6-1992

Characterization of Murine Interleukin-4 (IL-4) : Biological Activities and Initial Identification of Signal Transduction Mechanisms in IL-4-Mediated Macrophage Activation

Henkie Pierre Tan

ABSTRACT

CHARACTERIZATION OF MURINE INTERLEUKIN-4 (IL-4) BIOLOGICAL ACTIVITIES AND INITIAL IDENTIFICATION OF SIGNAL TRANSDUCTION MECHANISMS IN IL-4-MEDIATED MACROPHAGE ACTIVATION

by

Henkie Pierre Tan

IL-4, in the presence of macrophages (MΦs), has previously been demonstrated to be tumoricidal *in vitro* and *in vivo*, possibly through MΦ activation. In the current study, the biological activities of murine IL-4 on MΦs, and the initial identification of signal transduction mechanisms in IL-4-mediated MΦ activation were determined. Hamster MΦs were activated with IL-4 to study MΦ tumoricidal activity against hamster pancreatic adenocarcinoma *in vivo*. Through this initial study, it was observed that murine IL-4 is species specific and incapable of crossing the narrow phylogenetic barrier to hamster and perhaps rat (partially). The effects of murine IL-4 on allogeneic peritoneal and bone marrow-derived (14M1.4 cell line) MΦs were then evaluated. Results showed a marked, dose-dependent stimulation of chemiluminescent oxidative burst by IL-4-activated MΦs, an activity which was IL-4 monoclonal antibody reversible. In addition, it appeared that 14M1.4 MΦs were more responsive than peritoneal MΦs to IL-4 in that they responded quicker and released greater quantities of reactive oxygen intermediates. Based on this preliminary work, the 14M1.4 MΦs were used to study the yet unknown signal

transduction mechanisms in IL-4-mediated MΦ activation. By means of flow cytometric techniques and the membrane potential sensitive dye, $DIOC_6(3)$, it was shown that IL-4 caused no apparent biological but a statistically significant change in the membrane potential of MΦs. Using flow cytometry and indo-1AM, it was determined that intracellular calcium levels changed. An optimal concentration of IL-4 caused a rapid increase in the initial (inhibited by BAPTA-AM, a specific intracellular chelator of calcium) and then sustained (inhibited by EGTA) level of intracellular calcium. Through assessments of chemiluminescence oxidative burst activity, it was demonstrated that the initial and sustained increase in intracellular calcium was required and important for IL-4 enhancement of the oxidative burst by 14M1.4 MΦs. Increase in production of IP_3 but no change in levels of cAMP, immediate or delayed, were demonstrated using competitive binding assays. These findings were further supported by the fact that cholera toxin and prostaglandin E_2 (agents which increase cAMP), did not enhance chemiluminescence, nor did PMA, a potent activator of PKC. Surprisingly, IL-4-stimulated MΦs were not cytolytic to tumor cell targets; yet, IL-4 was directly cytostatic to some of the same cell lines.

LOMA LINDA UNIVERSITY
Graduate School

CHARACTERIZATION OF MURINE INTERLEUKIN-4 (IL-4) BIOLOGICAL ACTIVITIES AND INITIAL IDENTIFICATION OF SIGNAL TRANSDUCTION MECHANISMS IN IL-4-MEDIATED MACROPHAGE ACTIVATION

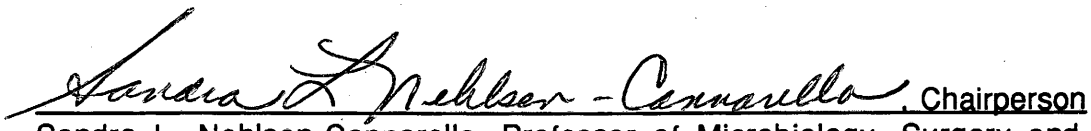
by

Henkie P. Tan

A Dissertation in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Microbiology

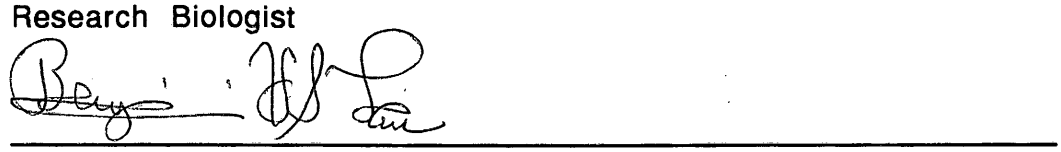
June 1992

Each person whose signature appears below certifies that this dissertation, in his/her opinion, is adequate in scope and quality, as a dissertation for the degree Doctor of Philosophy.

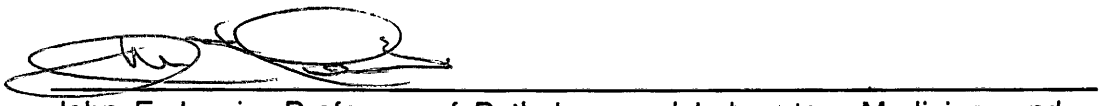


Sandra L. Nehlsen-Cannarella, Professor of Microbiology, Surgery and Pathology

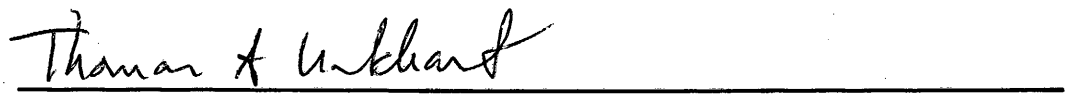
Christopher D. Cain, Assistant Research Professor of Surgery, and Research Biologist



Benjamin H. S. Lau, Professor of Microbiology and Surgery



John E. Lewis, Professor of Pathology and Laboratory Medicine, and Microbiology



Thomas A. Linkhart, Associate Professor of Biochemistry, Research Associate Professor of Pediatrics

ACKNOWLEDGEMENTS

I wish to extend my sincere appreciation to the following persons who contributed so much to my scientific nurturing and successful completion of the doctoral degree. In particular, I wish to thank Dr. Sandra L. Nehlsen-Cannarella, my mentor; Drs. Christopher D. Cain, Benjamin H. S. Lau, John E. Lewis and Thomas A. Linkhart, members of my dissertation committee; Drs. Carlos A. Garberoglio, Lauralynn K. Lebeck and Jeffrey M. Tosk, assistance in clinical design; and Doug Chritton and Judy Folz for helping with flow cytometry measurements.

I would like to acknowledge gifts from Dr. Steven Gillis (Immunex Corporation, Seattle, WA) and Sterling Winthrop Pharmaceuticals (Malvern, PA) for murine recombinant interleukin-4; Dr. C. Reynolds (National Cancer Institute-Frederick Cancer Research and Development Center, courtesy of Hazleton Laboratories, Vienna, VA) for anti-interleukin-4 monoclonal antibody; Dr. James Kettering (LLU Department of Microbiology) for the H238 murine fibroblast cell line; Drs. Benjamin H. S. Lau and Jeffrey M. Tosk (LLU Department of Microbiology) for the J774A.1 macrophage cell line; Dr. Barbara Chang (Medical College of Georgia) for the PDPaCa pancreatic adenocarcinoma cell lines; and Dr. Albert Zlotnik (DNAX, Palo Alto) for the 14M1.4 bone marrow-derived macrophage and L Cell fibroblast cell lines.

Finally, I thank Mom and Dad for their understanding, patience and love; and Dr. Robert Boucek for guiding me towards the path of research.

This work was supported in part by grants from the Immunobiology of Cancer Fund (grant no. 0313-4162) and Department of Surgery (LLUSMGI-60), Loma Linda University School of Medicine to Dr. Nehlsen-Cannarella.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 6

CHAPTER 7

Page

Page

CHAPTER 9

CHAPTER 10

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BAPTA-AM	(1,2-bis-[2-aminophenoxy]-ethane-N,N,N',N'-tetraacetic acid), acetoxymethyl ester
BMMΦ(s)	Bone marrow-derived macrophage(s)
$[Ca^{2+}]_i$	Intracellular free calcium concentration
CM	Complete medium
CT	Cholera toxin
DAG	1,2-diacylglycerol
$DiOC_6(3)$	3,3'-dihexyloxa-carbocyanine
GM-CSF	Granulocyte macrophage colony-stimulating factor
indo-1AM	([1-[2-amino-5-[carboxylindol-2-yl]-phenoxy]-2-2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid), acetoxymethyl ester
IP_3	D-myo-inositol-1,4,5-trisphosphate
LAK	Lymphokine-activated killer
min	minute
MΦ(s)	Macrophage(s)
MCSF	Macrophage colony-stimulating factor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide-adenine dinucleotide phosphate, reduced
PKC	Protein kinase C
PL(A_2, C, D)	Phospholipase (A_2, C, D)
PMN	Polymorphonuclear (leukocyte)

CHAPTER 1

INTRODUCTION

1.1. Structure and biochemical characteristics of IL-4 and IL-4 receptor

1.1.1. IL-4

IL-4, a cytokine, was originally described as a murine B cell growth factor (Howard et al., 1982) in the laboratory of William E. Paul at the National Institute of Allergy and Infectious Disease. The structure and biochemical characteristics of IL-4 have been reviewed (Ohara, 1989; Moller, 1989; Spits, 1992). Briefly, murine IL-4 complementary (c)DNA was first isolated from a mouse Th_2 cell library as a cDNA coding for a unique mouse interleukin that expresses B cell-, T-cell-, and mast cell-stimulating activities (Lee et al., 1986; Noma et al., 1986; Mosmann et al., 1986a, b). Subsequently, a human IL-4 cDNA was isolated by cross-hybridization from an activated human T cell clone cDNA library (Yokota et al., 1986). Both murine and human IL-4 genes occur as a single copy per haploid genome and are mapped on chromosomes 11 and 5, respectively (Otsuka et al., 1987; Arai et al., 1989). The IL-4 genes reside as part of a cluster of lymphokine genes that includes IL-3, granulocyte macrophage colony-stimulating factor (GM-CSF), and IL-5 (LeBeau et al., 1989; VanLeeuwen et al., 1989). This region is known to be deleted (del[5q]) frequently in patients with myeloid disorders. Like other lymphokine genes, such as IL-2, GM-CSF, interferon (IFN)-γ, and IL-5 genes, the IL-4 genes are composed of four exons and three introns (Otsuka et al., 1987; Arai et al., 1989). The mature polypeptides of murine and human IL-4 are

120 and 129 amino acid residues long, respectively, and both begin with a histidine residue. The predicted molecular weight of the nonglycosylated murine and human IL-4 proteins are 13,558 and 14,963, respectively (Trotta, 1992). The murine and human IL-4 proteins share about 50% homology. Amino acid positions 91 to 128 of human IL-4, share very little homology with the corresponding region of murine IL-4 at either amino acid or nucleotide sequence levels (Yokota et al., 1988; Arai and Arai, 1992). There are no predicted sites for phosphorylation in either murine or human IL-4, based on the sequence Arg-Arg-Ala-Ser-Leu, the recognition sequence for cyclic adenosine monophosphate (cAMP)-dependent protein kinase (Trotta, 1992). Murine and human IL-4 have alkaline isoelectric points of 9.7 and 10.5, respectively.

The secondary structure of murine and human IL-4 has been predicted to be significantly α-helical (64%) (Trotta, 1992). Human recombinant (r)IL-4 appears to have a highly stable tertiary structure which has been demonstrated to be stable to extreme pH (Windsor et al., 1991). In addition, human rIL-4 has recently been crystallized (Cook et al., 1991). Elucidation of the tertiary structure will constitute the first major step toward identifying the receptor-binding domain, and will provide the basis for the design of second-generation agonists and antagonists of human IL-4 with therapeutic potential.

1.1.2. IL-4 receptor

Interest in the IL-4 receptor (R) is reflected by the intense rate of publications, including Ohara, 1989; Banchereau and Galizzi, 1990; Maliszewski and Fanslow, 1990; Maliszewski et al., 1990; Jacobs et al., 1991; Garrone et al., 1991; Fernandez-Botran, 1991; Fanslow et al., 1991;

Miyajima et al., 1992; Harada et al., 1992; and Tan et al., 1992a. Only a single class of the high affinity IL-4R has been isolated. It has a K_d of 20-80 pM and is expressed on a wide variety of cells including nonhemopoietic cells such as fibroblasts, neuroblasts, keratinocytes, hepatocytes, and stromal cells (Lowenthal et al., 1988). The IL-4R on resting B cells is upregulated by lipopolysaccharide (LPS), anti-IgM, or IL-4 itself, and the IL-4R on resting T cells is likewise increased by stimulation with concanavalin (con) A or IL-4 (Lowenthal et al., 1988; Ohara and Paul, 1987). Chemical cross-linking revealed multiple cross-linked proteins of 140-, and 50- to 75-kDa (Ohara and Paul, 1987; Park et al., 1987). The smaller proteins appear to be degradation products of the 140-kDa protein. Gibbon IL-4R was purified by human IL-4 affinity chromatography. Purified gibbon IL-4R of 130-kDa bound IL-4 with a K_d of 35 pM (Galizzi et al., 1990a), indicating that a single molecular species binds IL-4 with high affinity. IL-4R cDNAs cloned from T cells, mast cells, and myeloid cells encode a protein of 140-kDa which binds IL-4 with high affinity when expressed in COS cells (monkey kidney cell line) (Mosley et al., 1989; Galizzi et al., 1990b; Harada et al., 1990; Idzerda et al., 1990). The mouse and human IL-4R, like the lymphokine, are about 50% identical at the amino acid level, and contain the common motif of cytokine receptors (Idzerda et al., 1990; Galizzi et al., 1990b; Harada et al., 1992). A mouse cDNA encoding a soluble extracellular domain of the IL-4R has been isolated, and shown to be capable of binding IL-4 with high affinity (Mosley et al., 1989). There is no consensus sequence for any known enzymes in the large cytoplasmic domains of 553 (mouse) and 569 (human) amino acid residues, but there are many prolines and serines in the cytoplasmic domains which could potentially be sites of phosphorylation. The human IL-4R molecules expressed in CTLL, a mouse T cell line, and

BaF3, a proB cell line, are capable of transmitting growth signals in response to human IL-4 (Idzerda et al., 1990; Harada et al., 1992).

1.2. Known biological activities of IL-4

The biological activities of IL-4 have been reviewed (Paul, 1987; Paul and Ohara, 1987; Moller, 1989; Spits, 1992). IL-4 was originally described as a murine B cell growth factor (Howard et al., 1982) and has subsequently been shown to have pleiotropic effects on multiple hematological and nonhematological cellular lineages (Monroe et al., 1988; Moller, 1989; Ohara, 1989; Banchereau et al., 1991; Tan et al., 1990, 1991a-d, 1992a, b). The host of functions ascribed to murine IL-4, on the basis of *in vitro* studies, includes stimulation (in conjunction with antigen or anti-IgM antibody) of B cell proliferation (Howard et al., 1982), T cell proliferation (Mosmann et al., 1986b), mast cell proliferation in conjunction with IL-3 (Mosmann et al., 1986b), stimulation of hematopoietic precursors of multiple lineages (Peschel et al., 1987; Rennick et al., 1989a, b), fibroblast proliferation (Monroe et al., 1988), macrophage (MΦ) activation (Crawford et al., 1987; Tan et al., 1990, 1991a, d, 1992b), and polymorphonuclear leukocyte (PMN) activation (Boey et al., 1989). IL-4 has also been shown to heighten the expression of class I (Stuart et al., 1988) and class II MHC antigens on MΦs (Crawford et al., 1987; Stuart et al., 1988), and class II MHC antigens on B cells (Noelle et al., 1984; Roehm et al., 1984), and to promote class switching for the production of IgE and IgG1 (Snapper and Paul, 1987; Lutzker et al., 1988; Tan et al., 1992a). Hart et al. (1989, 1991) and Gautam et al. (1992) have suggested that IL-4 may be a powerful, previously unrecognized, anti-inflammatory agent.

Of greatest potential relevance to the use of IL-4 in clinical trials are the findings related to activation of T cells, LAK cells, and MΦs. In this regard, IL-4 is a T cell growth factor stimulating the proliferation of antigen- and mitogen-stimulated T cells of both T helper and cytotoxic function, often working synergistically with IL-2 (Widmer et al., 1987). It appears to enhance the proliferation of resting thymocytes and peripheral blood T cells, maintains their viability, and induces the proliferation of activated T cells including lines and clones derived from tumor infiltrating lymphocytes (Kawakami et al., 1988; Spits et al., 1988). It also reduces the nonspecific cytolytic activity induced by IL-2 from peripheral blood lymphocytes and tumor (Kawakami et al., 1988, 1989). The ability of IL-4 to induce proliferation of activated T cells and to synergize with IL-2 in T cell growth is of potential importance in its clinical application.

1.3. Potential anti-tumor properties of IL-4

Interest in the potential anti-tumor properties of interleukin (IL)-4 stemmed from *in vitro* studies that had demonstrated the ability of murine IL-4 to activate MΦs (Crawford et al., 1987), lymphokine-activated killer (LAK) cells (Mule et al., 1987) and cytolytic lymphocytes (Widmer and Grabstein, 1987) to kill tumor cells. Very recently, it was shown that IL-4 inhibits *in vitro* cell growth of human melanomas and carcinomas of lung, gastric, breast or renal cell origins (Hoon et al., 1991, 1992). Additionally, the tumoricidal activity of IL-4 has been confirmed *in vivo* (Tepper et al., 1989; Bosco et al., 1990; Golumbek et al., 1991; Redmond et al., 1992). Tepper et al. (1989), while working in the laboratory of Philip

Leder[1], developed transfected murine IL-4 gene cell lines that spontaneously produce IL-4; when these cell lines were injected with transplantable tumors, the production of IL-4 by the IL-4 transfected cell lines correlated with the growth inhibition of tumor in Balb/c, athymic nu/nu (deficient T cell), and beige (bg/bg, deficient natural killer (NK) and cytotoxic T cell) mice. These investigators, in order to confirm that the production of IL-4 was indeed directly involved in tumor growth inhibition, injected anti-IL-4 monoclonal antibody (mAb) into tumor-burdened mice and found a reversal of tumor growth inhibition. However, more convincing data could have been presented had these authors measured the serum levels of IL-4 and correlated them to tumor growth inhibition. Tepper et al. (1989) also provided data indicating that IL-4 could cause the regression of established tumors. In addition, they found many MΦs and eosinophils at the site of tumor inhibition suggesting that the tumoricidal activity of IL-4 may be associated with the activation and effector function of these MΦs and eosinophils. Finally, recent clinical trials using IL-4 and IL-2 combination therapy in patients with melanoma, breast or renal cancer, have shown partial and complete tumor regressions (Wong et al., 1992; Lotze, 1992a, b).

