled Yac-1 target cells for 30 min at 4°C. AM or NK resistant P815 (murine mastocytoma) filler cells, were added to the wells and the cells were brought to 37°C and incubated for 1h or 3h. Introduction of AM at a ratio to LL of 1:1 resulted in the inhibition of cytotoxicity regardless of prior conjugate formation, at each time point (Figure 20). A significant inhibition of approximately 25% was seen as early as 1h into the cytotoxicity assay. At such an early time during the assay, there would be very little recycling of NK cells that

FIGURE 20. Effects of Conjugate Formation, Prior to AM Addition, on Inhibition.

LL were incubated with Cr^{51} labelled Yac-1 target cells, at 4°C in 96-well microtiter plates. AM were added to the wells at a ratio of 1:1 or 1:5 (AM:LL). Control experiments were performed with P815 cells, as filler cells, replacing AM. Cytotoxicity was determined and calculated as lytic units (LU_{20}) using four different E:T ratios. Data are expressed as percent basal activity observed with lymphocytes alone. Values represent the mean ± SEM of at least 3 experiments. *Significantly different from basal activity ($p \leq 0.05$).



PERCENT OF BASAL ACTIVITY
120
100
80
60
40
20
0
control
with AM
*
*
*
1:1
1:5
1:1
1:5
1:1
1h ASSAY
3h ASSAY
4h ASSAY

would tend to mask an inhibition of conjugate formation. Therefore, AM inhibits LL NK activity at a step in the lytic process subsequent to conjugate formation.

3. Enrichment of NK Cells in LL Population.

In order to examine the mechanism by which lung natural killer cells are inhibited by alveolar macrophages, a relatively pure population of natural killer cells was required. Two distinct methods were used to obtain lung lymphocyte populations enriched for NK cells: Percoll gradient centrifugation and magnetic removal of $CD3^+$ cells.

i) Percoll Gradient Centrifugation.

Large granular lymphocyte (LGL) morphology has long been associated with NK activity. This property can be exploited for the enrichment of NK cells using density gradient centrifugation. A self-generating Percoll gradient was used to obtain an enriched population of NK cells from the lung lymphocyte population. The fractions obtained were analyzed for LGL content by differential staining and for cytotoxicity. The most enriched fraction (F2) contained approximately 17.5% LGL compared to 6.6% in the control population (Table 8). Thus, there was slightly less than a 3-fold increase in the proportion of LGL. The enriched fraction was found to be enriched for NK activity as well as LGL. F2 cells were able to lyse Yac-1 targets 70% more effectively than the unseparated control population (Table 8).

TABLE 8. Percoll Fractionation of Lung Lymphocytes.

Fraction[a]	%LGL[b]	Enrichment of NK Activity[c]
Unseparated	6.64 ± 0.15	-
F1	2.08 ± 1.00	0.22 ± 0.13
F2	17.48 ± 0.92	1.70 ± 0.23
F3	8.78 ± 0.74	0.82 ± 0.03
F4	3.46 ± 0.86	0.39 ± 0.06
F5	2.18 ± 0.46	0.22 ± 0.03

Lung Lymphocytes were fractionated on a self-generating Percoll gradient. Individual fractions were washed and tested in a cytotoxicity assay.

[a] Fractions represent specific Percoll density bands numbered from the top of the gradient. The specific densities are given in **Materials and Methods.**

[b] %LGL was determined by differential analysis of at least 200 cells on Wright-Giemsa stained cytospin slides. The data represent the mean ± SEM of 5 experiments.

[c] Enrichment is the LU_{20} of the specific fraction divided by the LU_{20} of the unseparated population.

ii) Magnetic Selection of CD3 Negative LL.

In order to obtain a more highly enriched lung NK cell population than was possible with Percoll fractionation, the LL were separated according to expression of CD3. The lung lymphocyte population was labeled with anti-CD3-biotin and these cells were reacted with streptavidin Ferrofluid and removed after exposure to a magnet. The resultant $CD3^-$ population was found to contain approximately 47% LGL which was slightly less than a 5-fold enrichment over the unseparated population of about 10% LGL (Table 9).

The residual T cell component in the enriched population was determined by FACS analysis of CD5 expression to avoid possible errors due to antibody mediated down regulation of surface expression of CD3 during the isolation process. As shown in Figure 21, there is little expression of CD5 in the NK enriched population ($\approx$10%) compared to the unseparated population ($\approx$55%) after 18h incubation at 37 °C. Therefore, the NK enriched population appears to possess little T cell contamination.

C. Effects of AM Co-Culture, TGF-β_1, and PGE_2 on BLT-Esterase.

To further explore which post-binding step is inhibited by AM, enriched NK cell populations obtained by magnetic separation were co-cultured with AM and the levels of BLT-esterase activity were measured. BLT-esterase activity was measured in cells stimulated by PMA and calcium ionophore in

TABLE 9. Magnetic Separation using anti-CD3 and Ferrofluid.

	%LGL[a]	LU_{20}
Unseparated LL[b]	10.68 ± 1.39	11.97 ± 1.52
$CD3^-$ LL	47.42 ± 2.77*	10.38 ± 1.47

Nylon wool non-adherent lung lymphocytes were labelled with biotinylated anti-CD3 on ice for 45 min, washed 3x in 0.1% sodium azide, then incubated with streptavidin ferrofluid for 45 min on ice. The cells were washed 3x then exposed to the magnet for 10 min.

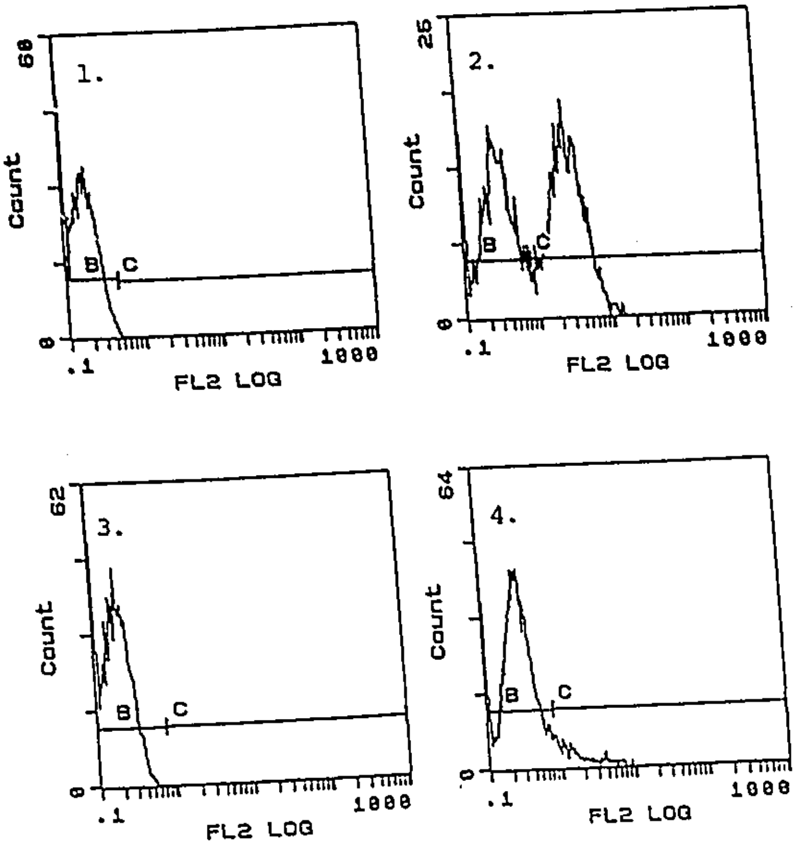
[a] %LGL was determined by differential analysis of at least 200 cells on Wright-Giemsa stained cytospin slides. These values were verified, in some cases, by FACS analysis of 3.2.3 reactive cells.