1.4. Possible mechanisms of IL-4-mediated tumoricidal activities

The current speculated mechanisms of IL-4-mediated tumoricidal activities are numerable. Given the results of *in vitro* studies, the (a) generation of cytotoxic T lymphocytes or (b) activation of LAK and (c) NK cells by IL-4, could be considered possible mechanisms *in vivo*. However,

---

[1] This data was presented in the American Association of Immunologists plenary lecture at the Federation of American Society of Experimental Biology meeting, 1991.

Tepper et al. (1989) have shown that the anti-tumor effect of IL-4 *in vivo* for several tumor types is operational in the athymic (nu/nu) host, suggesting that a primary role for cytotoxic T lymphocytes is unlikely.

Peace et al. (1988) found that murine rIL-4 could generate both T cell-like and NK-like LAK cells. However, a 500-fold (2 ng/ml) higher concentration of IL-4 was required compared to that needed to induce class II MHC antigen expression of B cells. On the contrary, several investigators have found that human rIL-4 not only suppressed IL-2-induced LAK activity but strongly inhibited the induction and function of LAK in tumor cell killing (Widmer et al., 1987; Spits et al., 1988; Gallagher et al., 1988; Gerosa et al., 1988; Keever et al., 1989), thus pointing to the existence of variability among the immune cells of different species in response to the species specific IL-4.

While a mechanism involving NK cells remains a theoretical possibility, the absence of lymphoid cells in the inflammatory lesions at the tumor rejection site in Tepper's (1989) studies argues against such a hypothesis. In addition, his preliminary studies indicate that the anti-tumor effect of murine IL-4 is still operational in the beige (bg/bg) mouse, which is deficient in NK activity (Roder and Duwe, 1979) and cytotoxic T cell functions (Saxena et al., 1982). These data are further supported by findings which indicate that human IL-4 inhibits the augmentation of NK cell activity by IL-2 (Spits et al., 1988; Keever et al., 1989), and the generation of NK cells (Migliorati et al., 1989).

Finally, the appearance of a striking number of eosinophils, as well as activated MΦs, at the tumor site *in vivo* suggests that mechanisms independent of the lymphoid system may be operative. The presence of activated MΦs at the site of IL-4 production raises the possibility that tumoricidal products are derived directly or indirectly from the MΦ.

Among the mediators released by MΦs are the reactive oxygen intermediates (O_2^-, H_2O_2, $HO^\bullet$, and $^1O_2$), which have been correlated with enhanced ability of MΦs to kill bacteria (Gabay, 1988), parasites (Wirth et al., 1989), and tumor cells (Wiltrout and Varesio, 1990). Other tumoricidal secretory products include IL-1 (Onozaki et al., 1985a, b; Lovett et al., 1986), tumor necrosis factor (TNF)-α (Urban et al., 1986), and macrophage colony-stimulating factor (MCSF) which, in turn, could activate MΦ to produce tumoricidal products such as interferon-α, TNF-α, and GM-CSF (Warren and Ralph, 1986; Grabstein et al., 1986a; Ralph and Nakoinz, 1987; Wieser et al., 1989). Although it has been claimed that murine IL-4 enhances the production of IL-1 (Wolpe et al., 1987) and TNF (Somers and Erickson, 1988), others (Hurme et al., 1988; Hart et al., 1989, 1991; Essner et al., 1989) have reported that species-specific human IL-4 inhibits IL-1 and TNF synthesis in monocytes and macrophages. While Crawford et al. (1987) have shown that IL-4 increases the Fc receptor expression of bone marrow-derived MΦs, Ralph et al. (1988) found that rIL-4 did not stimulate antibody-dependent cellular cytotoxicity by peritoneal MΦs. However, Fan et al. (1991) were able to strongly stimulate both rapid and slow antibody-dependent cellular cytotoxicity. These differences may be attributable to differences in target susceptibility. The tumoricidal activity of IL-4-activated MΦs through a non-specific mechanism remains a possibility (Alexander, 1973; Kurland et al., 1979).

The role of eosinophils in the IL-4-mediated anti-tumor effect also remains to be defined. Eosinophils are known to possess antibody-dependent and -independent phagocytic properties and play an important role in controlling certain parasitic infections (Weller and Goetzl, 1979), and their possible role as anti-tumor effectors has been suggested (Jong and Klebanoff, 1980). The presence of eosinophils in histologic sections of

human gastric and colonic malignancies has been associated with an improved prognosis (Iwasaki et al., 1986, Pretlow et al., 1983). In their review, Gleich and Adolphson (1986) have stated that eosinophils produce tumoricidal proteins and enzymes which include major basic proteins (Butterworth et al., 1979) and endoperoxidase enzymes. Nathan and Klebanoff (1982) have provided evidence that endoperoxidase enzymes act synergistically with H_2O_2, generated by MΦs, in destroying tumor cells. It is currently unknown whether the promotion of eosinophil chemotaxis *in vivo* is mediated directly by IL-4 or involves the secondary expression of other factors known to stimulate eosinophils, such as the eosinophil chemotactic factor A released by activated mast cells (Gleich and Adolphson, 1986), platelet aggregating factor (Braquet and Rola-Pleszczynski 1987), and eosinophil differentiation factor or IL-5 (Sanderson et al., 1986). While IL-4 in conjunction with IL-3 is known to stimulate mast cells *in vitro* (Arai et al., 1990), no increase in tissue mast cells was observed in Giemsa-stained tissue sections studied after administration of IL-4 *in vivo* (Tepper et al., 1989).

1.5. Significance of macrophage activation

The importance of the MΦ in the immune system can not be over emphasized, as indicated by a recent review entitled "Lymphocytes Play the Music but the MΦ Calls the Tune" (Solbach et. al., 1991). Animals will not survive in the absence of an intact mononuclear phagocytic system while they will if their lymphoid system is not completely functional (Esgro et al., 1990). The MΦ plays many roles including an active role in the immunosurveillance of cancer. The tumor cell killing by MΦs, which avoids damaging normal tissue, is apparently not associated with the development of tumor-specific resistance. The tumoricidal activity

appears to be independent of target cell characteristics such as immunogenicity, metastatic potential, and sensitivity to drugs (Esgro et al., 1990, Whitworth et al., 1990, Pak and Fidler, 1991). However, the destruction of tumor cells is, in part, due to the MΦ secretory repertoire (Adams et al., 1981; Nathan, 1982; Johnson et al., 1986; Lefkowitz et al., 1986; Hamilton and Adams, 1987; Adams and Hamilton, 1988a). Among the important mediators released by MΦs are the reactive oxygen intermediates, which have been correlated with an enhanced ability to kill bacteria (Gabay, 1988), parasites (Wirth et al., 1989), and tumor cells (Wiltrout and Varesio, 1990). The release of these highly reactive oxygen metabolites is enhanced by the MΦ activation factor IL-4 (Phillips et al., 1990; Redmond et al., 1990; Tan et al., 1990, 1991a, d). The importance of the reactive oxygen intermediates is further demonstrated in patients with chronic granulomatous disease where patients with this disease suffer various chronic inflammatory conditions and infections due to opportunistic and pathogenic microorganisms because their phagocytes fail to produce reactive oxygen intermediates (Curnette, 1992).

There are four well-characterized means of assessing MΦ activation that do not involve cellular interactions between MΦs and live target cells (Stein et al., 1991): 1) respiratory burst activity with chemiluminescence[2]; 2) MHC class II antigen expression; 3) mannosyl fucosyl receptor expression; and 4) MΦ cytokine release after activation with MΦ activation factors. The choice of assay depends on the need to discriminate between 1) resident and elicited, 2) resident and immunologically active, or 3) elicited and activated MΦs.

---

[2] Analysis of the individual reactive oxygen intermediates can also be tested. For example, superoxide anion can be measured by the reduction of cytochrome C and hydrogen peroxide by the dichlorofluorescin diacetate assay using flow cytometry (Robinson, 1990).

1.6. Macrophage activation by respiratory oxidative burst

When MΦs and other professional phagocytic cells (monocytes, PMNs and eosinophils) are appropriately stimulated by opsonized microorganisms, antigen-antibody complexes, chemotactic factors or other stimuli, a reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase-dependent respiratory oxidative burst is activated resulting in the release of reactive oxygen intermediates (Abo et al., 1991; Cross, 1992). These intermediates include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\cdot$). At the outer surface of the plasma membrane, O_2^- is released through the univalent reduction of molecular oxygen, H_2O_2 by spontaneous dismutation of O_2^-, and after a further univalent reduction of oxygen in H_2O_2, $OH^\cdot$ is generated (Ellis, 1990). Unstimulated MΦs have no detectable activity of the NADPH oxidase (Sasada et al., 1983; McPhail and Snyderman, 1983).

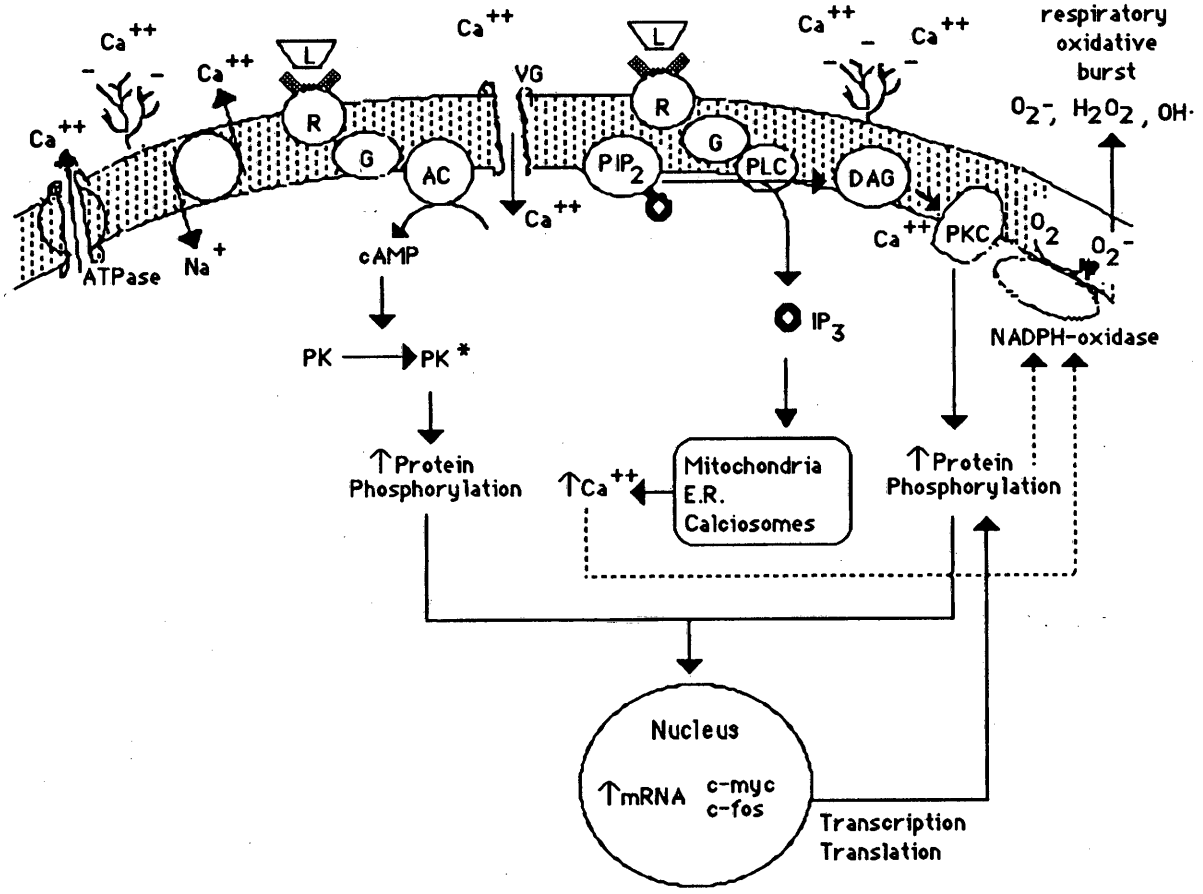
The NADPH oxidase (extensively reviewed by Babior, 1987, 1988; Segal, 1989; Gallin et al., 1991, Jesaitis and Dratz, 1992) constitutes a transmembrane electron transport chain containing a flavoprotein and a cytochrome b_{558}, with cytosolic NADPH as the donor and oxygen as the acceptor of a single electron. This cytochrome has been purified and was found to consist of two subunits, one 60- to 66-kDa and the other 22-kDa subunits (Pick et al., 1990). Recent experiments have shown that two cytosolic components of 47-kDa ($p47^{phox}$) and 67-kDa ($p67^{phox}$) form a complex with the cytochrome b_{558}. In guinea pig MΦs, it has been demonstrated that the two cytosolic components interact with yet a third cytosolic heterodimer, a small guanosine triphosphate (GTP)-binding protein $p21^{rac1}$ and the guanosine diphosphate (GDP)-dissociation inhibitor, rhoGDI (Abo et al., 1991).

The level of MΦ luminol-dependent chemiluminescence has been demonstrated to be proportional to the rate of reactive oxygen intermediates formation and thus directly reflects the activity of the NADPH oxidase (Allen, 1986; Wymann et al., 1987; Tosk et al., 1989).

1.7. Signal transduction in macrophage activation by respiratory oxidative burst

The identification of extracellular signals modulating cell behavior is central to cell biology. Progress in this area may ultimately identify pharmacologic agents capable of selectively modulating the function of various cells. MΦs are of particular interest as they have been shown to play a significant role in host defense against microbes and tumors, homeostasis, and disease such as carcinogenesis and atherogenesis. The function of MΦs in all these roles is stringently regulated by extracellular signals (Adams and Hamilton, 1987, 1988a; Adams, 1989). Such signals can stimulate the immediate execution of various complex functions which include destruction of tumor cells by the release of reactive oxygen intermediates (Adams and Hamilton, 1984, 1988a, b; Hamilton and Adams, 1987; Baggiolini and Wymann, 1990) or damage of normal cells and tissues in inflammatory diseases (McCord, 1992). Other extracellular signals can alter the potential of MΦs so that competence to respond to extracellular signals of the first type is markedly enhanced or diminished.

Physical and chemical changes in receptors, in the membrane itself, and in other cellular structures occur following ligand interactions with cell surface receptors (Fig. 1, Shapiro 1988; Jesaitis and Dratz, 1992). The earliest detectable biochemical changes occur in seconds after the


Ca++
Ca++
Ca++
Ca++
Ca++
Ca++
Ca++
L
L
R
R
G
G
AC
VG
PIP2
PLC
DAG
PKC
ATPase
Na+
cAMP
IP3
respiratory
oxidative
burst
O2-, H2O2, OH·
O2
O2-
NADPH-oxidase
PK → PK*
↑Protein
Phosphorylation
↑Ca++
Mitochondria
E.R.
Calciosomes
↑Protein
Phosphorylation
Nucleus
↑mRNA c-myc
c-fos
Transcription
Translation

increase in number of occupied receptor sites following exposure of cells to ligand. These include alterations in transmembrane Ca^{2+} and other ion fluxes resulting in a change in pH and membrane potential (Shapiro, 1981; Adams and Hamilton, 1987; Riches et al., 1988; Forman and Kim, 1989). The major signal transduction pathways utilized by receptors on eukaryotic cells are those that regulate activity of adenylate cyclase, polyphosphoinositide-phosphodiesterase, or tyrosine kinase (Rigley and Hicks, 1991). Emerging evidence (Klaus et al., 1987) indicates that these second-messenger systems feedback on each other in both a negative and positive way, suggesting that the summation of signals will drive an appropriate response. Thus, it appears that the underlying complexities controlling cell growth and activation lie not in the nature of the signal generated but the manner in which these signals impinge on each other. These difficulties are further compounded by the fact that, for IL-4-mediated MΦ activation, the types and modes of action of second and/or third messengers are currently unknown.

We do know, however, a little about the signal transduction pathways in phagocyte activation leading to the release of reactive oxygen intermediates by chemoattractants. The signal transductional events of leukocyte activation by reactive oxygen intermediates production has been reviewed extensively (Snyderman et al., 1986; Sadler and Badwey, 1988; Baggiolini and Wymann, 1990; Jesaitis and Dratz, 1992). Within seconds to minutes, there may be changes in cyclic nucleotide concentrations, increased protein phosphorylation, and alterations of membrane uptake of sugars, amino acids, and fatty acids. In polymorphonuclear phagocytes, it is known that receptor activation by chemoattractants induces conformational changes in one or more G proteins (Verghese et al., 1987; Baggiolini and Wymann, 1990; McPhail et al., 1992). The G protein then

interacts with and activates a target enzyme, usually a phospholipase (Snyderman et al., 1986; Gilman, 1987). Several phospholipases are activated by chemoattractants including phospholipases A_2, C, and D (Verghese et al., 1987; Bauldry et al., 1988; Agwu et al., 1989). It is not yet clear which phospholipases (A_2, C, D) are directly regulated by or interactive with a receptor-G protein, or whether the activation of the other phospholipases is due to signalling through second messengers generated by the first enzyme. The second messengers generated by phospholipase activation (McPhail et al., 1992) include: 1) inositol-1,4,5-trisphosphate (IP_3) (Berridge, 1987; Berridge and Irvine, 1989; Rana and Hokin, 1990) and 1,2-diacylglycerol (DAG) (Berridge, 1987; Uhing and Adams, 1989) by phospholipase C (PLC); 2) phosphatidic acid by PLD, and 3) arachidonic acid by PLA_2. Second messengers exert their signalling effects by either activating target enzymes/receptors or metabolizing to other products, some of which are inactive while others are active second messengers. IP_3 binds to receptors on intracellular calcium-storage organelles and releases calcium (Lew, 1989). Targets for calcium include protein kinase C (PKC) and calmodulin. DAG is a direct activator of PKC (Ashendel, 1985) and can also synergize with certain amphiphiles for activation of PKC, and can directly activate NADPH oxidase in a cell-free system (Bellavite et al., 1988). In addition, phosphatidic acid and DAG can be interconverted via the enzymes phosphatidic acid phosphohydrolase and DAG kinase. Arachidonic acid can also directly activate NADPH oxidase in a cell-free system (Heyneman and Vercauteren, 1984), as well as be metabolized to the lipoxygenase products leukotriene B_4 and 5-hydroxyeicosatetraenoic acid. These products may bind to their cell-surface receptors causing further phospholipase activation (Verghese et al., 1987; O'Flaherty et al., 1988).

The role of the kinases activated by second messengers in NADPH oxidase activation is still not clear. The correlative approaches and/or inhibitors used have, thus far, given contradictory results (Lambeth, 1988; Tyagi et al, 1988; McPhail et al., 1992). PKC can induce activation of NADPH oxidase in a cell-free system, but activation is much lower than that obtained with amphiphiles. Several oxidase components, including the cytosolic components $p47^{phox}$, $p21^{rac-1}$, and possibly cytochrome b_{558}, are phosphoprotein and targets for various protein kinases. However, convincing evidence that phosphorylation of any of these proteins modulates NADPH oxidase is lacking. Tyrosine kinase activity may also be important for signalling by chemoattractants. Tyrosine kinases are activated during stimulation by chemoattractants and cytokines, and inhibitors of tyrosine kinases block stimulation of the respiratory burst by these agonists (Gomez-Cambronero et al., 1989; Berkow et al., 1989). However, no oxidase components are yet known to be substrates for tyrosine kinases. Possibly, tyrosine kinases participate in an earlier transductional step in the activation, such as during phospholipase activation.

Later events, such as c-myc expression, synthesis of RNA, size increases, and expression of cell-surface activation antigens, involve more complex cell functions including activation of the nucleus and occur within hours or days (Callard, 1991). Recently, IL-4 has been demonstrated to induce the expression of c-myc and c-fos (Klemsz et al., 1989; Isfort and Ihle, 1990).

At present, it is not known whether the signal transductional events of IL-4-mediated MΦ activation by respiratory oxidative burst is similar to that of PMNs activated by chemoattractants. However, a single pattern of ionic events has been observed in most of the cell activation processes

which have been examined to date (Shapiro, 1981, 1988). Following ligand binding to cell surface receptors, there is a rapid influx of Ca^{2+}, usually accompanied by release of intracellular "membrane-bound" Ca^{2+}, and resulting in a transient rise in free cytoplasmic [Ca^{2+}], which lasts for a few minutes. There is also typically an increase in intracellular pH, resulting from Na^+/H^+ exchange or antiport, which can be inhibited by amiloride. Changes in membrane potential, when they are observed, can go either in the direction of depolarization (decrease in transmembrane potential) or hyperpolarization (increase in transmembrane potential) and appear to be due primarily to Na^+ and/or K^+ shifts across the membrane.

1.7.1. Role of membrane potential

Resting cells maintain large gradients between intracellular and extracellular concentrations of a variety of ions, including Ca^{2+}, K^+, Na^+, and Cl^- (Shapiro, 1981, 1988). The relative permeability of the membrane to K^+ ions is greater than that to other ions; the leakage of K^+ ions establishes an electron countergradient and the cytoplasm becomes electron negative with respect to the external medium. This K^+ electrochemical gradient (Gallin and McKinney, 1988) is the most significant contribution to the negative membrane potential of most mammalian cells. Maintenance of a large negative transmembrane potential has been postulated to be a control mechanism to arrest cells in an inactive stage, and changes in cell membrane potential (which occur in various cell types rapidly after binding of ligands to transmembrane receptors) have been suggested to be mediators of subsequent physiologic cellular responses. A confusing aspect of the study of lymphocyte membrane potential following antigen receptor activation has been the finding of either hyperpolarization (Tatham and Delves, 1984) or

depolarization (Cambier et al., 1986). These differential effects may be related to an inwardly directed calcium current (causing depolarization) and to activation of calcium-dependent K^+ channels (causing hyperpolarization). Heterogeneity among PMNs after activation has been reported with subpopulations which hyperpolarize and others which depolarize (Seligmann et al., 1984). Other reports indicate that there are species differences in the Ca^{2+}-sensitive K^+ channel activity; for example, that Con A-induced activity of the calcium-dependent K^+ channel hyperpolarizes mouse thymocytes but cannot do so in pig lymphocytes because the channel is already maximally activated (Felber and Brand, 1983). The role of membrane potential in IL-4-activated MΦs was determined by using a fluorescent membrane potential probe, 3,3'-dihexyloxa-carbocyanine ($DiOC_6(3)$, Shapiro et al., 1979; Lazzari et al., 1986; Jenssen et al., 1986; Dwyer and Cuchens, 1987). The resting membrane potential of adherent mouse peritoneal MΦs and J774 mouse MΦ cell line has been reported to be approximately -70mV (Sung et al., 1985).

In practice, a useful indicator should not itself perturb the membrane potential, either by its very presence or by cellular toxicity, and changes in the partitioning of the indicator should be readily detectable. $DiOC_6(3)$, first described by Hoffmann and Laris (1974), belongs to a family of cationic cyanine dyes (Appendix A), and is a lipophilic fluorescent (green) dye having a single negative charge delocalized over an extensive pi-electron system in a highly symmetric molecule. This charged lipophilic molecule serves as an indicator of membrane potential, as it partitions between the cell and surrounding medium according to the Nernst equation:

$$[C^+]_c/[C^+]_o=e^{-nFE/RT}$$

where C_c and C_o are the cytosolic and extracellular $DiOC_6(3)$ concentrations, E is the membrane potential, n (equals one) is the positive charge of the cation $DiOC_6(3)$ and F, R and T are the Faraday and gas constants, and temperature.

Once cells have been equilibrated with $DiOC_6(3)$, depolarization of the cells causes release of dye (decrease fluorescence in the cell) from the cells into the medium, while hyperpolarization makes the cells take up additional dye (increase fluorescence in the cell) from the medium (Jenssen et al., 1986). $DiOC_6(3)$ does not adequately represent the new value of membrane potential until equilibrium has again been reached; this process requires about 5 minutes (min, Shapiro, 1988).

1.7.2. Role of calcium and IP_3

Calcium plays an important role as a mediator of transduction of signals from the cell membrane; changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) are part of the regulation of diverse cellular processes (Whitfield, 1990). In PMNs, Ca^{2+} has been demonstrated to be required for the induction of respiratory burst since cells depleted of their Ca^{2+} storage pool fail to produce O_2^- or H_2O_2 in response to agonists unless extracellular calcium is supplied (Lew et al., 1984). However, it appears that influx of extracellular calcium is not essential for the induction of respiratory burst since the induction cannot be prevented by chelation of extracellular Ca^{2+}. Interestingly, the influx of extracellular calcium does contribute to the overall rise in $[Ca^{2+}]_i$ (VonTscharner et al., 1986).

Eukaryotic cells have an internal calcium ion concentration that is usually maintained at 100 nM (June and Rabinovitch, 1988), far below the extracellular environment, by regulation of calcium channels within their

plasma membranes, and of storage and release of Ca^{2+} from intracellular sites such as calciosomes, endoplasmic reticulum and mitochondria (Rasmussen, 1986; Volpe et al., 1988). The transfer of calcium across the plasma membrane is regulated by calcium channels which may be voltage gated or receptor operated, the $3Na^{+}/Ca^{2+}$ antiport, and the $Ca^{2+}/2H^{+}$-ATPase pump. Cellular calcium homeostasis is maintained by the Na^{+}/Ca^{2+} antiport (a high capacity, low affinity system) and the Ca^{2+}/H^{+}-ATPase pump (a low capacity, high affinity system); both extrude calcium in order to maintain $[Ca^{2+}]_i$ within a narrow range. Plasma membrane calcium influx is thought to be initiated by membrane depolarization which opens voltage-gated channels or by the binding of ligands to receptor operated channels. The binding of agonist to its specific membrane receptor causes the activation of a guanine nucleotide binding protein (Snyderman et al., 1986; Gilman, 1987) which, in turn, activates PLC. PLC causes the hydrolysis of a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, which yields water soluble IP_3 (Berridge, 1987; Berridge and Irvine, 1989; Rana and Hokin, 1990) and a lipid DAG (Berridge, 1987). IP_3 and perhaps additional products of IP_3 metabolism, then cause the release of calcium from intracellular stores while DAG, at the membrane and in conjunction with calcium ions, activate PKC (Kikkawa et al., 1989). The resulting phosphoprotein (phosphorylated by PKC) and Ca^{2+} may be necessary for the stimulation of NADPH-oxidase (Curnette, 1990; Nath and Powledge, 1990; Pick et al., 1990; Rotrosen et al., 1990; Segal, 1990) thus reducing O_2 to O_2^{-}. Ca^{2+} itself has a broad range of effects, activating a variety of enzyme systems, both as a cofactor and in conjunction with the calcium binding protein calmodulin. While it appears clear that the initial elevation of ionized calcium is due to the release of intracellular calcium stores, little is known about the regulation of the influx of calcium from

extracellular sources that is necessary to sustain the response. Recent evidence indicates two additional products of IP_3, inositol 1:2-cyclic 4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate, also have second messenger function in that they are active in mobilizing cellular stores of calcium (Zilberman et al., 1987; Luckhoff and Clapham, 1992). The production of inositol 1,3,4,5-tetrakisphosphate may be regulated in part by cytosolic calcium concentration.

1.7.2.1. Measurement of change in calcium with indo-1

An optimal indicator of $[Ca^{2+}]_i$ should span the range of calcium concentrations from 100 nM to above a micromolar, with greatest sensitivity to small changes at the lower end of that range. The response to transient changes should be rapid and the indicator should freely diffuse throughout the cytoplasm, but be easily and stably loaded. Finally, the indicator itself should have little or no effect upon $[Ca^{2+}]_i$ or on other cellular functions and must not be toxic to the cell. Grynkiewicz et al. (1985) have described a new family of highly fluorescent indicators of $[Ca^{2+}]_i$ which overcome most of these difficulties.

Indo-1 ([1-[2-amino-5-[carboxylindol-2-yl]-phenoxy]-2-2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid) has spectral properties and, therefore, is the choice indicator for flow cytometry applications, in which the limited excitation wavelengths make monitoring two different emissions more practical (Molecular Probes Handbook, 1989). The hypsochromic shift of indo-1 in absorption and excitation spectra upon binding Ca^{2+} is well understood (Tsien, 1980). In order for the nitrogens (together with their attached acetate groups and the ether oxygens) to envelope and chelate a Ca^{2+} ion (Appendix B), the nitrogen ring bonds would have to twist by nearly 90°, breaking the ring conjugation (485 nm) and

giving a spectrum of a simple benzene (405 nm). Indo-1 is asssociated with a 1:1 Ca^{2+} binding stoichiometry and binds with a rather quick association constant of 5 x 10^8 - 1x 10^9 $M^{-1}s^{-1}$ (Jackson et al., 1987). Rabinovitch et al. (1986) demonstrated that in lymphocytes loaded with indo-1, the loss of blue and violet fluorescence is about 10% while the ratio of violet to blue fluorescence loss is only about 4% at 25°C for 4 h. Thus, indo-1 loaded cells are remarkably stable.

1.7.2.2. Advantages of indo-1 over other $[Ca^{2+}]_i$ indicators

As compared to other $[Ca^{2+}]_i$ indicators, indo-1, 1) does not have as high an affinity for calcium and, therefore, does not perturb cellular calcium metabolism nearly as much as quin-2, for example; 2) is introduced into cells in the form of the permeant indo-1 acetoxymethyl ester (AM) form, which undergoes hydrolysis by non-specific esterase in living cells to yield the free dye; 3) has 30-fold greater quantum yield in terms of fluorescence than quin-2 at a given dye concentration (Grynkiewicz et al., 1985); 4) compared to quin2 is more selective for divalent cations (selects for Ca^{2+} 10^5 more than for Mg^{2+}) and is not quenched by heavy metals which are found in the cytoplasm of some cell lines; 5) undergoes substantial emission spectral shifts upon binding calcium; 6) makes ratiometric measurements of cytoplasmic $[Ca^{2+}]$ possible (which cancel out many extraneous factors including uneven illumination, light source noise and the effects of cell-to-cell variations in dye content; the degree of dye loading is independent of dye concentration, cell thickness, photobleaching and dye leakage, as long as free dye is washed away); 7) is remarkably nontoxic to cells subsequent to loading; and 8) discriminates between live and dead cells as the latter do not retain the hydrophilic impermeant dye.

1.7.3. Role of cAMP

It has been known for many years that an accumulation of cAMP exerts either a positive or negative effect on cell activation and growth (Klaus et al., 1987; Rigley and Hicks, 1991). However, it has recently been shown that IL-4 induces the activation of adenylate cyclase which leads to the increased expression of cluster of differentiation (CD)23 (high affinity receptor to IgE) on B cells (Finney et al., 1990). The requirement of adenylate cyclase activation for signal transduction has been well established in cells possessing receptors for growth factors and hormones (Sutherland and Rall, 1960; Rickley and Hicks, 1991). To determine, the role of the second messenger cAMP in the production of reactive oxygen intermediates in IL-4-mediated MΦ activation, a series of experiments were performed (Chapter 8, Tan et al., 1992b). It is known that cAMP-dependent protein kinase (Kammer, 1988; Taylor et al., 1990) can phosphorylate substrate protein containing serine, threonine, or tyrosine residues. Further introduction to the role of cAMP is briefly described in Chapter 8 (Tan et al., 1992b)

1.8. Known IL-4-mediated signal transduction

The mechanism of signal transduction by IL-4 is not clear and has recently been reviewed by Harada et al. (1992). Involvement of phosphatidylinositol turnover, increase in intracellular calcium, cAMP, and PKC activation in the IL-4 signal transduction pathway is controversial. Mizuguchi et al. (1986) demonstrated that IL-4 on resting B cells does not require elevation of inositol phospholipid metabolism or increased calcium. However, it has been demonstrated that IL-4 activates human B lymphocytes via a transient inositol lipid hydrolysis (Finney et al., 1990),

an increase in calcium (Finney et al., 1990; Ashida et al., 1990), and a delayed cAMP generation (Finney et al., 1990). Finney and colleagues suggested that this is in contrast to that of previous results because different sources of B lymphocytes were used, and the very rapid and transient increase in calcium may not have been recognized. Interestingly, Finney et al. (1991) and Galizzi et al. (1988) found that IL-4 does not have an appreciable effect on cAMP levels in IL-4-mediated CD23 expression by murine B cells or the human B cell line Jijoye, respectively. Justement et al. (1986) and Dancescu et al. (1992) demonstrated that IL-4 did not stimulate PKC translocation from cytosol to membrane in resting murine B cells and that IL-4 regulates the expression of CD20 by B cells through a PKC-independent pathway. In contrast, Chaikin et al. (1990) have reported that IL-4 increased PKC activity in the membrane fraction of mouse bone marrow-derived mast cells. One possible explanation for these dichotomous results is that IL-4 may bind to at least two functionally distinct types of receptors on target cells (Mosley et al., 1989; Finney et al., 1991). Recent data have suggested that there exist two separate signal transduction pathways stimulated by IL-4 in B cells, one that is dependent (IL-4-induced expression of CD23) and the other independent (IL 4-induced expression of sIgM) of the hydrolysis of phosphatidyl inositol 4,5-bisphosphate and generation of cAMP (Rigley et al., 1991). One could assume, therefore, that each signal transduction pathway is coupled to a distinct IL-4R subtype or that IL-4R signalling in different cells may involve distinct intracellular activation pathways (Nikcevich et al., 1992). An alternative explanation is that there may exist a single type of IL-4R (since only a single high affinity type has been isolated) that interacts with multiple signal transduction components (each different

type of cell using a unique component) which transmit different intracellular signals.

In some factor-dependent mouse myeloid cells, IL-4 clearly induces protein tyrosine phosphorylation within 10 min, suggesting a possible involvement of a tyrosine kinase in IL-4 signal transduction (Morla et al., 1988; Isfort and Ihle, 1990). However, Nikcevich et al. (1992) demonstrated that IL-4 stimulated tyrosine phosphorylation in some murine T cell lines but not in others. Collectively, these data suggest that IL-4R signalling in different cells may involve distinct pathways. Interestingly, Idzerda et al. (1990) demonstrated that the cytoplasmic domain sequence of human IL-4R possesses no sequence homologies with protein kinases or sequences found at phosphorylation acceptor sites for protein tyrosine kinases or PKC. They also demonstrated that the murine T cell line, CTLL, which is stably transfected with the human IL-4R cDNA, proliferated in response to human IL-4. These results indicate that the molecularly cloned human IL-4R protein is able to interact with the mouse signal transduction machinery. Interestingly, about 400 amino acid residues that form the C-terminus can be deleted without loss of the biological function of the human IL-4R in mouse stable transfectants, indicating that the restricted region of the IL-4R is required for interaction with a signal transducing molecule(s) for proliferation (Harada et al., 1992). Recently, IL-4 has been demonstrated to induce the expression of c-myc and c-fos (Klemsz et al., 1989; Isfort and Ihle, 1990), protooncogenes known to be involved in cell activation and proliferation.

1.9. Goals and objectives of the project

In view of the dismal clinical results in treating pancreatic cancer (Tan and Strong, 1986; Tan et al., 1987a, b), the goal of our early research

had been to develop *in vitro* and *in vivo* hamster models in which protocols applicable to the treatment of epithelial cancers, e.g. pancreatic cancer, could be tested. The hamster model was chosen because pancreatic cancer induced in Syrian Golden hamsters with the PC-1 transplantable pancreatic cancer cell line has been shown to share many characteristics with the equivalent human disease (Pour et al., 1981; Egami et al., 1989). A series of experiments were performed to activate hamster peritoneal MΦs with IL-4 to release free reactive oxygen intermediates (Chapter 4, Tan et al., 1991b).