[b] The data represent the mean ± SEM of 3 experiments.

* Significantly different ($p<0.05$) from unseparated lung lymphocytes.

FIGURE 21. Flow Cytometry Analysis of CD5 Expression of Lung Lymphocytes.

Nylon wool nonadherent lung lymphocytes and NK enriched lung lymphocytes were labelled with mouse anti-rat CD5 then phycoerythrin conjugated goat anti-mouse IgG (PE-IgG). The auto- fluorescence was determined in each case with the cells incubated with the secondary antibody alone. 1. represents the nylon wool nonadherent population incubated with the secondary antibody alone, 2. is the same cells labelled with anti-CD5 and PE-IgG, while 3. represents the NK enriched lung lymphocyte population incubated with the secondary antibody and, 4. the same cells labelled with anti-CD5 and PE-IgG.


1.
60
Count
0
.1
1000
FL2 LOG
B
C
2.
25
Count
0
.1
1000
FL2 LOG
B
C
3.
62
Count
0
.1
1000
FL2 LOG
B
C
4.
64
Count
0
.1
1000
FL2 LOG
B
C

order to measure the level available for secretion, independent of target cell derived signals. Table 10 shows that $CD3^-$ lung lymphocytes treated for 18h with 1 ng/mL TGF-β_1 or co-cultured with AM had significantly decreased levels of BLT-esterase activity (33.9% and 44.6% inhibition respectively). In contrast, treatment with 1 μg/mL of PGE_2 had no significant effect on BLT-esterase activity. These data indicate that AM inhibit the production or function of BLT-esterase (granzyme A) in lung lymphocytes, probably through the secretion of TGF-β. Furthermore, TGF-β and PGE_2 appear to inhibit lung NK activity by distinct mechanisms, since PGE_2 treatment had little effect on BLT-esterase activity.

D. Effects of AM Co-Culture, TGF-β_1, and PGE_2 on Granzyme B

In situ hybridization was used to determine whether the inhibition of granule enzyme function, suggested by the decrease in BLT-esterase activity, was a result of a specific down regulation of granule enzyme mRNA. $CD3^-$ lung lymphocytes were treated with 1 μg/mL PGE_2, 1 ng/mL TGF-β_1, co-cultured with AM, or incubated for 3h with media alone. These cells were hybridized on glass slides to cDNA probes for granzyme B (C11) and granzyme D (B10). Table 11 shows that the lymphocytes do not express granzyme D at detectablelevels, whereas granzyme B mRNA is detectable in approximately 23% of the cells. The number of cells

TABLE 10. Effects of Soluble Factors and Co-Culture with AM on BLT-Esterase Activity.

	Absorbance[a]	% Inhibition[b]
Untreated	0.147 ± 0.012	-
PGE_2	0.135 ± 0.004	8.2
TGF-β	0.097 ± .010*	33.9
LL-AM Co-culture	0.081 ± .010*	44.6

The effect of factors involved in NK inhibition by AM and co-culture on BLT-Esterase (Granzyme A) activity of $CD3^-$ lung lymphocytes. Lung lymphocytes separated by magnetic adherence of CD3 labelled cells were incubated for 18h with 1 μg/mL PGE_2, 1ng/mL TGF-β_1, or co-cultured 1:1 with AM. Cells were then stimulated with phorbol ester myristate and calcium ionophore to produce maximum exocytosis of granule factors. BTL-esterase activity was determined by measuring the colorimetric change, 1h after the addition of the substrate Nα-Cbz-Lys-thiobenzylester (BLT) and the indicator 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), in an optical plate reader at 405 nm.

[a] Absorbance represents the mean ± SEM of the values of 3 experiments.

[b] % Inhibition is 100% - (abs. treated/abs untreated x 100%)

* Significantly different ($p<0.05$) from lymphocytes treated with media alone.

expressing granzyme B mRNA was inhibited by treatment with TGF-β_1 and co-culture with AM (9.03% and 7.63% positive for expression respectively) but was not affected by treatment with PGE_2 (20.64%), as was the case for BLT-esterase activity. Therefore, it appears that AM inhibition of lung NK activity is a result of down regulation of the expression of granule components involved in the lytic process.

Table 11. Expression of Granzyme D or Granzyme B Measured by *In Situ* Hybridization of Treated CD3$^-$ Lung Lymphocytes.

	%B10 Positive (granzyme D)	%C11 Positive (granzyme B)
Untreated	0.40	23.53
PGE$_2$	4.09	20.64
TGF-β_1	1.09	9.03
AM Co-Culture	3.86	7.63

CD3$^-$ lung lymphocytes were isolated then treated with 1 μg/mL PGE$_2$, 1 ng/mL TGF-β_1, co-cultured with AM at a ratio of 1:1, or incubated with media for 3h. The cells were then attached to baked slides by cytospin and fixed with acetone. Cells were hybridized with S^{35} labelled B10 or C11 cDNA probes. The labelled slides were dipped in emulsion and exposed for 2 weeks at 4°C then developed. Cells were analyzed on a computerized image analysis system. Cells were defined as positive if they were at least 3 times brighter than background.

DISCUSSION

Natural killer activity has been observed in the lung of human (31), mouse (34), and rat (32). There is accumulating evidence from several sources that these tissue-associated NK cells are under local regulation independent of systemic NK activity (5,6,8). However, little is known of the regulation of these cells, particularly in terms of direct interaction with purified cytokines likely to be found in the tissue compartment or interaction with regulatory cells. This study was designed to characterize lung-associated natural killer activity and its regulation *in vitro* in the rat model.

Characterization of Lung Lymphocyte NK Activity

Lymphocytes with NK activity exist in at least 3 distinct populations in the lung: bronchoalveolar lavage (BAL) cells (9), lung intracapillary (LIC) cells (3,4), and interstitial lymphocytes (LL) (5,32). Bronchoalveolar lavage cells represent the most accessible of the populations, particularly in humans. However, the majority of BAL cells are alveolar macrophages. AM content of BAL approaches 100% in the Wistar rat, the model system used in this study, and lymphocyte infiltration is most often the result of alveolar disorder rather than the resting state. Furthermore, the presence of significant proportions of AM in the lymphocyte population is likely to cloud the interpretation of the *in vitro* regulation. LIC do not suffer from these drawbacks but it is difficult to differentiate these cells from blood lymphocytes since they are obtained from the vascular side of the pulmonary capillaries. The observed differences between these cells and blood lymphocytes may be a result of stimulation and adherence rather than a lung specific difference in differentiation or stimulatory state. I have chosen to study NK activity in the lung interstitium of the rat.

This model provides relatively large numbers of lung resident lymphocytes with minimal macrophage (AM) contamination. The macrophages present do not seem to have a regulatory effect because 24h incubation of these cells, once they have been depleted of macrophages, does not result in an increase in cytotoxicity, however, further experiments would be necessary to verify this hypothesis. The interstitial lymphocytes have been prepared by perfusion of the pulmonary vasculature and extent of blood cell contamination was monitored visually in terms of 'redness' of the tissue from red blood cells. However, it is likely that a certain proportion of LIC are present in the preparation because LIC have been reported to adhere to the capillary epithelium after flushing of the capillaries to remove RBCs. The proportion of LIC in the LL preparation is impossible to assess and as such are included in the definition of lung-associated lymphocytes used in this study.

One major experimental drawback to studying lung interstitial lymphocytes is the requirement for enzymatic digestion of the lung tissue to free useful numbers of cells.

It was important to determine whether this treatment was responsible for the NK activity in the LL preparation. This was examined by isolating lung lymphocytes in the absence of enzymatic digestion by mechanical dissociation. Three separate methods were employed giving three distinct populations. Each population demonstrated natural killer activity against the NK target, YAC-1. The populations, however, differed a great deal in the lymphocyte to macrophage ratio. The NK activity of the different populations was directly proportional to the number of lymphocytes and inversely proportional to the percentage of macrophages (AM). The contribution of AM-mediated inhibition of NK activity in these populations cannot be easily assessed. However, the fact that these populations contained NK activity indicates that the enzymatic treatment was not responsible for the observed NK activity in the LL population.

The presence of lung resident lymphocytes with NK activity led to the investigation of whether this activity was susceptible to methods typically used to remove NK cells. *In vivo* administration of asialo GM-1 antiserum and *in vitro* treatment with this antiserum and complement have been used to eliminate NK activity in a variety of rodent NK studies (5,6,8). The NK activity of the LL population under study was completely eliminated by this treatment. This is a good indication that the cytolysis of YAC-1 by LL was a result of NK cell lysis. However, asialo-GM1 has been shown to interact to a limited extent with monocytes (150). Since there was very little contamination of macrophages and polymorphonuclear cells (PMN) in the lung lymphocyte population, the contribution of these cells to the cytotoxic effect is unlikely. In addition, monoclonal antibody 3.2.3, that recognizes a rat NK marker that does not cross react with macrophages (142), was found in similar proportion in the lung and the spleen, providing additional evidence that NK cells are present in the lung lymphocyte population. As added support for the hypothesis that NK cells were responsible for the observed lytic activity, the LL were treated *in vitro* with L-leucine methyl ester, a lysosomotropic agent known to abolish NK activity (141). This treatment almost completely eliminated the NK activity in the study population. Together these data show that the lung lymphocyte population possesses a lytic potential susceptible to protocols designed to eliminate NK cells and is likely to contain a population of lung resident NK cells.

The assumption underlying many of the studies of the regulation of natural killer activity is that all NK cells, regardless of their origin will behave similarly. This assumption was tested using the lung lymphocyte population and lymphocytes obtained from the spleen and peripheral blood. These populations were found to be comprised of similar proportions of lymphocytes. However, lung lymphocytes were significantly more effective in lysing YAC-1 targets than either the spleen or the peripheral blood cells. This was not a result of a higher proportion of NK cells in the lung since the lung and the spleen were found to contain equivalent amounts of 3.2.3 positive cells. Therefore, the lung NK population appears to possess a higher lytic capacity on a per cell basis. The reason for this phenomenon has not been investigated further but it suggests that there are quantitative differences in the functional state of the resting cell populations tested. It is possible that this difference is a result of the methods used to isolate the cells, however, every opportunity was taken to harmonize the isolation procedures. The major difference between the procedures was the enzymatic digestion of the lung tissue. If this were to result in an increase in basal activity, presumably through the digestion of inhibitory surface structures, then there should be a depression of NK activity upon regeneration of these structures. There was no such change after 24h incubation in culture media in any of the isolated cell populations. Therefore, the difference in basal activity is likely a result of local regulation and/or maturation. Interestingly, it has been reported that lung lymphocytes possess higher, basal, BLT-esterase activity than PBL (102).

The comparison of the NK activity in the lung, spleen, and peripheral blood was continued by assessing the responsiveness to signals previously shown to augment human peripheral blood NK activity such as IL-2 (84), IFN-γ (85), and IFN-α/β (85). The responsiveness of LL to IL-2 was comparable to PBL but lower than SL. IFN-γ was unable to augment NK activity in the lung or spleen populations, in contrast to the significant increase in peripheral blood NK activity. IFN-α/β was equally effective in enhancing PBL NK activity as IFN-γ but had a more pronounced effect on LL and SL NK activity. These data are in agreement with a previous report that IFN-γ was much less active than IL-2 or IFN-α/β in augmenting spleen NK activity (106). Taken together, these results suggest that lung, spleen, and peripheral blood NK activities vary substantially in their cytokine responsiveness profile and lend support to the hypothesis that regulation is tissue dependent. In this respect, a final comparison was made between lung and spleen NK activity on the interaction of these cells with alveolar macrophages and macrophage supernatants. Alveolar macrophages co-cultured with lung lymphocytes for 18h resulted in a significant inhibition of NK activity while similar co-culture with spleen lymphocytes resulted in an augmentation of NK activity. Since the AM population was identical in both cases, the differences must have occurred at the level of the lymphocyte populations, suggesting tissue specific regulation. There are examples in the literature where monocytes have enhanced human peripheral blood NK activity (120,121), however, AM have been exclusively associated with inhibition of NK activity (123,125,132). Our observation of a macrophage stimulatory effect on splenic NK may be related to the methods of co-culture used. In the present study there was no physical contact between the lymphocytes and AM which was not the case in the other studies cited. Perhaps AM release a potent inhibitory factor upon direct interaction with NK cells or possess a signal on their cell surface that inhibits NK cells coming in contact with it (125). Regardless of the reason for the difference between the observed interactions and the reported ones, these data clearly indicate that the lymphocytes play a role in the regulation of inhibition by AM. LL appear to stimulate AM to secrete inhibitory factors while SL may stimulate AM to secrete enhancing factors, based on the functional effects of co-culture as opposed to addition of conditioned media from the same concentration of AM. The interaction of SL with AM was not examined further, thus additional experiments would be required to test this hypothesis. However, co-culture supernatant from LL with AM showed an increase in the concentration of the inhibitory factors, TGF-β and PGE_2 compared to AM incubated under similar conditions without LL. Furthermore, the finding that both lymphocyte populations respond similarly to the inhibitory supernatants from higher concentrations of AM suggests that the difference is not simply a result of differential responsiveness to AM products. Interaction with AM, therefore, illustrates another important difference in the regulation of lung NK activity in comparison to the spleen.

The lung, spleen and peripheral blood NK cells have been shown to differ significantly in intrinsic cytotoxicity, the responsiveness to certain cytokines, and interaction with alveolar macrophages. Therefore, the assumption that NK cells in the easily accessible compartments of the spleen and blood can be used to represent NK activity from other tissue sources has been demonstrated to be invalid. This is in accordance with other studies indicating a compartmentalization of tissue-associated NK activity (5-7). Furthermore, these data indicate that a more comprehensive examination of the local regulation of lung NK activity is warranted, to provide insight into this neglected aspect of NK activity. The local regulation of lung NK activity was examined both in relation to cytokine mediated enhancement and the interaction with alveolar macrophages.