In mid 1987, it was not known whether murine IL-4 is species-specific or will cross narrow phylogenetic barriers for biological activity on hamster MΦs. However, it was found later that there is no species cross reactivity between murine IL-4 and human cells, and human IL-4 and murine cells (Mosmann et al., 1987; Ohara and Paul, 1987) although both human rIL-4 and murine rIL-4 cDNA clones share 50% homology at the amino acid level (as inferred from nucleotide sequence; Yokota et al., 1988) and exhibit similar activities in many instances. Later, Leitenberg and Feldbush (1988) found that murine IL-4 will not induce class II MHC antigen expression on rat B cells. To determine the species specificity of murine IL-4, a series of experiments was undertaken to determine if murine IL-4 could activate hamster and rat peritoneal MΦs (Chapter 4, Tan et al., 1991b).

Next, the biological activity of IL-4 on murine peritoneal MΦs was further characterized (Chapter 5, Tan et al., 1990, 1991d). In this study, the MΦ-activating properties of murine IL-4 on the formation of reactive oxygen intermediates was evaluated by monitoring zymosan-triggered chemiluminescent oxidative burst. It was reported that IL-4 induced a dose-dependent enhancement of chemiluminescence in murine peritoneal

MΦs triggered with zymosan. In contrast, IL-4 inhibited the zymosan-triggered chemiluminescence in the J774 murine MΦ cell line.

Further work was undertaken to develop a model which could suitably replace the use of murine peritoneal MΦs (Tan et al., 1991a, 1992b). The bone marrow-derived MΦ (BMMΦ) cell line, 14M1.4, responds to IL-4 in a manner similar to murine peritoneal MΦs but has advantages that include shorter kinetics for response, better reproducibility, shorter preparation time, and avoids the use of animals. These characteristics enabled me to study the signal transduction mechanism of MΦ activation by IL-4 (Chapter 6-8; Ralph, 1981, 1986; Tan et al., 1991c; Tan et al., 1992b). Thus, in using the 14M1.4 BMMΦ and J774 MΦ cell lines as models, it became possible to study the contrasting effect of IL-4 on the respiratory oxidative burst. The yet unknown signal transduction mechanisms of IL-4-mediated MΦ activation was determined at the second/third messenger levels. Using the 14M1.4 MΦs, the change in membrane potential (Chapter 6) and the role of IP_3 and calcium (Chapter 7), and cAMP (Chapter 8) as possible mediators of transduction signals in IL-4-activated MΦs were investigated. An attempt to correlate the tumoricidal activity to the release of reactive oxygen intermediates in IL-4-mediated MΦ activation in 14M1.4 and J774 MΦs was done by assaying cytolysis and cytostasis of tumor target cells (Chapter 9).

Beyond the scope of this dissertation, the long-term goal of this *in vitro* study is to determine a protocol whereby mononuclear phagocytes are activated *in vivo* by IL-4 to kill host tumor cells. Difficulty in studying this is expected, however, because it is known that IL-4 (and other lymphokines), when administered systemically, manifest a very brief half-life. For example, in cancer patients receiving IL-2 (Koths and Halenbeck, 1985), the most well studied lymphokine, and IL-4 (Custer and Lotze,

1990), the α distribution phases are approximately 5 and 8 min, respectively, followed by apparent β clearance phases of approximately 30-40 and 48 min, respectively. One way to circumvent the problem of a short half life would be to deliver liposome encapsulated IL-4. These liposomes would be recognized and engulfed by MΦs, the first line of immune defense (Cohn, 1986). Of course, the success of this proposal will work only if MΦs possess cytosolic receptors for IL-4. The concept of using MΦ activating factors, e.g. MΦ lipopolysaccharide derivatives, encapsulated in liposomes has been demonstrated to be successful (Kleinerman et al., 1983; Kleinerman and Fidler, 1985; Sone et al., 1985; Fidler, 1985, 1989). If such a treatment regimen were applicable in vivo, it might comprise a new therapy regimen for patients diagnosed as having cancer. Another alternative might be to conjugate IL-4 to tumor-specific or tumor-associated mAb. As early as 1985, Oldham had shown that mAb diffuses well into the tumor module and suggested that it could provide an appropriate delivery system.

More immediate goals (not included in this dissertation) would be the identification of the specific mechanism(s) by which IL-4-activated MΦs kill tumor cells. Since chemiluminescence is a reflection of the entire release of many reactive oxygen intermediates by activated MΦs, it is not known specifically which reactive oxygen intermediates (for example, hydrogen peroxide vs superoxide anion) is released from IL-4-mediated activated MΦs. It would be interesting to note which of these reactive oxygen intermediates are required for IL-4-mediated MΦ tumoricidal activity. In addition, it has previously been demonstrated that cytolytic proteases are necessary for MΦs to bind tumor cells and to develop competence for cytolysis of neoplastic targets (Adams, 1980; Johnson et al., 1983). Recently, reactive nitrogen intermediates generated by

enzymatic deimination of L-arginine by murine MΦs (Hibbs et al., 1987; Stuehr and Nathan, 1989) and a tumoricidal glycoprotein (170-kDa) secreted from cultured murine bone marrow-derived MΦs (Schwamberger et al., 1992) have been implicated in MΦ tumoricidal effector mechanisms. Thus, it would be interesting to note if IL-4-activated MΦs kill tumor cells by the reactive oxygen and nitrogen intermediates pathway or by yet uncharacterized tumoricidal proteins, or both.

CHAPTER 2

REGULATORY ROLE OF CYTOKINES IN IGE-MEDIATED ALLERGY*

Henkie P. Tan[1], Lauralynn K. Lebeck[2], and Sandra L. Nehlsen-Cannarella[3]

[1]Student who conceptualized, reviewed the literature and prepared the manuscript

[2]Student's research instructor who supervised the literature review and manuscript preparation

[3]Student's research committee chairman who supervised the literature review and manuscript preparation

---

* **Tan, H. P., L. K. Lebeck, and S. L. Nehlsen-Cannarella.** 1992a. Regulatory role of cytokines in IgE-mediated allergy. *J. Leukocyte Biol.* (In press)

Regulatory Role of Cytokines in IgE-mediated Allergy

Henkie P. Tan, Lauralynn K. Lebeck, and Sandra L. Nehlsen-Cannarella

Immunology Center (H.P.T., L.K.L., S.L.N.-C.) and Departments of Microbiology (H.P.T., S.L.N.-C.), Surgery (L.K.L., S.L.N.-C.), and Pathology (L.K.L, S.L.N.-C.), Loma Linda University, School of Medicine, Loma Linda, California

Key words: IL-4, IFN-γ, interleukin, lymphokine, growth factor

MAILING ADDRESS
Henkie P. TAN
Immunology Center, Department of Surgery
Loma Linda University
School of Medicine
LOMA LINDA, CA 92354

Tel: (714)-824-0800 Extn 5152
FAX: (714)-824-4819

Reprint requests: Henkie P. Tan, Immunology Center, Department of Surgery, Loma Linda University, School of Medicine, Loma Linda, CA 92354.

RUNNING TITLE

Cytokines in IgE-mediated Allergy

2.1. ABSTRACT

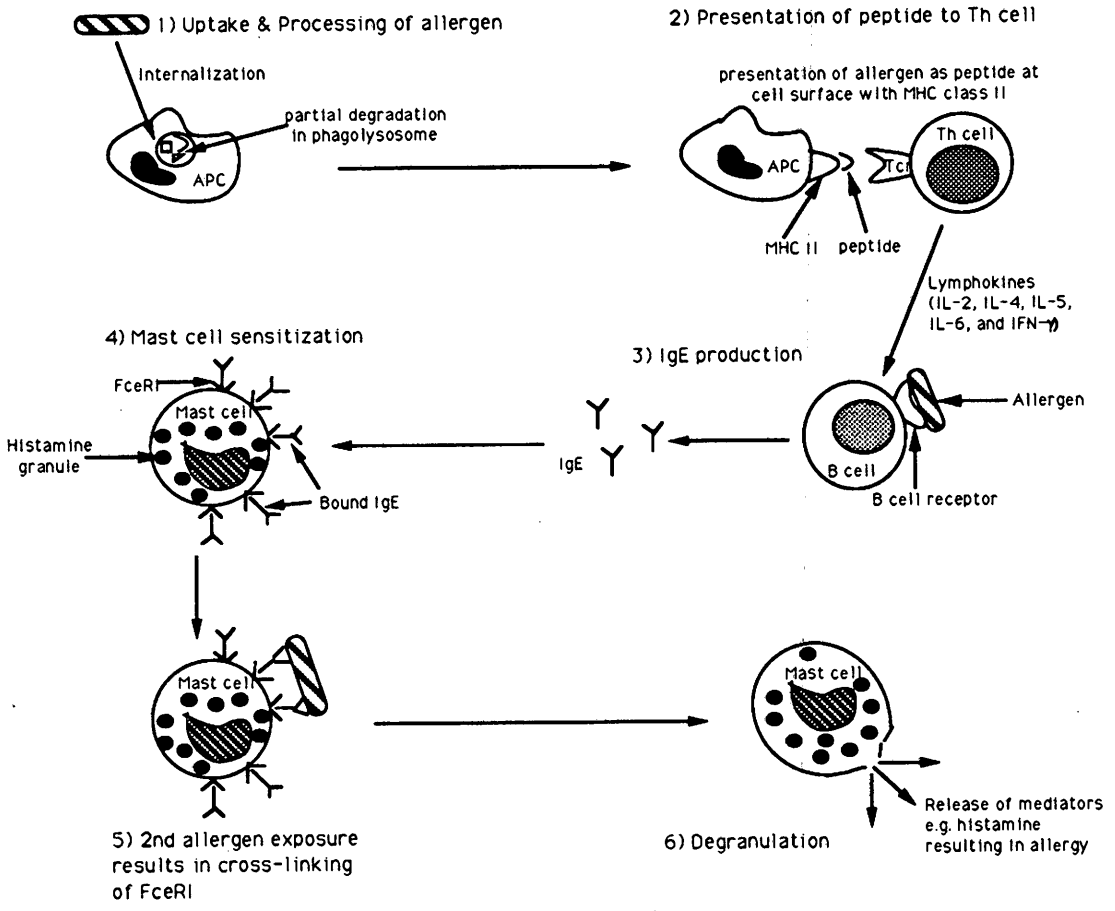
The discovery of IgE is considered the most important contribution, to date, in the field of clinical allergy. Studies in rodents and humans have suggested that IgE production could be regulated by antigen-specific helper and suppressor T cells, and by isotype-specific factors having affinity for IgE. In recent years, the synthesis of IgE has been shown to be regulated, in part, by a cytokine network. This review summarizes the cytokines that upregulate (IL-4, IL-5 and IL-6) and downregulate (IFN-γ and IL-2) the production of IgE. Emphasis is placed on IL-4 and IFN-γ, two lymphokines known to play a major, but reciprocal, role in IgE synthesis. Increased insight into the various IgE control mechanisms by cytokines and their receptors will eventually lead to improved treatment strategies in the clinical management of IgE-mediated allergy.

2.2. INTRODUCTION

The incidence of patients presenting with IgE-mediated allergic diseases is increasing. Currently, these diseases affect about 10% of the world population and represent a major socioeconomic problem (1). Allergic diseases are characterized by a consistently elevated synthesis of IgE upon stimulation by environmental allergens. The recognition of allergen by the immune system, in a simplistic fashion, is accomplished by class II-MHC-positive cells and specific T helper lymphocytes (2). The T cells, following activation, induce B cells to produce allergen specific IgE (3). The secreted IgE binds preferentially to high affinity Fcε receptors (FcεRI) on mast cells and basophils, thus sensitizing them. The FcεR are cross-linked upon a second encounter with the allergen. This sequence of events (summarized in Fig. 1) results in the release of histamine, leukotrienes, and other pharmacologic mediators from the sensitized cells and is responsible for Type I immediate hypersensitivity.

2.3. THE REGULATORY ROLE OF IL-4

Coffman and colleagues (4) first reported that the T cell-derived lymphokine IL-4 was able to induce IgE production *in vitro* by co-stimulation of murine B cell blasts. IL-4, originally described as a B cell growth factor, has subsequently been shown to have pleiotropic effects on multiple hematologic and nonhematologic cellular lineages (5,6,7). The IgE inducing effect of IL-4 initially observed *in vitro* was confirmed by *in vivo* studies showing that the administration of an IL-4 monoclonal antibody inhibited 99% of the primary IgE response (8). This group further demonstrated that IL-4 is unequivocally required for an *in vivo* primary IgE response and that IL-4 also plays a major role in the secondary chronic


1) Uptake & Processing of allergen
Internalization
partial degradation in phagolysosome
APC
2) Presentation of peptide to Th cell
presentation of allergen as peptide at cell surface with MHC class II
APC
Th cell
MHC II
peptide
Lymphokines (IL-2, IL-4, IL-5, IL-6, and IFN-γ)
3) IgE production
Allergen
B cell
B cell receptor
IgE
4) Mast cell sensitization
FceRI
Mast cell
Histamine granule
Bound IgE
Mast cell
5) 2nd allergen exposure results in cross-linking of FceRI
Mast cell
Release of mediators e.g. histamine resulting in allergy
6) Degranulation

production of IgE. The secondary response was not completely inhibited by anti-IL-4 and is thought to be due to the formation of a memory pool of IgE-producing cells.

Direct evidence linking IL-4 to the allergic response was provided by Tepper *et. al.* (9) when they fused the IL-4 gene to an immunoglobulin promoter/ enhancer gene and made a transgenic mouse model that incorporated this modified product. Overexpression of IL-4 resulted in a marked increase in serum IgE levels and histopathologic features seen in typical allergic reactions. Supporting evidence that IL-4 plays a key role in the regulation of IgE synthesis was soon provided by additional reports in humans (10). Exogenous IL-4 (100U/ml), could induce IgE synthesis by peripheral blood mononuclear cells (PBMC) in normal individuals but not in atopic patients (11). The important response parameter being that of a baseline serum IgE level of <10,000 IU/ml (12). One explanation for these findings might be that atopic individuals are near a maximal stimulation level *in vivo* and are, therefore, less responsive than normal healthy individuals to the further addition of IL-4.

Another important role unique to IL-4 is the induction of low affinity Fcε receptors (FcεRII or CD23) expressed on normal human B lymphocytes, a function originally thought to be critical to the production of IgE by B cells (13,14). A relatively low concentration (3-5 U/ml) of IL-4 is sufficient for CD23 expression but the concentration of IL-4 required for immunoglobulin class switching to IgE synthesis is much higher than physiologic levels (15,16). The concentration of IL-4 needed (5-10 U/ml) for IgE synthesis can be diminished to near physiologic levels by the addition of IL-5 (17).

A two signal model for the induction of IgE has been proposed (14,18). One of the two signals is delivered by IL-4, and is IgE isotype specific. However, it appears that IL-4 alone is not sufficient to induce IgE synthesis in B lymphocytes. The other mandatory signal is primarily provided by physical interactions between T and B cells either through the TCR and/or surface molecules expressed on activated T cells. This signal can also be furnished by an increasing list of B cell activators that include EBV infection (19), anti-CD40 antibody (20), and hydrocortisone (21). Again, neither EBV infection, hydrocortisone, nor anti-CD40 alone induced IgE synthesis in the absence of IL-4.

2.4. THE REGULATORY ROLE OF IFN-γ

Many studies have demonstrated that IFN-γ downregulates IgE production and IL-4-promoted CD23 expression *in vitro*. As early as 1986, Coffman *et. al.* (4) provided data that suggested IgE production could be inhibited by IFN-γ. In subsequent years, Pene and colleagues (10) showed that IFN-γ can inhibit IL-4-mediated IgE production by normal human lymphocytes. The role of IFN as an inhibitor of IgE production is further supported by recent clinical trials. Treatments with IFN-γ *in vivo* significantly lowered the IgE serum levels of hyper-IgE syndrome patients (22). Similar treatments in atopic dermatitis patients showed clinical improvement and a reduction in the spontaneous *in vitro* synthesis of IgE; no effect on serum IgE levels was noted (23). Taken together, these data would suggest that IFN-γ is a potent suppressor of the IgE response *in vitro* and that it is likely to exert a similar effect *in vivo*.

2.5. THE REGULATORY ROLE OF OTHER CYTOKINES

IL-2 has recently been demonstrated to be required for IL-4 production by T cells (24) and unpublished data of Pene (25) suggested that IL-2 may upregulate IL-4-induced IgE synthesis. However, patients with severe atopic dermatitis receiving IL-2 intravenously showed a clear therapeutic effect although there were no significant changes in serum IgE, or in *in vitro* IgE production (26). This therapeutic effect has been supported by *in vitro* experiments of Spiegelberg (27) in which IL-2 was seen to inhibit IL-4-induced IgE synthesis of PBMC from normal and atopic dermatitis patients.

Other lymphokines, in particular, IL-5 and IL-6, have been demonstrated to upregulate the IgE response induced by IL-4 (17,25,28). That IL-6 enhanced the production of IgE appears to explain the potentiating effect of monocytes (29).

Other growth factors such as TGF-β and IFN-α have been reported as inhibitors of IL-4-promoted IgE production and also of IL-4-promoted CD23 expression by B cells (10,30,31).

Mast cells play an important role in the efferent arm of the allergic response. As outlined in the introduction, these cells bind IgE through the FcεRI and are subsequently activated by allergens leading to the release of allergic mediators. Mast cells have been shown to produce cytokines, including IL-3, IL-4, IL-5, and IL-6 (32). This ability of mast cells to secrete and respond to cytokines has important consequences in autoregulation within the allergic response; IL-3 and IL-4 stimulate the growth of mast cells while IL-5 stimulates the growth of eosinophils (33).