Enhancement of Lung NK Activity by Selected Cytokines.

The choice of cytokines to examine the *in vitro* stimulation of lung natural killer activity was made based on published reports of NK stimulating activity in other systems. IL-2, IFN-γ, and IFN-α/β had been extensively studied in the peripheral blood and the spleen and were considered important regulators of NK activity. It was considered important to include monokines that may be produced by regulatory AM in the lung and have been shown to possess NK augmenting activity. For this reason IL-1 and TNF-α were added to the list.

Therefore, *in vitro* effects of IL-1, IL-2, IFN-γ, IFN-α/β, and TNF-α on lung NK activity were examined upon 18h pretreatment.

IL-2 was found to be the most efficient enhancer of lung NK activity among those examined. Relatively small doses of IL-2 produced significant enhancement and the maximal stimulation was reached with a dose as little as 50 U/mL. This is consistent with the observed kinetics of NK activation by IL-2 in the human peripheral blood (152). However, the degree of stimulation was reportedly much higher (800%) than determined in this study (50%) for lung NK, and the concentration of IL-2 required to reach maximal stimulation was also higher. The reason for these quantitative differences is difficult to assess, however, it may be a result of organ-related differences in the NK population. Consistent with this hypothesis is the more recent finding that human lung lymphocytes from biopsy samples were stimulated approximately 100% by 24h treatment with 100 U/mL IL-2 (101). This human lung preparation clearly represents a more suitable model for comparison than the PBL NK activity. The relatively small difference seen in IL-2 stimulation between this and our study may reflect the likely presence of substantial peripheral blood contamination and the underlying disease in the biopsied lung (PBL were found to be marginally more responsive than LL).

IFN-α/β was found to stimulate lung NK activity to a level similar to IL-2, although much higher concentrations of IFN-α/β were required (1000 U/mL). Platsoucas et al. (85) found similar levels of activation in human peripheral blood NK activity using high concentrations of IFN-α (2000 U/mL). In contrast, Brunda et al. (106) found massive enhancement of murine spleen NK activity using human recombinant IFN-α. However, the degree of enhancement may have been more a reflection of very low basal activity than a high induced NK activity, so the differences may not be as significant as they first appear.

Treatment of lung lymphocytes with a combination of IFN-α/β and 10 U/mL of IL-2 resulted in a synergistic enhancement of NK activity. However, this effect was only observed at low doses of IFN-α/β, concentrations that have little stimulatory activity on their own. Brunda et al. (106) observed a similar "more than additive" response to doses of recombinant IL-2 and recombinant IFN-α in spleen NK activity. However, they report this effect over a much broader range of concentration. It is difficult to analyze the reason for the differences reported, since Brunda et al. report their NK activity in terms of specific cytotoxicity at specified target to effector ratios rather than lytic units and the ratios chosen (200/1) are much higher than any employed in this study.

It is of interest to note that the maximum stimulation of lung NK activity by IL-2, IFN-α/β, or a combination of the two is similar. This may represent a physical limit to the stimulation of the lung lymphocyte population. However, it is unlikely to be a limitation imposed by the assay system because spleen NK activity was shown to exceed this limit. It is conceivable that lung lymphocytes, which possess a higher basal NK activity than either spleen or peripheral blood, are limited in their ability to respond to external stimulation by some organ-associated process. This limitation may reflect a stimulation or maturation step in the lung that is absent in the other lymphocyte populations. In contrast to the response to IFN-α/β, lung NK activity was found to be unresponsive to treatment with IFN-γ, and IFN-γ did not enhance IL-2 mediated stimulation. This is entirely consistent with the findings of Brunda et al. (106) for murine spleen NK activity. However, Nuntirooj et al. (34) report significant enhancement of murine lung NK activity by *in vitro* IFN-γ, although, not to the same extent as *in vivo* treatment with IFN inducers. There are some differences in the isolation procedures which may, in part, account for this difference. One such difference was that the vasculature of the murine lung was not perfused prior to isolation of the lymphocytes. This is likely to result in a greater contamination of blood lymphocytes in the lung lymphocyte population and these cells, which we have shown to respond to IFN-γ, may be responsible for the augmentation. Alternatively, Nuntirooj et al. (34) used a partially purified IFN-γ preparation rather than a recombinant preparation employed in this study. It is possible the partially purified IFN-γ contained other stimulatory factors, such as IL-2 or IFN-α/β.

Recombinant TNF-α stimulated lung natural killer activity in a dose-dependent manner similar to IFN-α/β, however, TNF-α was only about half as effective in terms of the maximal stimulation. This is in direct contrast to the finding of Talmadge et al. (153) that TNF-α stimulation of murine spleen NK activity occurs upon *in vivo* treatment but not *in vitro* treatment. However, it is entirely consistent with the observations of Ostensen et al. (86), who found that rTNF-α treatment of human PBL resulted in a small but significant augmentation of NK activity.

Treatment of lung lymphocytes with TNF-α and a low dose of IL-2 resulted in a synergistic enhancement of NK activity at doses of TNF-α that were not effective in augmenting NK activity alone. Thus TNF-α resembled IFN-α/β in terms of the interaction with IL-2, as well as its activity alone. Ostensen et al. (86) reports similar findings with human PBL. It is difficult to reconcile the differences between the findings of Talmadge et al. (153), Ostensen et al. (86) and the present study but there seems to be a common finding that TNF-α has an immunostimulatory effect on NK activity.

There was no consistent effect of IL-1 on lung NK activity alone or in conjunction with low dose IL-2. Dempsey et al. (111) reported similar findings for treatment of human

PBL with IL-1 alone but found that IL-1 was capable of enhancing IL-2 mediated stimulation. Additionally, Dinarello et al. (83) observed enhancement of human PBL NK activity after treatment with rIL-1 and synergism between rIL-1 and rIL-2. The differences between the present study and the others cited may reflect differences in effector cell origin, either tissue or species related, or may reflect differences in stimulation protocol.

Many other cytokines have been reported to affect NK activity and LAK activity since the design of these experiments. However, it is impractical to attempt to characterize the effects of each new cytokine on lung NK activity in a single study.

This study has provided evidence that lung NK activity differs from the activity of other lymphocytes in terms of responsiveness to cytokines and has characterized that responsiveness. Of greater interest than pursuing the characterization of the ever expanding list of cytokines is the local, cell-mediated, regulation of lung NK activity.

The Interaction of Lung Lymphocytes and Alveolar Macrophages.

Cells of the monocyte/macrophage lineage have long been implicated in the regulation of natural killer activity (32,33,123,125,) as have several monocyte/macrophage products (128,134,136,138). However, the mechanism of the inhibition and the factors directly involved have not been satisfactorily explored. The interaction of alveolar macrophages with lung interstitial lymphocytes, in their *in situ* environment, has received even less consideration despite the unique characteristics of this system. This study was designed to answer the questions surrounding this interaction. First, the modulation of lung NK activity was explored with respect to the kinetics of the interaction. Once it was determined that the interaction resulted in inhibition, the factors involved in the inhibition were investigated. This investigation employed specific inhibitors and the identity of the factors were verified through the use of exogenous source of the factors and assaying the co-culture supernatant for the presence of the candidate factors. Finally, the mechanism of action of the inhibition was examined as to whether it took place at the level of conjugate formation, or at a critical post-binding step, notably granzyme production and/or secretion.

Although alveolar macrophages are important and potent immunoregulators in the lung, they are not normally in direct contact with lung interstitial lymphocytes. There is considerable evidence indicating that alveolar macrophages inhibit PBL natural killer activity by a method requiring cell-to-cell contact (124,153). Therefore, it was important to maintain this compartmentalization wherever possible in studying the interaction of AM and LL. This anatomical isolation was simulated through the use of removable tissue culture inserts. These inserts provided a two-chamber system which prohibited physical interaction but permitted chemical signalling. There is an additional practical advantage to this two-chamber system when compared to the typical mixing experiments, in that AM can be removed at the end of the co-culture period. The separation of the AM and the LL at the end of the co-culture period prevents non-specific interference of the AM in subsequent assays, particularly the chromium-51 release assay.

Alveolar macrophages inhibit lung interstitial lymphocyte NK activity in a time-dependent manner. Interestingly, there appear to be two temporally distinct phases of increase in inhibition. These kinetics are consistent with the hypothesis that at least two independent factors are involved in the inhibition where one is released immediately or shortly after co-culture begins and a second which requires synthesis. Alternatively, both factors may be released quickly but the later acting factor may operate by a mechanism that requires a significant period of time to be observed. The kinetics, further, show that macrophage-mediated inhibition of lung NK activity is reversible, in that removal of AM and subsequent incubation of LL leads to the recovery of over 90% of the basal activity. This clearly demonstrates that the inhibition is a result of specific functional regulation rather than the result of non-specific damage to the effector cells.

The search for the identity of the inhibitory factors began with prostaglandin E_2 (PGE_2). PGE_2 has been shown to inhibit NK activity *in vitro* in several cell systems (128,155,156) and indirectly through *in vivo* administration of the cyclooxygenase inhibitor, indomethacin (148,157). PGE_2 is believed to cause an increase in intracellular cAMP (155). cAMP then interacts with cAMP-protein kinase A which inhibits the inositol phosphate turnover and protein kinase C activation that occurs after binding to the target cell (158,159). In this study, it was found that the addition of indomethacin at the beginning of the 18h co-culture suppressed the inhibition of lung NK activity by alveolar macrophages, at concentrations of indomethacin that did not affect NK activity. PGE_2-mediated inhibition of NK activity in a co-culture situation where AM were removed and AM products washed away prior to exposure of effectors to the target cells, seems to contradict the recent work of Fulton and Chong (133) who showed that PGE_2-mediated inhibition of NK activity takes place at the target cell rather than the effector cell level. In order for that mechanism to be operating in this system, either the lymphocytes are storing PGE_2 or indomethacin is suppressing the inhibitory effect through a PGE_2 independent pathway. Given the short biological half-life of PGE_2 it is highly unlikely that the lymphocytes took up and stored sufficient quantities of PGE_2 to release upon introduction of target cells.

It is likely that indomethacin treatment inhibited the production of other cyclooxygenase metabolites of arachidonic acid. Several metabolites of the cyclooxygenase pathway other than PGE_2, such as, PGE_1, PGA_1, and PGA_2, have been shown to inhibit natural killer activity (128). A contribution of these other factors to the inhibitory effect has not been formally disproved. However, there are two pieces of indirect evidence suggesting that PGE_2 may be responsible for the indomethacin-mediated suppression of inhibition. First, the observation that PGE_2 concentration decreased with indomethacin treatment and that this correlated with reduced inhibition indicates that PGE_2 may be involved. Second, the supernatants from co-culture experiments contained much more PGE_2 than supernatants from AM cultured alone, which were not inhibitory. Therefore, it is likely that the differences between the observations reported here and those of Fulton and Chong (133) are a function of the differences in the lymphocyte systems involved.

It is interesting to note that it was not possible to completely suppress the inhibition of lung NK activity with indomethacin. This indicated that a second factor may be involved in the observed inhibition. The effect of exogenously added PGE_2 on lung NK activity was explored and correlated to the concentration of PGE_2 found in the co-culture supernatant to test this possibility. It was found that the amount of PGE_2 in the co-culture supernatant was not sufficient to account for the degree of inhibition that took place. Furthermore, the effect of exogenous PGE_2 was potent when added directly into the lytic assay but was insignificant after 18h pre-treatment. This may indicate that PGE_2 is responsible for the early inhibition seen in the co-culture. However, it does not discount the possibility that PGE_2 is also involved in the inhibition at later time points. In the co-culture situation, AM were present throughout the incubation time, thus there was a constant source of fresh PGE_2 in contrast to the experiments where PGE_2 was added. Nonetheless, it is attractive to envision a situation where PGE_2 would play a major early role and only a minor role later in the kinetics of AM inhibition of lung NK activity.

TGF-β_1 was found to account for much of the remaining inhibitory potential of the co-culture AM. The evidence for this is indirect but still compelling. Supernatants from $4x10^6$ AM/mL which have been shown to have an inhibitory effect similar to co-culture, despite lower concentrations of PGE_2, were pre-incubated with neutralizing antibody to TGF-β_1. The inhibitory capacity of these supernatants was decreased by more than half after treatment, suggesting that TGF-β_1 is produced by AM in a form and concentration sufficient to inhibit lung NK activity. More indicative of a role for TGF-β_1 in the inhibition of lung NK activity by AM in co-culture was the observation that there was a sufficient concentration of TGF-β_1 in the co-culture supernatant to account for 20% inhibition. In accordance with these results, TGF-β has been shown to be a potent inhibitor of human peripheral blood NK activity (94,138,160). It has been shown to act at a post-binding step probably by interfering with signal transduction (94,138) and/or gene expression (126,160), at least after IL-2 stimulation.

The assumption that the treatment of LL with supernatant of 4×10^6 AM/mL was analogous to co-culture of LL with AM was not rigorously tested. However, it was found that each of the conditions resulted in significant production of PGE_2 and TGF-β_1, as well as resulting in comparable levels of inhibition. These similarities were sufficient to support the use of this supernatant in conjunction with neutralizing antibodies to screen cytokines for their potential role in AM-mediated inhibition. An actual role for the cytokine was then assessed by directly measuring the cytokine in the co-culture supernatant and comparing that to the response of LL to exogenous doses of the cytokine.

In contrast to the results with PGE_2, treatment of lung lymphocytes with exogenous TGF-β_1 resulted in inhibition of lung NK activity after 18h pre-treatment but had little effect when added directly into the assay. This is consistent with the action of TGF-β in down-regulating the expression of important components of the lytic cycle, as was found for granzyme A and B, discussed below. In agreement with this proposed function is the recent finding that TGF-β inhibits the induction of perforin and granzyme A production upon IL-2 treatment (94).