2.6. THERAPY WITH RECOMBINANT SOLUBLE CYTOKINE RECEPTORS

Rational therapeutic approaches are now possible due to our understanding of the mechanisms that trigger IgE synthesis in B cells. IL-4 activates IgE producing B cells through an interaction with a specific membrane glycoprotein, the IL-4 receptor (IL-4R) (34). With the advent of recombinant soluble (s)IL-4R molecules [mouse (35,36); human (37)], it is conceivable that sIL-4R can be used to modify IL-4 production in patients with IgE-mediated allergy. The working hypothesis is that sIL-4R will competitively bind and offset the effect of excess IL-4, thus downregulating IgE synthesis by B cells.

Ideally, recombinant soluble receptors could provide the means for a specific and potent immunosuppression. Soluble IL-4R, having at least 100- to 1000-fold higher affinities for IL-4, would be superior to IL-4 and IL-4R monoclonal antibodies in that the soluble receptors are smaller and will allow for better tissue distribution (38). For example, in a cardiac allograft model, a 0.015mg/kg total dose of sIL-4R was found to inhibit 50% of the peripheral lymph node response to allogeneic cells *in vivo*, whereas a 300mg/kg total dose of anti-IL-4 or 18.8 mg/kg total dose of anti-IL-4R was required to cause the same amount of inhibition (39). In general, soluble receptors would not be recognized as foreign by the immune system such as in the case of monoclonal antibodies raised in different species (40). Additionally, they do not have specific structures reacting with immune cells as would the Fc portion of immunoglobulins.

Low levels of sIL-4R have been detected in the blood and urine of mice and in concentrated culture supernatants of purified murine T and B cells (38,41). The presence of sIL-4R in these body fluids may in fact be a physiologic control mechanism to fine tune the production of IL-4. Low

concentrations of purified sIL-4R specifically inhibited IL-4-mediated proliferation *in vitro*, suggesting its utility as a possible therapeutic agent (36,37). The finding that IL-4 inhibitory agents in humans might abrogate the IgE response without affecting the levels of other isotypes (8) provided support for the therapeutic application of sIL-4R. Recent *in vivo* results have indicated that the kinetics and affinity of IL-4 binding to the recombinant sIL-4R are similar to the native membrane bound IL-4R (36). It is important to note that therapeutic intervention with IL-4R should not be used to completely abrogate IgE antibodies *in vivo* since it has been demonstrated that IL-4-induced IgE antibodies may be beneficial (42).

Taken together, IL-4 appears to be the major cytokine that upregulates IgE production since the other cytokines, by themselves, had no effect on IgE synthesis. These molecules however could enhance the IL-4-induced IgE production. With an increased insight into the various IgE control mechanisms by cytokines and their corresponding receptors, it is conceivable that improved treatment strategies in the clinical management of IgE-mediated allergy can be formulated.

2.7. ACKNOWLEDGMENTS

This work was supported by grants from the Immunobiology of Cancer Fund and a research grant from the Department of Surgery, Loma Linda University, School of Medicine.

2.8. REFERENCES

1. Vercelli, D., Geha, R.S. (1989) The IgE system. *Ann. Allergy* **63**, 4.11.
2. Romagnani, S., Maggi, E., Parronchi, P., Macchia, D., Piccinni, M. P. (1991) Regulation of IgE synthesis by CD4+ human T-cell subsets. *Res. Immunol.* **142**, 63.67.
3. Bahna, S. L. (1989) A 21-year salute to IgE. *Ann. Allergy* **62**, 471.478.
4. Coffman, R. L., O'Hara, J., Bond, M. W., Carty, J., Zlotnik, A., Paul, W. E. (1986) B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* **136**, 4538.4541.
5. Moller, G. (1988) IL-4 and IL-5: Biology and genetics. *Immunol. Rev.* **102**,1.212.
6. Banchereau, J., DeFrance, T., Galizzi, J. P., Miossec, P., Rousset, F. (1991) Human interleukin 4. *Bull. Cancer Paris* **78**, 299.306.
7. Tan, H. P., Nehlsen-Cannarella, S. L., Garberoglio, C. A., Tosk, J. M. (1991) Recombinant interleukin-4 enhances the chemiluminescent oxidative burst of murine peritoneal macrophages. *J. Leukocyte Biol.* **49**, 587.591.
8. Finkelman, F. D., Holmes, J., Katona, I. M., Urban, J. F., Beckmann, M. P., Park, L. S., Schooley, K. A., Coffman, R. L., Mosmann, T. R., Paul, W. E. (1990) Lymphokine regulation of in vivo isotype selection. *Annu. Rev. Immunol.* **8**, 303.333.
9. Tepper, R. I., Levinson, D. A., Stanger, B. Z., Campos-Torres, J., Abbas, A. K., Leder, P. (1990) IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice. *Cell* **62**, 457.467.

10. Pene, J., Rousset, F., Briere, F., Chretien, I., Bonnefoy, J. Y., Spits, H., Yokota, T., Arai, N., Arai, K., Banchereau, J., De Vries, J. (1988) IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E_2. *Proc. Natl. Acad. Sci. USA.* **85**, 6880.6884.

11. Vollenweider, S., Saurat, J.-H., Rocken, M., Hauser, C. (1991) Evidence suggesting involvement of interleukin-4 (IL-4) production in spontaneous in vitro IgE synthesis in patients with atopic dermatitis. *J. Allergy Clin. Immunol.* **87**, 1088.1095.

12. Rousset, F., Robert, J., Andary, M., Bonnin, J.-P. Souillet, G., Chretien, I., Briere, F., Pene, J., DeVries, J. E. (1991) Shifts in interleukin-4 and interferon-γ production by T cells of patients with elevated serum IgE levels and the modulatory effects of these lymphokines on spontaneous IgE synthesis. *J. Allergy Clin. Immunol.* **87**, 58.69.

13. DeFrance, T., Aubry, J. P., Rousset, F., Vanbervliet, B., Bonnefoy, J. Y., Arai, N., Takebe, Y., Yokota, T., Lee, F., Arai, K., De Vries, J., Banchereau, J. (1987) Human recombinant interleukin 4 induces Fcε receptors (CD23) on normal human B lymphocytes. *J. Exp. Med.* **165**, 1459.1467.

14. Romagnani, S. (1990) Regulation and deregulation of human IgE synthesis. *Immunol. Today* **11**, 316.321.

15. Ishizaka, K. (1989) Basic mechanisms of IgE-mediated hypersensitivity. *Curr, Opin. Immunol.* **1**, 625.629.

16. Gauchat, J.F., Lebman, D. A., Coffman, R. L., Gascan, H., DeVries, J. E. (1990) Structure and expression of germline ε transcripts in human B cells induced by interleukin 4 to switch to IgE production. *J. Exp. Med.* **172**, 463.473.

17. Coffman, R. L., Seymour, B. W. P., Lebman, D. A., Hiraki, D., Christiansen, J. A., Shrader, B., Cherwinski, H. M., Savelkoul, H. F., Finkelman, F. D., Bond, W. M., Mosmann, T. R. (1988) The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev.* **102**, 5.28.

18. Vercelli, D., Geha, R. S. (1991) Regulation of IgE synthesis in humans: A tale of two signals. *J. Allergy Clin. Immunol.* **88**, 285.295.

19. Thyphronitis, G., Tsokos, G. C., June, C. H., Levine, A. D., Finkelman, F. D. (1989) IgE secretion by Epstein-Barr virus-infected purified human B lymphocytes is stimulated by interleukin 4 and suppressed by interferon gamma. *Proc. Natl. Acad. Sci. USA* **86**, 5580.5584.

20. Jabara, H. H., Fu, S. M., Geha, R. S., Vercelli, D. (1990) CD40 and IgE: Synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med.* **172**, 1861.1864.

21. Jabara, H. H., Ahern, D. J., Vercelli, D., Geha, R. S. (1991) Hydrocortisone and IL-4 induce IgE isotype switching in human B cells. *J. Immunol.* **147**, 1557.1560.

22. King, C. L., Gallin, J. I., Malech, H. L., Abramson, S. L., Nutman, T. B. (1989) Regulation of immunoglobulin production in hyperimmunoglobulin E recurrent-infection syndrome by interferon-γ. *Proc. Natl. Acad. Sci. USA* **86**, 10085.10089.

23. Boguniewicz, M., Jaffe, H. S., Izu, A., Sullivan, M. J., York, D., Geha, R. S., Leung, D. (1990) Recombinant gamma interferon in treatment of patients with atopic dermatitis and elevated IgE. *Am. J. Med.* **88**, 365.370.

24. Ben-Sasson, S. Z., LeGros, G., Conrad, D. H., Finkelman, F. D., Paul, W. E. (1990) IL-4 production by T cells from naive donors: IL-2 is required for IL-4 production. *J. Immunol.* **145**, 1127.1136.

25. Pene, J. (1989) Regulatory role of cytokines and CD23 in the human IgE antibody synthesis. *Int. Arch. Allergy Appl. Immunol.* **90**, 32.40.

26. Hsieh, K. H., Chou, C. C., Huang, S. F. (1991) Interleukin 2 therapy in severe atopic dermatitis. *J. Clin. Immunol.* **11**, 22.28.

27. Spiegelberg, H. L. (1990) Fc receptors for IgE and interleukin-4 induced IgE and IgG4 secretion. *J. Invest. Dermatol.*. **94**(Suppl. 6), 49S.52S.

28. Vercelli, D., Jabara, H. H., Arai, K., Yokota, T., Geha, R. S. (1989) Endogenous interleukin-6 plays an obligatory role in interleukin-4 dependent human IgE synthesis. *Eur. J. Immunol.* **19**, 1419.1424.

29. Maggi, E., Del Prete, G.F., Parronchi, P., Tiri, A., Macchia, D., Biswas, P. (1989) Role of T cells, IL-2 and IL-6 in the IL-4-dependent in vitro human IgE synthesis. *Immunology* **68**, 300.306.

30. Gordon, J., Katira, A., Strain, A. J., Gillis, S. (1991) Inhibition of interleukin 4-promoted CD23 production in human B lymphocytes by transforming growth factor-β, interferons or anti-CD19 antibody is overridden on engaging CD40. *Eur. J. Immunol.* **21**, 1917.1922.

31. Wu, C., Brinkmann, V., Heusser, C., Cox, D., Delespesse, G. (1991) Transforming growth factor β (TGF- β) is a potent modulator of human IgE synthesis. *J. Allergy Clin. Immunol.* **87**, 281 (abstr.).

32. Plaut, M., Pierce, J. H., Watson, C. J., Hanley-Hyde, J., Nordan, R. P., Paul, W. E. (1989) Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores. *Nature* **339**, 64.67.

33. Arai., K.-I., Lee, F., Miyajima, A., Miyatake, S., Arai, N., Yokota, T. (1990) Cytokines: Coordinators of immune and inflammatory responses. *Annu. Rev. Biochem.* **59**, 783.836.

34. Banchereau, J., Galizzi, J. P. (1990) Receptors for interleukin 4, interleukin 5 and interleukin 6. *Ann. Ist. Super. Sanita.* **26**, 453.467.

35. Maliszewski, C. R., Sato, T. A., Vanden Bos, T., Waugh, S., Dower, S. K., Slack, J., Beckmann, M. P., Grabstein, K. H. (1990) Cytokine receptors and B cell functions: I. Recombinant soluble receptors specifically inhibit IL-1- and IL-4-induced B cell activities in vitro. *J. Immunol.* **144**, 3028.3033.

36. Jacobs, C. A., Lynch, D. H., Roux, E. R., Miller, R., Davis, B., Widmer, M. B., Wignall, J., Vanden Bos, T., Park, L. S., Beckmann, M. P. (1991) Characterization and pharmacokinetic parameters of recombinant soluble interleukin-4 receptor. *Blood* **77**, 2396.2403.

37. Garrone, P., Djossou, O., Galizzi, J.-P., Banchereau, J. (1991) A recombinant extracellular domain of the human interleukin 4 receptor inhibits the biological effects of interleukin 4 on T and B lymphocytes. *Eur. J. Immunol.* **21**, 1365.1369.

38. Fernandez-Botran, R. (1991) Soluble cytokine receptors: Their role in immunoregulation. *FASEB J.* **5**, 2567.2574.

39. Fanslow, W. C., Clifford, K. N., Park, L. S., Rubin, A. S., Voice, R. F., Beckmann, M. P., Widmer, M. B. (1991) Regulation of alloreactivity in vivo by IL-4 and the soluble IL-4 receptor. *J. Immunol.* **147**, 535.540.

40. Dykewicz, M. S., Cranberg, J. A., Patterson, R., Rosen, S. T. Shaughnessy, M. A., Zimmer, A. M. (1990) Human IgE, IgG and IgA antibody responses to T101, a murine monoclonal antibody against

human lymphocytes: Implications for pathogenesis, risk and avoidance of adverse immunologic reactions. *Int. Arch. Allergy Appl. Immunol.* **92**, 131.137.

41. Fanslow, W. C., Clifford, K., Vanden Bos, T., Teel, A., Armitage, R. J., Beckmann, M. P. (1990) A soluble form of the interleukin 4 receptor in biological fluids. *Cytokine* **2**, 398.401.

42. Hagan, P., Blumenthal, U. J., Dunn, D., Simpson, A. J. G., Wilkins, H. A. (1991) Human IgE, IgG4 and resistance to reinfection with Schistosoma haematobium. *Nature* **349**, 243.245.

CHAPTER 3

MATERIALS AND METHODS OF UNPUBLISHED WORK

3.1. Reagents

Bovine serum albumin (BSA)-globulin free, gramicidin, luminol, penicillin, saponin, streptomycin, trypsin, valinomycin, zymosan, EGTA, LPS, PLC, ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), phorbol myristate acetate (PMA), prostaglandin (PG)E_2, sodium dodecyl sulfate (SDS), 3-isobutyl-1-methylxanthine, tri-n-octylamine, 1,1,2-trichlorotrifluoroethane, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St Louis, MO). Anhydrous DMSO was purchased from Aldrich (Milwaukee, WI); Proteose peptone from Difco (Detroit, MI); $DiOC_6(3)$, indo-1AM and BAPTA-AM (1,2-bis-[2-aminophenoxy]-ethane-N,N,N',N'-tetraacetic acid, acetoxymethyl ester) from Molecular Probes (Eugene, OR); Roswell Park Memorial Institute (RPMI) 1640, Hanks' balanced salt solution (HBSS), fetal calf serum (FCS), phosphate-buffered saline (PBS) from Irvine Scientific (Santa Ana, CA); Ionomycin from Calbiochem (San Diego, CA); [methyl-^{3}H]-thymidine and Biofluor emulsifier cocktail from New England Nuclear (Boston, MA); and IP_3 and cAMP [^{3}H] assay system from Amersham (Arlington Heights, IL).

Murine rIL-4 was kindly provided by Dr. Steven Gillis (Immunex Corporation, Seattle, WA, and Sterling Winthrop Pharmaceuticals, Malvern, PA). This protein had a specific activity of 10^8 U/mg; 1 U is the concentration of IL-4 required to half-maximally stimulate the uptake of [methyl-^{3}H]-thymidine by 5 X 10^4 resting splenic B cells costimulated

with 5 μg/ml of purified anti-IgM antibody, a standard anti-immunoglobulin comitogenesis assay (Grabstein et al., 1986b).

Anti-IL-4 was obtained from TexStar Monoclonals (Dallas, TX) and Genzyme (Boston, MA). It is an $IgG_{1\kappa}$ affinity-purified mAb from 11B115E3 hybridoma culture supernatant. 11B115E3 is a high producer subclone of 11B11 which is the original clone Ohara and Paul (1985) produced against murine rIL-4 in Lew/Sn rats. This anti-IL-4 mAb blocked the biological activity of IL-4 as measured in HT-2 proliferation, B cell differentiation factor-γ, and B cell growth factor I anti-Ig-costimulation assays. Anti-IL-4 has an IC_{50} (50% inhibition of a 50% maximal response in a IL-4 anti-Ig-costimulation assay) of 0.006 μg/ml. For neutralization of mouse IL-4 bioactivity, it was found that 8 μg of the anti-IL-4 mAb was sufficient to inhibit 100% of the bioactivity of 100 U rIL-4 (Tan et al., 1991d). Anti-IL-4 obtained later from NCI-FCRDC, courtesy of Hazleton Labs (Vienna, VA), had similar biological activity.

3.2. Animals and cell lines

Male Balb/c mice, Spraque Dawley rats, and Syrian golden hamsters, 8-12 weeks old, purchased from Charles River Laboratories (Wilmington, MA), were pre-screened and found to be negative for bacterial pathogens, parasites and subclinical viral infections.

The cell lines used were: 1) L1210, a mouse lymphocytic leukemic cell line from American *Type Culture* Collection; 2) PDPaCa, a hamster poorly differentiated pancreatic carcinoma cell line (Chang et al., 1987); 3) H238, a murine fibroblast cell line (Boyd and Orme, 1975); 4) J774A.1, a mouse monocyte-MΦ cell line from American *Type Culture* Collection TIB 67, was generously provided by Drs. Lau and Tosk of LLU, Department of

Microbiology; and 5) 14M1.4, a BMMΦ cell line, was a gift from Dr. A. Zlotnik (DNAX, Palo Alto, CA).

3.3. Cell culture conditions

Nondetectable endotoxin (<0.01 ng/ml) RPMI 1640 medium (Moore et al., 1968; Ham and McKeehan, 1979, Irvine Scientific, Santa Ana, CA, Appendix C) was supplemented with 10% FCS (nondetectable endotoxin, Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, and 100 µg/ml streptomycin (hereafter known as complete medium, CM). CM for each experiment was sterilized with 0.22 µm filter and made fresh frequently since L-glutamine is unstable and has a half-life in medium at 4°C of 3 weeks, and at 37°C of 1 week. Overall health of the cultures were ascertained daily by inspecting all cultures by inverted phase microscopy. All cell lines lines were subcultured every 4-6 days for two months. To avoid potential problems developing in subcultures maintained for prolonged periods, a large number of ampules of cells were frozen and new stock cultures initiated (Appendix D) approximately every 2 months. When the cells were confluent (about once every 5 days), they were split (Appendix E) to create fresh subcultures of approximately 1:5 to 1:10 cells in fresh CM. Media were changed approximately 2-3 times a week, using prewarmed CM.