The temporal difference in the response of lung NK activity to exogenous treatment with TGF-β_1 and PGE$_2$ mirror the two factors hinted at by the kinetics of inhibition in the co-culture system. It seems likely that PGE$_2$ or some other prostaglandin is responsible for the early inhibition and may contribute to the effect at later time points while TGF-β_1 is responsible for most of the later inhibition.

The possible contribution of platelet-derived growth factor in the inhibition was investigated by the same method as TGF-β_1, however, neutralizing antibody to PDGF was found to have no effect on the inhibition. PDGF is known to be produced by human monocytes (135) and to have an inhibitory effect *in vitro* on human peripheral blood NK activity (136). However, the inhibition is thought to take place at the level of conjugate formation (136). The inhibition under study here has been shown to take place at a post-conjugation step, therefore, it would be unlikely for PDGF to play a significant role. The fact that PDGF is not involved in the inhibition is not necessarily inconsistent with published reports. The ability of alveolar macrophages to produce PDGF under appropriate stimulatory conditions is not addressed, nor is the inhibitory effect of PDGF upon NK activity. It has, however, been determined that PDGF is not involved in the AM-mediated inhibition of lung NK activity in this system.

The final factor examined for its potential involvement in the inhibition of lung NK activity in this system was nitric oxide. The contribution of nitric oxide was assessed by measuring the production of nitrite, a stable metabolite, in the co-culture supernatant that was inhibited by treatment with a nitric oxide synthetase inhibitor, N^G-monomethyl-L-arginine. There was an absence of nitric oxide production in co-culture supernatant despite the production in LPS stimulated AM culture supernatants. The absence of nitric oxide production in the co-culture may be related to the production of TGF-β which has been shown to inhibit nitric oxide production by murine peritoneal macrophages (161).

There is considerable evidence of a lymphocyte requirement for the production of a measurable inhibition. The supernatants from AM cultured in the absence of LL were unable to mimic the inhibitory effects of a similar number of AM co-incubated with LL. It is unlikely that this is a result of dilution of the factor(s) since the same concentration of AM was used in either situation. It is probable that there is the production of a signal by the lung lymphocytes that stimulates an increase in the production and/or release of inhibitory factors by the AM, when in co-culture. There is evidence for this both in the production of PGE_2 and TGF-β_1, where the co-culture supernatant has been shown to contain substantially more of these factors than supernatant from AM cultured alone. Natural killer cells have been shown to stimulate macrophage function in other systems through the production of cytokines (162,163). Furthermore, it is known that lymphocytes and NK cells, in particular, are able to produce a battery of cytokines under the proper conditions, including: granulocyte-macrophage colony-stimulating factor (163), interleukin-1 (164), interleukin-2 (165), interleukin-4 (166), interferon-γ (166), and tumor necrosis factor-α (167), among others. Many of these cytokines have been shown to have an effect on macrophage function and the others can easily be envisioned to have an indirect effect through the stimulation of secondary and tertiary factors.

The lung lymphocyte population used in this study was a compromise between a wish to simulate the *in situ* composition of the lung and the need to remove as many contaminating macrophages as possible. A consequence of this compromise was that the lung lymphocyte population was composed of 98% lymphocytes but only about 12% of the total were natural killer cells. The advantage of having the immunologically relevant populations intact were obvious in terms of understanding the reactivity of the lung lymphocyte population. However, the heterogeneous nature of the lung lymphocyte population was a disadvantage in terms of assessing specific cell interactions and mechanisms of action.

For these reasons, we attempted to enrich the lung lymphocyte population for natural killer cells. This was accomplished by the selective removal of CD3 positive cells by specific labelling and magnetic adherence. The resulting population was almost 50% LGL and 3.2.3 positive (NK cells).

The cytotoxic T lymphocyte (CTL) contamination was of particular interest because T lymphocytes share several properties with NK cells and could thus obscure the results. The T cell depletion was monitored by FACS analysis with CD5, a pan T marker. The proportion of T cells was reduced from greater than 50% in the unseparated cells to approximately 10% in the NK enriched population. This enrichment of NK cells was deemed sufficient to undertake studies of the mechanism of action of AM inhibition.

As outlined in the introduction, there are several steps involved in the lytic activity of NK cells, any one of which is a potential point of inhibition. However, the evidence obtained with the unseparated lung lymphocyte population indicated that the inhibition took place at a post-conjugation step and that the effect was mediated by TGF-β and PGE$_2$. It has been shown that TGF-β inhibits the lytic activity of peripheral blood NK cells stimulated with IL-2 (94) and T-LAK activity (160) by suppressing the IL-2 induction of the expression of lytic molecules. Therefore, we began the search for the mechanism of AM inhibition by focusing on the expression of lytic molecules.

Granzyme A has been implicated in the lytic potential of NK cells by virtue of its localization in secretory granules and its involvement in target cell apoptosis (168). The expression of granzyme A, which has been shown to be the major granzyme source of BLT-esterase activity (168) was monitored by the standard BLT-esterase assay. It was found that TGF-β treatment and AM co-culture result in an equivalent decrease in functional granzyme A in lung NK cells while PGE_2 treatment had no effect. Malygin et al. (94) reported a suppression of granzyme A and PFP induction by TGF-β but not PGE_2, in peripheral blood NK cells stimulated with IL-2. However, they were unable to determine whether TGF-β acted in a similar manner on resting NK cells because granzyme A could not be reproducibly detected by immunohistochemistry in unstimulated cells. The results reported here clearly show that granzyme A activity is reduced in lung NK cells treated with TGF-β or co-cultured with AM. Our ability to detect granzyme A by the functional assay may, in part, be due to the amplification of the signal in this sort of assay in comparison to immunohistochemical staining. An organ-associated difference may also be playing a role in this difference. There is evidence that lung natural killer cell granules contain significantly higher levels of BLT-esterase activity when compared to peripheral blood NK cells (102).