Mycoplasma contamination (Callard et al., 1987)

Mycoplasma contamination can affect tumoricidal assays in many different ways. In particular, interpretation of [methyl-^{3}H]-thymidine incorporation results may be impossible if the responding cell lines are contaminated. To prevent mycoplasma contamination, in addition to using sterile techniques and working within a laminar flow hood which had been

exposed to UV light when not in use, the laminar flow cabinet was frequently fumigated with formaldehyde overnight. MYCOTRIM TC (Hana Biologics, Alameda, CA) was used to screen and monitor continuously passaged cells for mycoplasma contamination (Appendix F) once every two months and again before freezing stocks of cell lines. Each new lot of serum, trypsin, and medium, as well as newly acquired cell cultures, were tested for mycoplasma contamination before use. Fortunately, there was no known mycoplasma contamination throughout the entire research training.

3.4. Macrophage preparations.

3.4.1. J774 macrophages

This cell line was adapted to culture from a tumor which arose in a female Balb/c mouse in 1968 during a plasmacytoma induction program (Hirst et al., 1971; Ralph et al., 1975). The J774 MΦs, similar to that of murine peritoneal MΦs, have been demonstrated to be chemotactic and phagocytic (Snyderman et al., 1977). J774 MΦs adhere to plastic and exhibit antibody-dependent cellular cytotoxicity, produce lysozyme and possess F_c receptors (Ralph et al., 1975; Ralph et al., 1976; Snyderman et al., 1977; Kaplan and Morland, 1978). However, this cell line releases higher levels of acid phosphatase and β-glucoronidase and is more sensitive to LPS than normal murine peritoneal MΦs, indicating that the tumor cells are more "activated" (Morland and Kaplan, 1978). It has been shown that cell growth can be inhibited by dextran sulfate and LPS (Ralph and Nakoinz, 1977), and that IL-1 is synthesized continuously (Ralph et al., 1976) by this line.

MΦs were grown in CM at 2-3 x 10^5 cells/ml (Adams and Edelson, 1981) in 25 x 125 mm disposable sterile polystyrene petri dishes that contained a thin, transparent flourocarbon Teflon film (FEP, type A; Welch Fluorocarbon, Medway, MA) allowing MΦs to adhere rapidly yet disengage easily without trypsinization (VanderMeer et al., 1981; Helinski et al., 1988). The Teflon film had been vacuum-formed as to provide a liner for the petri dish.

3.4.2. 14M1.4 macrophages

14M1.4, a BMMΦ cell line, is derived from adherent bone marrow cells of Balb/c origin (Zipori et al., 1984). These cells were termed MΦs because they exhibit the morphology of MΦs and are able to phagocytize, process and degrade antigens. They also possess enzyme characteristics of MΦs such as alpha-naphthyl butyrate esterases, acid phosphatases, and peroxidases (Diesselhoff-denDulk and VanFurth, 1981). This cell line requires MCSF to survive and replicate at low cell concentrations ($\leq$ 2 x 10^4/ml); however, at higher cell concentrations, it is able to survive without external sources of MCSF (Zipori et al., 1984; Tan et al., unpublished observation). In all experiments, 14M1.4 MΦs were grown at a density of >1 x 10^5/ml (Adams and Edelson, 1981) in the absence of MCSF, and in teflon-coated petri dishes as with J774 MΦs (VanderMeer et al., 1981; Helinski et al., 1988).

3.4.3. Peritoneal macrophages

Peritoneal exudate cells (MΦ-rich) were collected from Balb/c mice, Sprague Dawley rats, and Golden Syrian hamsters which had received 3 ml sterile 3% proteose peptone (Difco, Detroit, MI) i.p. 3 days previously. Cells were collected by peritoneal lavage (Appendix G) with CM containing

10 U/ml heparin (Ruco and Meltzer, 1978; Meltzer, 1981a; Herbert et al., 1986; Crawford et al., 1987). Three percent proteose peptone had been prepared by dissolving 15 g of proteose peptone in 500 ml of pyrogen-free PBS, dispensed into 15 ml polypropylene tubes, autoclaved (15 psi, 121°C, 15 min), cooled, and stored at 25°C. MΦs harvested from 1-3 animals (>3-20 X 10^6 MΦs per animal) were pooled and washed 3 times in CM and centrifuged for 5 min at 700*g* at 4°C. Aliquots were taken for differential and total cell counts, the remaining cells were centrifuged at 700*g* for 5 min at 4°C and resuspended to 1.2 x 10^6 MΦs/ml. Wright-stained smears were prepared by cytocentrifugation of cell suspension and cells were enumerated on a light microscope (Meltzer, 1981b). Cell preparations consistently contained >90% MΦs. The viability of cells used in all experiments was >90% as determined by trypan blue dye exclusion.

3.5. In vitro macrophage-mediated [methyl-^{3}H]-thymidine cytotoxicity and cytostasis assays

3.5.1. Effector cells

3.5.1.1. J774 macrophages

Resting J774 MΦs have been shown to possess approximately 234 to 355 IL-4R per cell (Lowenthal et al., 1988; Feldman and Finbloom, 1990). Miner et al. (1983) have shown that polyinosilic-polycytidylic acid-activated J774 MΦs were cytolytic to murine B16 melanoma variant subline B16-F1 (poor lung colonization potential) at 26% lysis, B16-F10 (good lung colonization potential), 12%, and B16-B14b (good lung-, ovary-, and brain colonization), 15%. Thioglycolate-elicited peritoneal (C57BL/6 mice) MΦs show similar cytolytic activity as compared with J774 MΦs (24%, 16%, and 17% respectively). J774 MΦs were not cytolytic (0%) when

not activated with polyinosilic-polycytidylic acid. Interestingly, Tepper et al. (1989) found that IL-4-producing cells were able to prevent the growth of C57Bl/6 B16-F0 melanoma in their *in vivo* mixed tumor transplantation assay.

3.5.1.2. Murine peritoneal macrophages.

It is important that young MΦs be elicited from the circulatory system since it has been shown that resident peritoneal MΦs (or fixed tissue MΦs in any site) may not be the precursors for activated tumoricidal cells (Ruco and Meltzer, 1978). Furthermore, resident MΦs respond poorly, if at all, to lymphokine. Ralph et al. (1988) have found that thioglycolate-elicited MΦs (58% cytotoxicity), not stimulated with lymphokines, are more effective than peptone-elicited MΦs (3% cytotoxicity) in mediating antibody-dependent cellular cytotoxicity. Therefore peptone-elicited MΦs were chosen to show that the tumoricidal activity obtained, if any, is due to the stimulation of the MΦs by IL-4.

3.5.1.3. 14M1.4 bone marrow-derived macrophages

This is a MΦ cell line that has just recently been established (Zipori et al., 1984) and no study using this cell line as an effector for tumor target cell lysis has been published as yet. However, as mentioned in the discussion of Chapter 8 (Tan et al., 1992b), IL-4 can induce class I and II MHC antigen expression in this cell line. Similar upregulation of MHC antigens was seen in normal bone marrow MΦs but not in other MΦ cell lines, including P388D1, J774.1, PU5.1.8, and WEHI-3, nor in thioglycolate-elicited peritoneal MΦs (Zlotnik et al., 1987; Stuart et al., 1988). 14M1.4 MΦs have been demonstrated to possess about 2300 IL-4R per MΦ (Lowenthal et al., 1988).

3.5.2. [Methyl-^{3}H]-thymidine labelling of target cells

Target tumor cells used for radiolabelling were in exponential growth phase and about 50% confluent. It is essential to obtain a complete growth cycle through the initial lag phase, logarithmic or exponential growth, and finally a plateau at confluence so as to develop a characteristic growth curve for each cell type against which treated cultures can be compared. Confluent or overconfluent cultures often do not incorporate radioactive nuclear labels as well as subconfluent cultures (Vodinelich and Lennox, 1987), possibly because extracellular signals through cell receptors indicate to the cells that confluency has been reached and growth stimulation mediated by intake of extracellular nutrients (including radioactive label) must be downregulated. In addition, the spontaneous release of radioactive label from unhealthy cultures will often be much higher than normal, which makes interpretation of data difficult. The use of a nuclear label avoids the potential pitfall of a high spontaneous release of cytoplasmic label (such as ^{51}Cr); use of [methyl-^{3}H]-thymidine also does not affect target cell proliferation as has been reported for [^{125}I] uridine (Meltzer, 1981c).

The procedure for labelling tumor targets with [methyl-^{3}H]-thymidine is detailed in Appendix H. Briefly, subconfluent target cells were trypsinized, washed, and labelled with [methyl-^{3}H]-thymidine for about 18 h. Unincorporated radiolabel was removed by washing, and tumor targets adjusted to 1.2 X 10^5 cells/ml.

3.5.3. The [methyl-^{3}H]-thymidine cytolytic and cytostatic assays

Cells were activated with murine rIL-4 and tested for tumoricidal activity towards various tumor target cells (Johnson and Adams, 1986):

1) H238, a mouse fibroblast tumor cell line; 2) L1210, a mouse lymphocytic leukemia cell line; 3) PDPaCa, a hamster pancreatic carcinoma cell line.

MΦs were added to 6 mm 96-well culture plates (Costar, Cambridge, MA), 1.2 X 10^5 MΦs in 0.1 ml aliquots, and incubated for 2 h. To ensure a uniform distribution of cells in the wells, the plates are shaken gently several times at right angles before they are placed in the incubator for the MΦ adherence step (this action helps avoid uneven distribution of cells around the periphery of the wells due to the vortex created by moving the cultures from work area to incubator). For peritoneal MΦ preparations, after 2 h incubation at 37°C, non-MΦ, nonadherent cells were removed (Mosier, 1981) by pipetting 0.2 ml of cold CM into each well, vigorously swirling the fluid with the pipet, and withdrawing by aspiration. The monolayers were washed in all directions very vigorously, and the thoroughness of the resultant wash was routinely and consistently monitored by microscopy. Since, the washing step is critical to all of these assays in terms of achieving the desired number and purity of effectors as well as appropriately bound targets, a minimum of 3 washes were performed.

Adherent MΦs (>95% MΦs for peritoneal preparations) that are >90% viable were cultured with murine rIL-4 (40 U and 200 U in 50 µl volume) for 4 h. Fifty µl of 1.2 X 10^5 [methyl-^{3}H]-thymidine prelabelled target tumor cells/ml or 6 X 10^3 target tumor cells (giving an effector-to-target cell ratio of 20:1) were added and incubated at 37°C in 5% CO_2. To determine the duration of the effect of IL-4 cytotoxicity, the released radioactivity was measured after incubations of 24, 48 and 72 h duration. Every assay was consistently performed using the experiment design drawn in Appendix I.

Radiolabeled target tumor cells were used as spontaneous release controls (A1-A3, A7-A9 of Appendix 1). Wells containing control MΦs and radiolabelled target tumor cells were used for background cytotoxicity controls (A4-A6, A10-A12 of Appendix 1). Target cells lysed with SDS in water were used to estimate total maximal release cell-incorporated counts (Vodinelich and Lennox, 1987). Cytotoxicity (by cytolysis) was estimated by measurement of radioactivity released into culture fluids at 48 h in triplicate wells and expressed as percent of total SDS counts. Aliquots of (0.1 ml) culture supernatants were removed and transfered to scintillation vials containing 0.04 ml of 0.5% SDS, mixed with 10 ml Biofluor emulsifier cocktail, and counted by liquid scintillation spectrometry (Parckard Tri-Carb 4530, Downers Grove, IL). The control wells (i.e., tumor cells alone) were treated with 0.1 ml of 5% SDS, and 0.15 ml of this supernatant was transferred to scintillation vials. Specific tumor target cellular cytotolysis mediated by IL-4 activated MΦs and IL-4 directed cytostasis were calculated as follows:

$$\% \text{ Cytotolysis} = \frac{\text{cpm (test) - cpm (B)} \quad X \quad 100\%}{\text{cpm (MR-SR)}}$$

where SR = spontaneous release,
B = background cytotoxicity, and
MR = maximal release.

$$\% \text{ Cytostasis} = [1-(E-T)/C-T)] \text{ X } 100\%$$

where T = no. of target cells added per well,
C = no. of target cells in control wells at the conclusion of the experiment, and
E = no. of target cells remaining after IL-4 directed cytostatic activity.

3.6. MTT colorimetric cytostasis assay

This colorimetric assay is a modification of the thymidine incorporation assay and is based on the ability of viable cells to absorb tetrazolium salts and to cleave the tetrazolium ring by active mitochondria (Mosmann et al., 1983). The MTT test has previously been described (Mosmann et al., 1983; Shalaby and Palladino, 1986; Gallagher et al., 1988). Indicator cells (tumor targets) are added at 2000 cells per 50 µl per well to standard and test cultures. To determine if IL-4 will inhibit the growth of tumor cells, various concentrations of IL-4 (4-1667 U) were added in a volume of 50 µl. After 40 h of incubation, MTT solution (50 µg MTT in 10 µl PBS) was added to each 100 µl cell suspension contained in each well of the assay, and the plates were further incubated at 37°C for 4 h. Acid-isopropanol (100 µl of 0.04 N HCL in isopropanol) was added to all wells and thoroughly mixed to dissolve all dark blue crystals. After all crystals were dissolved (5 min), the plates were read on a Dynatech MR 700 Microelisa reader, using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99. Plates were read within 1 h of the addition of acid-isopropanol, and optical densities recorded.

3.7. Measurement of IP_3

IP_3 in MΦ suspension was measured using a [^{3}H]-labelled IP_3 competitive assay kit by Amersham. For a detailed procedure see Appendix J. Briefly, IL-4 and PLC (positive control, Imamura et al., 1990) was added to MΦ suspensions, and the change in IP_3 concentration measured. IP_3 has a tendency to associate with proteins in a charge-dependent manner, therefore, extraction procedures using perchloric acid

were used. Then the extracted IP_3 samples were neutralized by a mixture of tri-n-octylamine and 1,1,2-trichlorotrifluoroethane and centrifuged to separate the water-soluble IP_3. This water-soluble IP_3 was measured by Amersham's [^{3}H]-labelled IP_3 competitive assay kit. Plasticware was used to minimize losses of phosphorylated inositol species.

3.8. Measurement of change in membrane potential

The membrane potential dye $DiOC_6(3)$, has been used previously to load almost any cell (Shapiro, 1988) and in particular PMNs (Lazzari et al., 1986) and MΦs (Valet et al., 1981; Jenssen et al., 1986). Briefly, $DiOC_6(3)$ is dissolved in DMSO and stored in the dark at 25°C as a 1 mM stock solution. Ten µM working solution $DiOC_6(3)$ was made by diluting the stock solution with analytical grade ethanol 100X. Prior to stimulation with IL-4, one ml of cell suspension (1 x 10^6/ml) in CM was incubated with 5 µl of 10 µM working $DiOC_6(3)$ (final concentration of 50 nM $DiOC_6(3)$) for 0 to 25 min at 25°C and kinetics determined every minute for dye equilibration. Since the dye is lipophilic, it tends to be absorbed by the cell membrane. As a result the cell concentration was held as constant as possible. After incubation, the cell suspension was introduced into the flow cytometer, with temperature and flow rate held constant since this is an equilibrium staining procedure. This dye adsorbs onto the sample tubing within the flow cytometer, and the adsorption may reach equilibration very slowly (resulting in a slowly rising baseline of cellular fluorescence). To minimized this problem, the tubing was pretreated with the dye solution alone. Kinetics of $DiOC_6(3)$ uptake by MΦs were determined by incubating dye with MΦs every min from 0 to 15 min.

3.8.1. Membrane potential data analysis

After the addition of stimuli, data were collected on 2000 cells at each 10 s interval for 10 min. The fluorescence distribution for $DiOC_6(3)$ was obtained using a Becton Dickinson FACScan with an argon laser emitting 4 mW at 488 nm (blue light) to excite the $DiOC_6(3)$. Green fluorescence at 530 nm emission was used for estimation of potential-dependent dye uptake. Unlike the calcium ratio measurement, large cells which bind more dye will appear more fluorescent, independent of their membrane potential. Artifacts (debris) and unstained cells (usually below 3%) were excluded by appropriate electronic discriminators. As an internal standard for fluorescence intensity, FITC-labelled monosized latex particles of 1.83 μm diameter (Polysciences, Warrington, PA) were measured before and after each series of experiments. As with indo-1 calcium analysis, the resulting fluorescence profiles were analyzed and displayed as histograms and as ratio of mean fluorescence vs. time.

3.8.2. Controls

Controls include an untreated cell sample (in RPMI 1640, a high-Na^+, low K^+ [5×10^{-3} M] medium), a sample of cells hyperpolarized by addition of 50 μl of 0.5 mM (5 mg in 8.9 ml absolute ethanol, with a final concentration of 25 μM) valinomycin, and another sample depolarized by addition of 25 μl of 2 mM (100 mg in 26.6 ml ethanol, with a final concentration of 50 μM) gramicidin. In this way, the cells were established to have a nonzero potential difference across their cell membranes, and the indicator dye used will cause the cells to respond to potential changes in either direction from the control value. Valinomycin, which is lipophilic, forms a complex with K^+ ions and can thus readily

transport them across cell membranes. Addition of valinomycin thus effectively increases the cellular potassium permeability to the point at which membrane potential is determined almost entirely by the transmembrane $[K^+]$ gradient. If $[K^+]_o$ is low, valinomycin addition hyperpolarizes the cells; if $[K^+]_o$ is high, valinomycin addition depolarizes the cells, and if $[K^+]_o=[K^+]_i$, valinomycin addition does not change membrane potential. To ensure that the ethanol ($\leq$ 50 µl) used to dissolve valinomycin and gramicidin had no effect on the change in membrane potential of MΦs, 50 µl ethanol was added to a cell suspension containing equilibrated dye, and the change in membrane potential was measured.