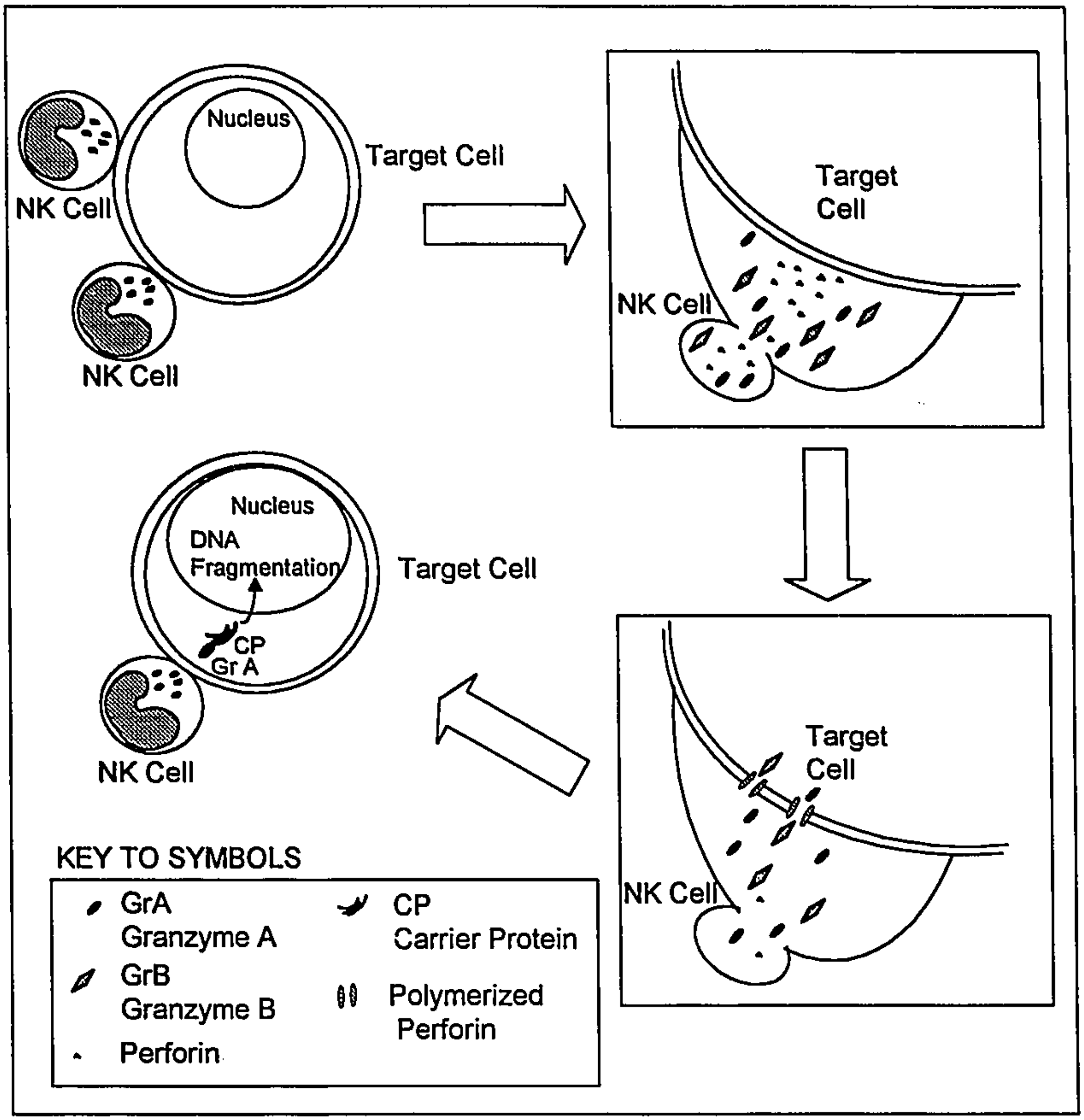
Granzyme A has been associated with the lytic activity of activated CTL (160) and these cells may contribute to the observed BLT-esterase activity. However, T cells were only a minor constituent of the enriched NK population. Additionally, the rats employed in this study were pathogen-free and thus would not be expected to possess significant levels of activated CTL. Furthermore, it has been shown that in unprimed mice, BLT-esterase activity is localized in the NK cell rather than the T cell or B cell population (170). Therefore, TGF-β and AM co-culture results in the down-regulation of granzyme A, and if the effect on granzyme B, discussed below, can be extrapolated to this case, the regulation takes place at the mRNA level.

Granzyme B is another granule enzyme associated with the lytic activity of NK cells (171) and may operate synergistically with granzyme A in inducing target cell apoptosis (172). Granzyme B expression has been shown to be more rapidly induced in the presence of stimulatory factors, such as IL-2 and IL-12, than granzyme A (170) and thus may be particularly relevant for the modulation of NK activity. The expression of granzyme B was determined at the mRNA level by *in situ* hybridization. It was found that the number of lymphocytes expressing detectable quantities of granzyme B mRNA was reduced by approximately 50% in the presence of TGF-β or co-culture with AM while PGE_2 treatment had no effect on its expression. An attempt was made to use granzyme D, a granule protease not associated with target cell lysis, as a control for irrelevant down-regulation. Granzyme D was not expressed within the limits of detection in the lung NK population. Thus, it has not been demonstrated here whether the down-regulation of granzyme B expression is a specific effect of TGF-β on NK cells or a generalized effect on mRNA expression. However, an effect specific for granzyme B was not anticipated, rather a more generalized effect on components involved in lysis was expected. To this end, the finding that granzyme B mRNA expression was reduced in treated lung NK cells provides a strong suggestion that it is the point at which TGF-β and AM inhibit lung NK activity.

The data discussed above provide evidence that AM inhibit lung NK cell activity at the level of expression of mRNA for molecules involved in target cell lysis. The correlation between the down regulation of granzymes and the inhibition of NK activity lends support to the hypothesis that NK cells induce apoptosis in target cells through the secretion of granule contents. It has recently been reported (173) that 'non-activated' NK cells lyse target cells through necrosis while IL-2 stimulated NK cells operate through apoptosis. Our study does not address this in a direct way. However, Lui et al. (80) have reported that unstimulated NK cells induce DNA fragmentation in target cells. The difference between these studies appears to be the population employed. Knight et al. (173) used human peripheral blood lymphocytes separated by a commercial lymphocyte separating medium, whereas, Lui et al. (80) employed spleen cells passed through a nylon wool column and further enriched by complement-mediated elimination of specific sub-populations. Thus, the unstimulated population of Knight et al. (173) was more likely to contain non-lymphocytes capable of phagocytozing the apoptotic bodies and obscuring the results.

FIGURE 22. Model of the Mechanism of Apoptosis.

The mechanism of action of these granule proteins is not completely known. However, it is plausible that the granule component, perforin, is exocytosed and polymerizes on the surface of the target cell. The polymerized perforin then would provide channels of access for the other granule components. Once inside the target cell the granzymes would be shuttled to the nucleus and there activate the apoptotic program, resulting in DNA fragmentation. The mechanism by which this occurs is still unknown. However, the granzymes do not contain nuclease activity. Thus, they may activate endogenous endonuclease(s), initiate a cascade resulting in the activation of endonuclease(s), and/or cleave chromosome-associated proteins exposing the DNA to enzymes that are already present in the nucleus.


Nucleus
Target Cell
NK Cell
NK Cell
Target
Cell
NK Cell
Nucleus
DNA
Fragmentation
Target Cell
CP
Gr A
NK Cell
Target
Cell
NK Cell
KEY TO SYMBOLS
GrA
Granzyme A
CP
Carrier Protein
GrB
Granzyme B
Polymerized
Perforin
Perforin

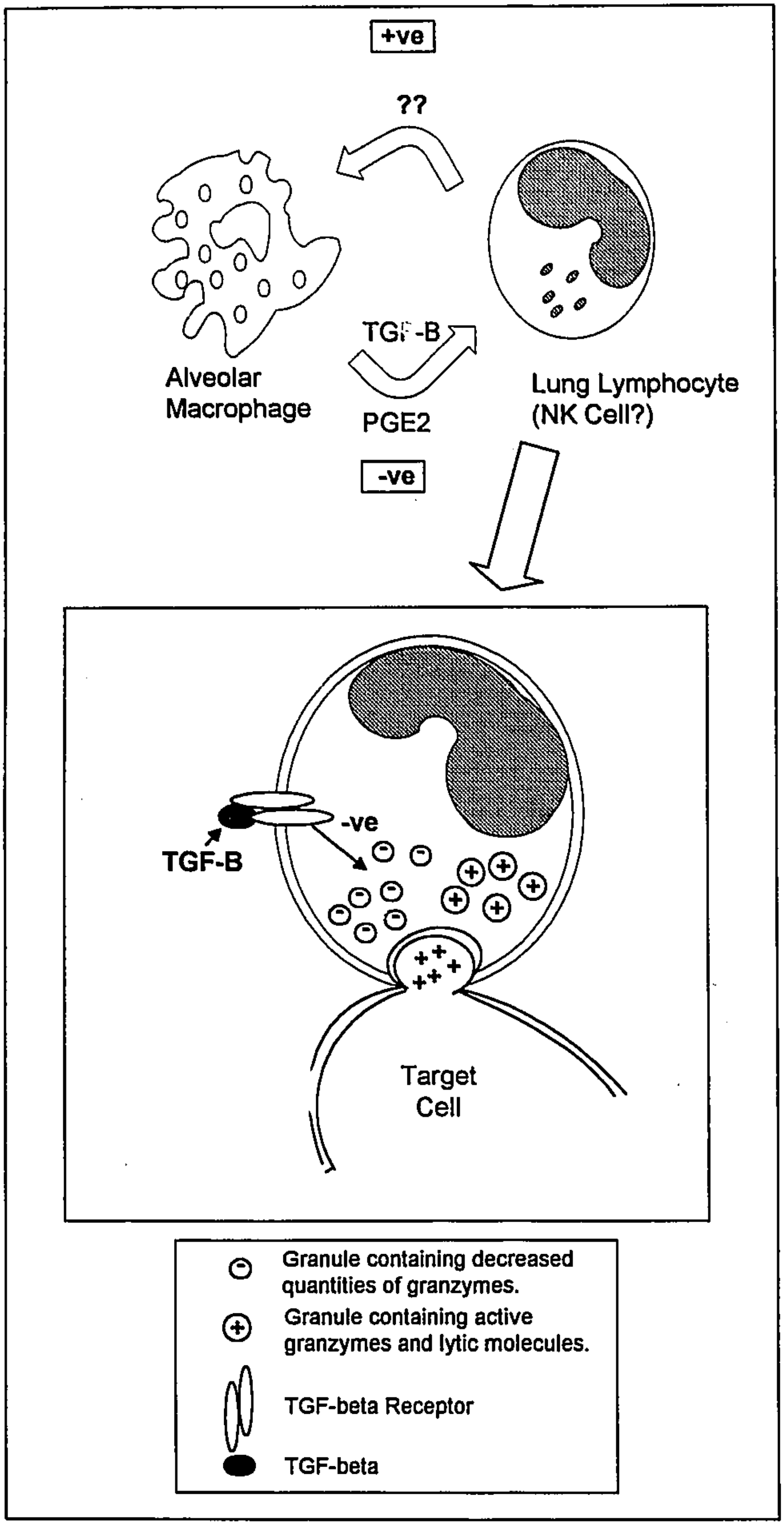
In the model of apoptosis, Figure 22, the entry of granzymes into the target cells is most likely accomplished by the polymerization of perforin on the target cell surface and the resultant production of membrane pores (174). Thus, inside the target cell the granzyme must be transported to the site of action. The site of action is generally assumed to be the nucleus of the target cells where DNA degradation takes place. It has been demonstrated that granzyme A can bind to the nuclear-cytoplasmic shuttle protein nucleolin (175). Nucleolin and similar target cell proteins, likely provide access to the nucleus.

Once in the target cell nucleus, the ultimate function of the granzyme remains unknown. Granzymes do not possess nucleolytic activity of their own, thus target cell molecules may again play a role. It has been postulated that the granzymes activate the endogenous 'suicide program' of the target cell probably through cleavage of proto-nucleolytic enzymes. However, this hypothesis does not take into account the diversity of granzymes and their substrates. It is possible that the granzymes cleave DNA-associated proteins, exposing the strands to endogenous DNAses (174). In either case the target cell undergoes the morphological changes associated with apoptosis: chromatin condensation, cell membrane blebbing, DNA fragmentation, formation of apoptotic bodies and lysis (75, 176).

Many of the questions related to the interaction of lung

FIGURE 23. Model of the Interaction between Lung NK Cells and Alveolar Macrophage.

Co-culture of alveolar macrophages with lung NK cells results in the production and secretion of soluble factors, including PGE_2 and TGF-β by the AM. The secretion is influenced by the presence of the NK cell by some as yet unknown mechanism. TGF-β inhibits lung NK activity by suppressing the production of granule proteins thought to be involved in the lytic cycle, such as granzyme A and B.


+ve
??
Alveolar Macrophage
TGF-B
PGE2
Lung Lymphocyte (NK Cell?)
-ve
-ve
TGF-B
Target Cell
Granule containing decreased quantities of granzymes.
Granule containing active granzymes and lytic molecules.
TGF-beta Receptor
TGF-beta

NK cells and alveolar macrophages have been addressed in this study. A model for the mechanism of inhibition of lung NK activity, illustrated in Figure 23, has been proposed from these data.

The inhibition of lung NK cell activity by AM requires a functional activation of the macrophage by the lymphocytes, likely the NK cells themselves, in an undefined manner that involves at least one soluble factor. The AM responds to the lymphocyte signal by secreting increased amounts of PGE_2 and TGF-β. Each of these factors is an effective inhibitor of NK activity at a post-conjugation step in the lytic cycle. Inhibition by PGE_2 was found to be independent of the production of granzymes and presumably other granule proteins. The rapidity of action of PGE_2 supports this hypothesis. The most reasonable site of action, though not addressed in this study, is at the point of secretion of lytic factors, which has been proposed elsewhere (155).

The kinetics of inhibition of lung NK activity indicate that TGF-β is responsible for a substantial portion of the inhibitory effect. TGF-β was shown to operate at the level of production of lytic granule proteins. TGF-β appears to suppress the expression or, in some way, limit the availability of specific messenger RNA for granzymes and perhaps others. This, in turn, translates into a functional reduction in granzyme activity.

This mechanism of action presumes a model for NK activity dependent upon the delivery of granule serine proteases into the target cell and a lytic role for the granzymes. Conversely, the correlation between inhibition of granzyme production and NK activity lends support to such a mechanism of lysis.

SUMMARY and CONCLUSIONS

It has been shown that rat lung lymphocytes possess significant levels of NK activity which differs from the NK activity found in the spleen and peripheral blood in terms of basal activity and response to biological response modifiers. Lung lymphocytes were found to be responsive to stimulation by IL-2, IFN-α/β, and TNF-α but not IL-1 or IFN-γ. Lung NK activity was shown to be suppressed by alveolar macrophages by a mechanism involving the induction of prostaglandin E_2 and TGF-β production. The inhibition was found to be reversible and to take place at a post-binding step in the lytic cycle. When the lung lymphocyte population was enriched for NK cells, it was found that TGF-β and AM but not PGE_2, inhibited the BLT-esterase, granzyme A, activity of these cells. Furthermore, the expression of granzyme B mRNA was suppressed by TGF-β and AM treatment of the NK cell enriched lung lymphocyte population. Taken together, our results indicate that alveolar macrophages suppress lung NK activity, at least in part, through the production of TGF-β which inhibits the production of factors required for lysis, eg. granzyme A and B.

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