3.8.3. Limitations and possible problems

The cyanine dyes are recognized to have a variety of toxic and inhibitory effects, as well as certain limitations inherent in the use of cationic probes (see review by Chused et al., 1986). These dyes act to uncouple oxidative phosphorylation, deplete cellular ATP, block Ca^{2+}-dependent K^+ conductances, and cause depolarization of the resting membrane potential of lymphocytes and PMNs. These toxicities can be reduced by use of lower dye concentrations. In addition, mitochondrial potentials are highly negative and cyanine dye association with mitochondria can be a substantial component of the total fluorescence signal. A high ratio of dye/cell has been reported to decrease the partitioning of cyanine dye in mitochondria (Chused et al., 1986). These problems can be circumvented by using low dye concentrations, ($\leq$ 50 nM) and assaying on the flow cytometer (Shapiro, 1981).

Finally, the dye concentration in suspensions of cells in a cyanine dye may be poorly buffered, in that the major fraction of dye may be cell-associated. Extrusion of dye from a subpopulation of cells may increase

the available extracellular dye, which then may be taken up by another cell population (independent of changes in membrane potential) merely due to changes in concentration of dye in the medium. Thus, the cyanine dyes, under the best conditions, will not yield absolute values of membrane potential to the nearest millivolt (Shapiro, 1988).

3.9. Measurement of change in intracellular calcium.

3.9.1. Flow cytometry assay with indo-1

A primary advantage of flow cytometry is that by analysis of large numbers of individual cells, heterogeneity in cellular characteristics may be observed that would not be apparent in a bulk spectrofluorimetric assay. Increased sensitivity to small changes may also be possible by detection of alterations in a small proportion of cells, which might otherwise be masked by the unresponsive population. Rabinovitch et al. (1986) have demonstrated that the flow cytometric assay with indo-1 can detect less than a 0.3% subpopulation of cells responding to a stimulus of $[Ca^{2+}]_i$ flux.

Thioglycolate-elicited murine peritoneal MΦs and J774 MΦs in suspension and monolayer culture have been demonstrated to possess resting $[Ca^{2+}]_i$ of 110-140 nM and 85-120 nM, respectively, as measured by quin2 and fura-2 fluorescence spectrophotometry (DiVirgilio et al., 1988a). For both MΦ populations, the resting $[Ca^{2+}]_i$ was consistently 25% lower in monolayer cultures than with suspension cultures. In addition, the increase in $[Ca^{2+}]_i$ (when activated with IgG) is reproducible in suspension cultures but not in monolayer cultures. Thus, MΦs for these experiments utilized MΦs from a suspension culture. It was also noted by DiVirgilio et al. (1988a) that the increase in $[Ca^{2+}]_i$ caused by ligation of

Fc receptors was mainly due to release of Ca^{2+} from intracellular stores in J774 as opposed to influx of extracellular Ca^{2+} in thioglycolate-elicited MΦs, probably due to differences in MΦ populations in response to the same stimuli. In the presence of probenecid, which prevents the leakage of indo-1 (especially known to occur in J774 MΦs) Greenberg et al. (1988) demonstrated in a spectrofluorimetric assay that the $[Ca^{2+}]_i$ of J774 MΦs was about 160 nM (range of 130-210 nM).

3.9.2. Limitations and possible problems

1) It has been suggested that indo-1AM may be incompletely deesterified within some cell types (e.g. endothelial cells; Luckhoff, 1986). Since the ester has little fluorescence spectral dependence upon Ca^{2+}, the presence of this dye form could lead to falsely decreased estimates of $[Ca^{2+}]_i$. However, results of calibration experiments can be used to monitor for this possibility. 2) It is usually observed that not all cells, even of a specific immunophenotypic subset, respond to a particular signal, and of those that do, the values of $[Ca^{2+}]_i$ are quite heterogeneous. Some of this heterogeneity is due to differences in cell subsets. It is not known to what extent the residual heterogeneity in $[Ca^{2+}]_i$ represents the effect of oscillations of $[Ca^{2+}]_i$ within individual cells as a function of time (Rasmussen, 1989; Berridge and Moreton, 1991). Since flow cytometric analysis does not allow for repeated examination of a cell sample, other techniques (e.g. digital microscopy; Wilson et al., 1987) must be used to address this question. 3) Indo-1, fura-2 and other organic anion fluorescent dyes that are sequestered in the cytoplasmic vacuoles or endosomes of MΦs, are efficiently secreted from the cells (Greenberg et al., 1988; DiVirgilio et al., 1988b; Lipman et al., 1990). The secretion of these dyes into the extracellular medium is thought to be mediated through

organic anion transporters and can be inhibited by probenecid. The 14M1.4 MΦs appear not to possess efficient organic anion transporters for indo-1 because fluorescence from these MΦs, as indicated by flow cytometric measurements, did not decrease after a period of 10 min (Tan et al., unpublished observation).

3.9.3. Reagents

The choice of medium in which the cell sample is suspended for analysis is dictated primarily by the metabolic requirements of the cells, subject only to the presence of mM concentrations of calcium (to enable calcium agonist-stimulated calcium influx) and reasonable buffering. Although the new generation of Ca^{2+} indicator dyes are not highly sensitive to small fluctuations of pH over the physiologic range, bicarbonate buffered solutions can impart uncontrolled pH shifts. To circumvent the above problems, RPMI 1640 with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 0.42 mM total calcium concentration are used (see Table I). The use of phenol red as a pH indicator does not impair

Table I

Calcium concentration and pH of RPMI 1640

	RPMI 1640 CM	RPMI 1640
pH* :	7.370	7.479
Ionized $[Ca^{2+}]$,* :	390 μM	250 μM
Total $[Ca^{2+}]$** :	600 μM	420 μM

Measured with the *Ciba-Corning 288 Blood Gas System and the **Kodak EktaChem 700 Analyzer.

the detection of indo-1 fluorescence signals (Shapiro, 1988).

3.9.4. Instrumental technique

Since the indo-1 excitation maximum is within 330-350 nm, depending upon the presence of calcium (June et al., 1987), the indo-1 fluorescence was excited by UV light (150-200 mW) with a fluorescence-activated cell sorter (FACS) 440, 5W argon laser that can be tuned to a range of 351-365 nm, very close to the excitation maximum (Tsien, 1989a). The free dye maximum emission is at 482-495 nm (blue) while the Ca^{2+}-bound dye maximum emission is at 398-405 nm (violet) (Grynkiewicz et al., 1985). The emitted light was split with a 440 nm dichroic splitter. Free indo-1 emission was measured at 495 nm with a 20 nm bandpass filter, and Ca^{2+}-bound indo-1 emission at 405 nm with a 35 nm bandpass filter. The ratio of F_{UV405}/F_{UV495} is the measure of $[Ca^{2+}]_i$.

3.9.5. Controls

A negative control (very low $[Ca^{2+}]_i$) was created by incubating indo-1AM loaded cells in 15 mM EGTA (30 µl 0.5M stock solution, made from 0.5706 g/3 ml Ca^{2+}-free PBS, to 1 ml cell suspension) containing 0.0016% (w/w) saponin (20 µl 0.08% stock solution, made from 0.0008 g/g Ca^{2+}-free PBS, to 1 ml cell suspension) for 1 h at 37°C in 5% CO_2 (Griffioen et al. 1989). This concentration of EGTA and saponin was sufficiently low to allow $\geq$ 90% of the MΦs to remain viable for at least 2 hours after preparation. A positive control (very high $[Ca^{2+}]_i$) was generated by adding 5 µM ionomycin (5 µl of 1 mM stock solution prepared from 1 mg ionomycin/1.4 ml DMSO) to indo-1 loaded cells. Ionomycin-treated cells reach near-saturating levels of $[Ca^{2+}]_i$ (Rabinovitch et al., 1986).

3.9.6. Loading of indo-1 (Rabinovitch et al., 1986; Lazzari et al., 1986; Baud et al., 1987; June and Rabinovitch, 1988; Lopez et al., 1989; Bernardo et al., 1990)

One mM (1 mg in 0.992 ml anhydrous DMSO) indo-1AM stock solution was aliquoted to ten 100 µl vials and stored dessicated in the dark at -20°C. Three microliters of indo-1 were removed from an aliquot and diluted with 1 ml CM. MΦs were washed twice and resuspended to 2 x 10^7 cells/ml in medium (prewarmed to 37°C from here onward). Cells (1 ml) were loaded by incubation with 1 ml of indo-1AM solution (3 µM) to yield a final concentration of 1.5 µM. After the cells were incubated for 15 min at 37°C in the dark, the cell suspension was diluted 7-fold (to reduce toxicity of dye to cells) with 11 ml medium containing 1 ml 30 mM probenecid (8.56 mg probenecid/ml PBS, a final concentration of 2.5 mM), and incubated an additional 15 min for a total of 30 min at 37°C (to allow complete intracellular hydrolysis of indo-1AM to indo-1 free acid, Greenberg et al., 1988)[1] . Cells were then washed twice at 4°C to remove unincorporated indo-1AM, preferably to 10 nM or lower, and resuspended in fresh medium at 2.5 x 10^6/ml. Cells were kept in the dark to prevent any significant leakage of the dye (the efficiency of cellular dye uptake and trapping is about 20%). Fifteen minutes before analysis, indo-1-loaded cells were diluted to 1 x 10^6/ml with CM and equilibrated at 37°C in 5% CO_2. The effects of stimulation on indo-1-loaded MΦs were analyzed by

---

[1] It was found later, that probenecid was not needed as 14M1.4 MΦs do not appear to possess anion transporters that secrete indo-1 efficiently. Indo-1 loading appears to be complete by the end of 30 min incubation as determined by observing no change in fluorescence assessed by flow cytometric analysis. It has been demonstrated that loading is more rapid in monocytes than in lymphocytes due to variation in intracellular esterase activity (June and Rabinovitch, 1988). The lower limit of intracellular concentrations of indo-1 is determined by the sensitivity of fluorescence detection of the flow cytometer and the upper limit is determined by avoidance of buffering of $[Ca^{2+}]_i$ by the presence of the calcium chelating dye itself.

flow cytometry at a rate of 400 cells/s at 37°C[2]. In order to obtain values for resting cells, each sample was run for 60 s before addition of a stimulant (50 µl IL-4 to give a final concentration of 200 U/2 x 10^5 MΦs, or 50 µl ionomycin to give a final concentration of 5 or 50 µM). After addition of the stimulants, data were recorded for a period of 10 min. The flow of cells was halted at this time, and the sample line was flushed to remove stimulated cells before analysis was resumed. Physiologic variation has been documented to be <10% (Rabinovitch et al., 1986) and autofluorescence (fluorescence of unloaded and dead cells) about 5-10% (Ransom et al., 1987).

3.9.7. Determination of source of increased $[Ca^{2+}]_i$

Of primary interest is the source of increased $[Ca^{2+}]_i$. Is it from the release of intracellular storage or from an extracellular influx? The release Ca^{2+} from intracellular stores, independent of extracellular Ca^{2+} influx, can be determined by adding BAPTA-AM, a specific intracellular calcium chelator (Gelfand et al., 1987). Likewise, the increase in $[Ca^{2+}]_i$ from an extracellular influx can be determined by adding 5 mM EGTA (final concentration) to the cell suspension to abolish the usual extracellular-to-intracellular gradient. This reduces the extracellular ionized $[Ca^{2+}]$ nearly 100-fold, to about 15 nM (June et al. 1986) in the presence of RPMI 1640 medium containing 10% heat-inactivated serum and a total calcium concentration of 0.600 µM and ionized calcium of 0.39 µM.

---

[2] Regulation of the temperature of the cell sample is essential, as transmembrane signaling and calcium mobilization are temperature-dependent and active processes. If cells are allowed to cool before they flow past the laser beam, $[Ca^{2+}]_i$ will often decline, so that either the sample input tubing was warmed, or narrow-gauge tubing and high flow rates (e.g., ≥ 50 µl sample/min) was used to keep transit times from the sample uptake port to the flow cell chamber minimized.

3.9.8. Calcium data analysis

Five parameters were recorded: volume (forward angle scatter), free dye by blue fluorescence (fluorescence 1), bound dye by violet fluorescence (fluorescence 2), granularity (side scatter), and time. The blue and violet fluorescence were displayed on a linear scale. The photomultiplier setting for fluorescence was chosen so that the mean ratio for resting cells was between channels 80 and 160 (full scale 1024). Data analysis was performed using the CICERO calcium analysis software (Cytomation, Englewood, CO). Data from indo-1 analyses were stored in 'list mode' for later analysis in which the y-axis represented the bound (violet, 405 nm) to free (blue, 495 nm) indo-1 fluorescence ratio and the x-axis represented elapsed time. The isometric displays were analyzed by CICERO and the mean of the indo-1 violet (405 nm) to blue (495 nm) fluorescence ratio or the percentage of responding cells against time were calculated. The percentage of responding cells was calculated as the proportion of cells having an indo-1 ratio of more than two standard deviations above the mean value for control cells. There were 120 data points on the x-axis (time) on plots of either mean indo-1 ratio or the percentage of responding cells. Each of these data points represents the average value of approximately 2000 cells analyzed during the 5 s that constitute each data point.

3.10. Statistical analysis

Where appropriate, data were expressed as the mean ± standard error of the mean (SEM). In experiments where only two groups were compared, the significance of differences were assessed using a two-tailed Student *t* test (Lutz, 1986) in the Macintosh SE StatWorks™ program. For multiple

group comparisons, the significance of overall difference between the group means was determined by one-way analysis of variance (ANOVA, Lutz, 1986); and between individual groups by Tukey's test for honestly significant difference. Significance between experiments and controls were defined as probability (p) < 0.05. Statgraphics software version 3.0 (STSC, Inc., Rockville, MD) was used for analyses.

CHAPTER 4

FURTHER EVIDENCE OF MURINE RECOMBINANT IL-4 SPECIES SPECIFICITY AS MEASURED BY CHEMILUMINESCENT OXIDATIVE BURST IN MACROPHAGES*

Tan, H. P.[1], C. A. Garberoglio[2], B. H. S. Lau[3], and S. L. Nehlsen-Cannarella[4]

[1]Student who conducted all experiments, collected the data, and prepared the manuscript

[2]Student's clinical research instructor who supervised the research and manuscript preparation

[3]Student's research committee member who supervised the research and manuscript preparation

[4]Student's research committee chairman who reviewed the experimental design, supervised the research, reviewed the data, and supervised the manuscript preparation

---

* **Tan, H. P., C. A. Garberoglio, B. H. S. Lau, and S. L. Nehlsen-Cannarella.** 1991b. Further evidence of murine recombinant IL-4 species specificity as measured by chemiluminescent oxidative burst in macrophages. *Proc. Am. Assoc. Cancer Res. 32:1388.* (abstr., Appendix L) [Please note that part of this abstract has been integrated into this chapter]

FURTHER EVIDENCE OF MURINE RECOMBINANT IL-4 SPECIES SPECIFICITY AS MEASURED BY CHEMILUMINESCENT OXIDATIVE BURST IN MACROPHAGES

4.1. ABSTRACT

We provide further evidence that murine IL-4 is a highly species specific lymphokine that will not cross even the narrow phylogenetic barrier to hamster and perhaps only partially to rat. The release of reactive oxygen intermediates in hamster peritoneal MΦs incubated with IL-4 (over a range of 4-1000 U/ml) for 24, 48, and 72 h were similar to that of non-stimulated controls. However, murine IL-4 induced a small but significant dose-dependent, monoclonal anti-IL-4 irreversible[1] stimulation of reactive oxygen intermediates in rat peritoneal MΦs.

4.2. INTRODUCTION

IL-4, in the presence of macrophages (MΦs), has previously been demonstrated to be tumoricidal *in vitro* and *in vivo*, possibly through MΦ activation (Crawford et al., 1987; Tepper et al., 1989; Golumbek et al., 1991; Redmond et al., 1991; Wong et al., 1992; Lotze, 1992a, b). In mid 1987, it was not known whether murine IL-4 is species specific or that it will cross narrow phylogenetic barriers and has any biological activity on hamster MΦs. The initial goal of this research proposal[2] was to activate hamster MΦs with IL-4 to study MΦ tumoricidal activity against hamster pancreatic adenocarcinoma *in vivo*. Therefore, to determine if hamster

---

[1] Although non-specific stimulation by IL-4 cannot be ruled out.

[2] Immunobiology of Cancer Fund, Loma Linda University, School of Medicine.

cells will response to murine IL-4, hamster MΦs were cocultured with the cytokine and the IL-4-enhanced chemiluminescent oxidative burst was measured. In addition, to further delineate the species specificity of murine IL-4, rat MΦs were also exposed to the cytokine.

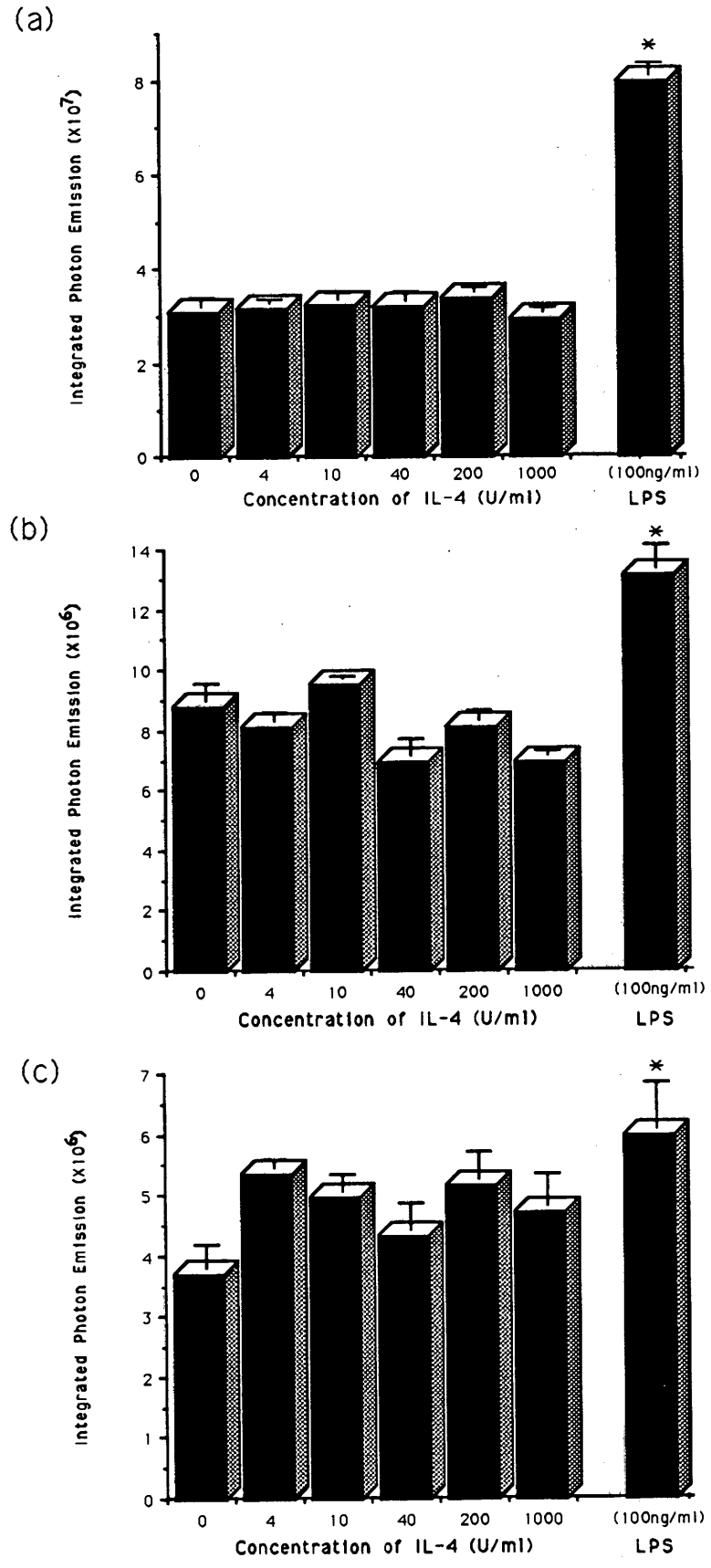
4.3. MATERIALS AND METHODS

Chemiluminescence assays were performed in an automated luminometer (Picolite model 6500, Packard Instrument Co., Downers Grove, IL) interfaced with a PC-compatible computer as previously described (Tosk et al., 1989; Tan et al., 1991d (Chapter 5), 1992b (Chapter 8)). Peritoneal MΦs were obtained from Golden Syrian hamsters and Sprague Dawley rats which had received 3 ml sterile 3% proteose peptone i.p. 3 days previously (Section 3.4.3. of Materials and Methods in this thesis). MΦs were incubated with various concentrations of IL-4 (4-1000 U), incubated for 24, 48, and 72 h and assayed for chemiluminescence. LPS was used as a positive control (Pabst et al., 1982; Tosk et al., 1989).

4.4. RESULTS

4.4.1. Effect of murine IL-4 on hamster peritoneal macrophages

To test if murine IL-4 has any activity on hamster cells, a MΦ activation assay was used to measure the amount of chemiluminescence released by MΦ production of reactive oxygen intermediates. MΦs from peritoneal exudates of golden Syrian hamsters were induced with proteose peptone and treated with various concentrations of IL-4 (0-1000 U) for 24, 48, and 72h. As shown in Fig. 1, there was no effect ($p > 0.05$) of IL-4 on the oxidative burst of hamster peritoneal MΦs when incubated with all concentrations of IL-4 for 24 h (Fig. 1a), 48 h (Fig. 1b) and 72 h (Fig. 1c).


(a)
Integrated Photon Emission (X10⁷)
0
2
4
6
8
*
0
4
10
40
200
1000
(100ng/ml)
Concentration of IL-4 (U/ml)
LPS
(b)
Integrated Photon Emission (X10⁶)
0
2
4
6
8
10
12
14
*
0
4
10
40
200
1000
(100ng/ml)
Concentration of IL-4 (U/ml)
LPS
(c)
Integrated Photon Emission (X10⁶)
0
1
2
3
4
5
6
7
*
0
4
10
40
200
1000
(100ng/ml)
Concentration of IL-4 (U/ml)
LPS

LPS at 100 ng/ml (with or without IL-4) was used as a positive control to induce an enhanced chemiluminescence (Pabst et al., 1982; Tosk et al., 1989). The chemiluminescence of control MΦs triggered with zymosan alone (without IL-4) was from 10^6-10^7 integrated photon emission.

4.4.2. Effect of murine IL-4 on rat peritoneal macrophages

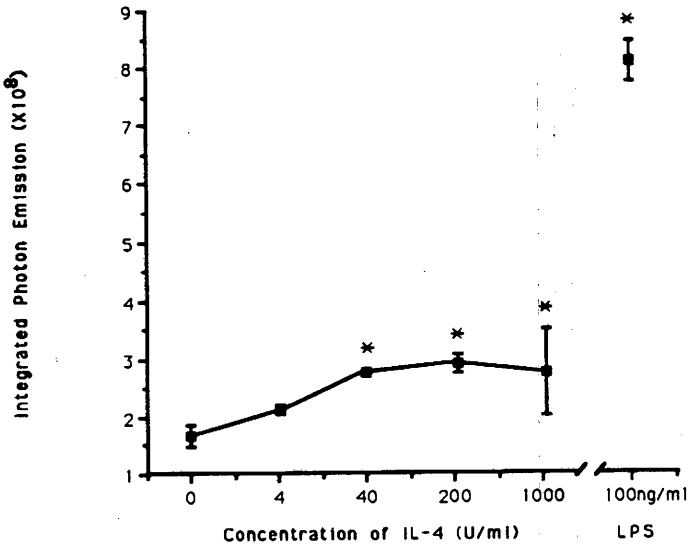
To test if murine IL-4 has activity on rat cells, a chemiluminescence assay was conducted on proteose peptone-elicited peritoneal MΦs from Sprague Dawley rats after incubation with various concentrations of IL-4 (0-1000 U) for 24, 48, and 72h. In contrast to the effect of murine IL-4 on hamster MΦs, murine IL-4 induced a small but significant ($p < 0.05$) dose-dependent stimulation of reactive oxygen intermediates in rat peritoneal MΦs (Fig. 2). After 24 h and 48 h incubation with IL-4, MΦs released significant ($p < 0.05$) amounts of reactive oxygen intermediates at 40-1000 U IL-4 (Fig. 2a, b). With longer IL-4 incubation time (72 h), MΦs showed an enhanced chemiluminescence only with 1000 U IL-4 (Fig. 2c). LPS (100 ng/ml) was used as a positive control (Gardner et al., 1992). However, with 72 h IL-4 incubation time, LPS no longer enhanced the chemiluminescence due to the toxicity of LPS and resulting decreased cell viability.

To test if the effect of murine IL-4 on rat MΦs was wholly dependent on the cytokine, anti-IL-4 mAb was used to block cytokine activity. The stimulation of reactive oxygen intermediate production by IL-4 action on the MΦs was anti-IL-4 irreversible (Fig. 3).

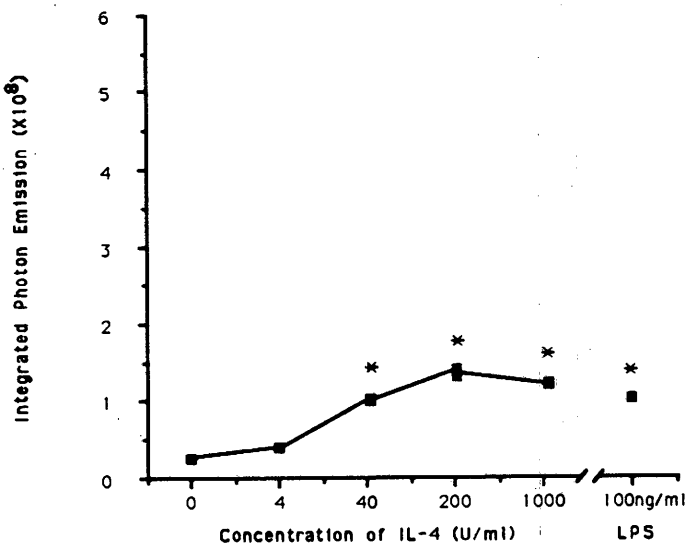
4.5. DISCUSSION

Pancreatic cancer is the fourth most common cause of cancer death in the United States and is recognized as the most lethal of all cancers

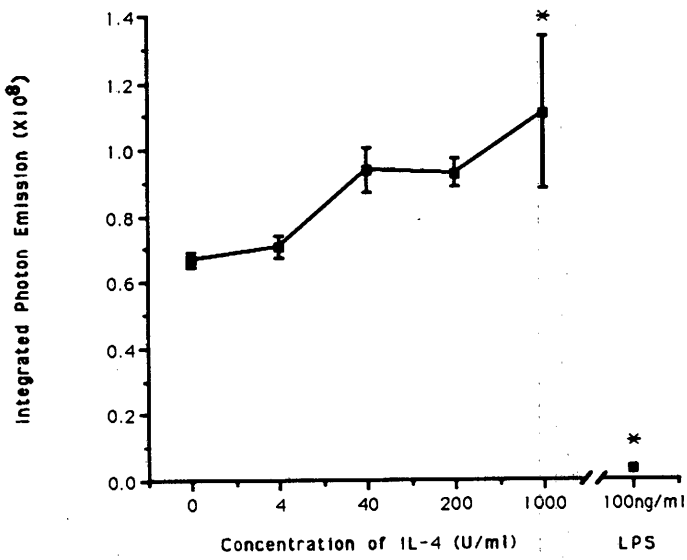
(a)

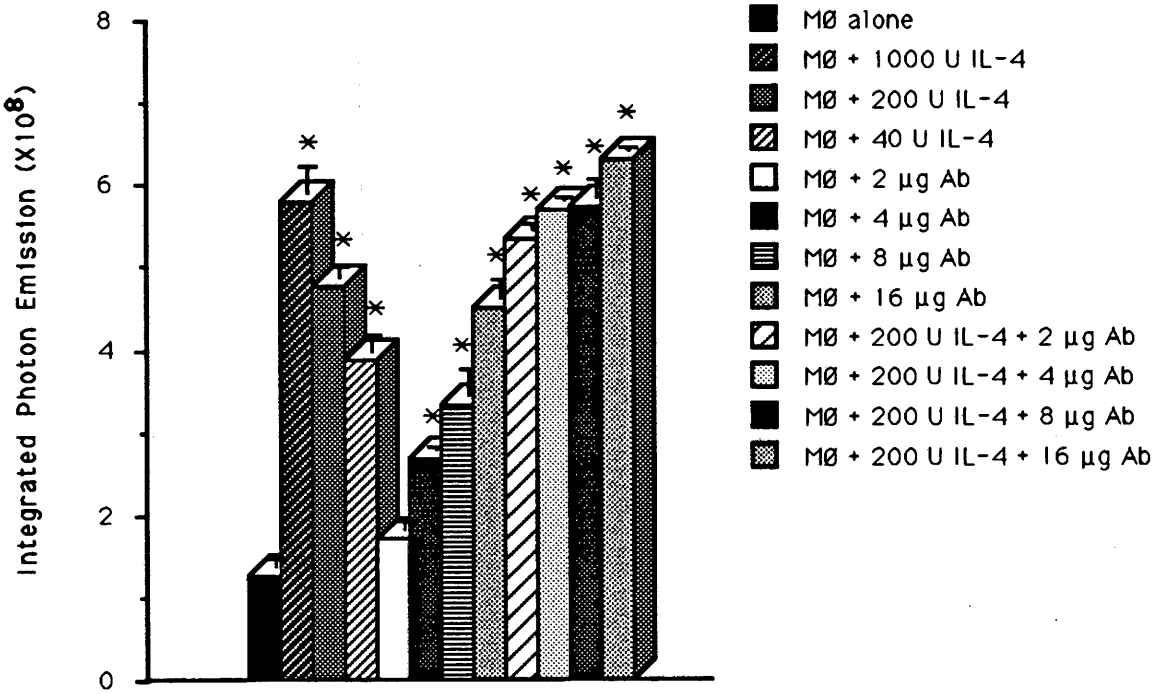

Integrated Photon Emission (X10⁸)
9
8
7
6
5
4
3
2
1
0
4
40
200
1000
100ng/ml
Concentration of IL-4 (U/ml)
LPS

(b)


Integrated Photon Emission (X10⁸)
6
5
4
3
2
1
0
4
40
200
1000
100ng/ml
Concentration of IL-4 (U/ml)
LPS

(c)


Integrated Photon Emission (X10⁸)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
4
40
200
1000
100ng/ml
Concentration of IL-4 (U/ml)
LPS


Integrated Photon Emission (X10⁸)
8
6
4
2
0
MØ alone
MØ + 1000 U IL-4
MØ + 200 U IL-4
MØ + 40 U IL-4
MØ + 2 µg Ab
MØ + 4 µg Ab
MØ + 8 µg Ab
MØ + 16 µg Ab
MØ + 200 U IL-4 + 2 µg Ab
MØ + 200 U IL-4 + 4 µg Ab
MØ + 200 U IL-4 + 8 µg Ab
MØ + 200 U IL-4 + 16 µg Ab

(Silverberg and Lubera, 1987). Even with current technological treatment modalities, we found, in a retrospective chart review, a low overall median survival of approximately 3.9 months (Tan et al., 1986, 1987a, b). The goal of this early research was to develop *in vitro* and *in vivo* hamster models whereby protocols applicable to the treatment of epithelial cancers, e.g. pancreatic adenocarcinoma, can be tested. The Syrian Golden hamster pancreatic cancer model was used since this adenocarcinoma, induced by the transplantable PC-1 tumor cell line, had been demonstrated to share morphological, biological and immunological characteristics with the human disease (Pour et al., 1981; Egami et al., 1989). To initiate this study, we performed the chemiluminescent oxidative burst assay to determine if IL-4-stimulated hamster peritoneal MΦs are activated to release free reactive oxygen intermediates (Tan et al., 1991b). The activation of MΦs and their release of free reactive oxygen intermediates, including O_2^- and H_2O_2, have been correlated with an enhanced ability to kill bacteria (Gabay, 1989), parasites (Wirth et al., 1989), and tumor cells (Wiltrout, 1990).

In mid 1987, it was not known whether murine IL-4 is species specific or that it will cross narrow phylogenetic barriers and has any biological activity on hamster MΦs. However, it was found later that there is no species cross reactivity between murine IL-4 and human cells, and human IL-4 and murine cells (Mosmann et al., 1987; Ohara and Paul, 1987) although both human rIL-4 and murine rIL-4 cDNA clones share 50% homology at the amino acid level as inferred from nucleotide sequence (Yokota et al., 1988) and exhibit similar activities in many instances. Later, Leitenberg and Feldbush (1988) found that murine IL-4 will not induce class II MHC antigen expression on rat B cells. Our results (Tan et al., 1991b) extends this observation of murine rIL-4 as a highly species

specific cytokine that will not cross even the narrow phylogenetic barrier to hamster and perhaps only partially to rat. The species specificity of the ten currently available interleukins, as standardized by the WHO-IUIS Nomenclature Subcommittee on Interleukin Designation (1992), is reviewed in Appendix O.

Interestingly, LPS suppressed the oxidative burst of IL-4-stimulated rat peritoneal MΦs when incubated for ≥ 48 h. This was also seen when murine 14M1.4 bone marrow-derived MΦs were stimulated with LPS for incubation times greater than the optimal period for chemiluminescence (Chapter 8, Tan et al., 1992b). It appeared that LPS when incubated for a longer period of time was toxic to these MΦs, as observed by the slight decrease in viability. It has been demonstrated that LPS will enhance the oxidative burst of J774 MΦs (Tosk et al., 1989) and kill tumor cells *in vitro*, but not of bone marrow-derived MΦs (Tan et al., 1992b; Keller et al., 1992). Another finding was that the stimulation of reactive oxygen intermediates by IL-4 on rat peritoneal MΦs was anti-IL-4 irreversible indicating that non-specific stimulation of the MΦs by IL-4 cannot be ruled out. Indeed, one can see that non-specific stimulation does occur (Fig. 3) probably at the Fcγ receptor (Johnston et al., 1976) of rat MΦs through the binding of anti-IL-4, an IgG molecule.

CHAPTER 5

RECOMBINANT IL-4 ENHANCES THE CHEMILUMINESCENT OXIDATIVE BURST OF MURINE PERITONEAL MACROPHAGES¥

Henkie P. Tan[1], Sandra L. Nehlsen-Cannarella[2], Carlos A. Garberoglio[3], and Jeffrey M. Tosk[4]

[1]Student who conducted all experiments, collected the data, and prepared the manuscript

[2]Student's research committee chairman who reviewed the experimental design, supervised the research, reviewed the data, and supervised the manuscript preparation

[3]Student's research clinical advisor who supervised the research and manuscript preparation

[4]Student's research instructor who reviewed the experimental design, supervised the research, reviewed the data, and supervised the manuscript preparation

---

¥ **Tan, H. P., S. L. Nehlsen-Cannarella, C. A. Garberoglio, and J. M. Tosk.** 1991d. Recombinant IL-4 enhances the chemiluminescent oxidative burst of murine peritoneal macrophages. *J. Leukocyte Biol. 49:587.*

RECOMBINANT IL-4 ENHANCES THE CHEMILUMINESCENT OXIDATIVE BURST OF MURINE PERITONEAL MACROPHAGES

HENKIE P. TAN, SANDRA L. NEHLSEN-CANNARELLA, CARLOS A. GARBEROGLIO, and JEFFREY M. TOSK

Immunology Center (H.P.T., S.L.N.-C.) and the Departments of Microbiology (H.P.T., S.L.N.-C.), Surgery (S.L.N.-C., C.A.G.), Pathology (S.L.N.-C.), and Psychiatry (J.M.T.), Loma Linda University, School of Medicine, and Neuroimmunology Laboratory, Psychiatry Service, Jerry L. Pettis Memorial Veterans' Hospital (J.M.T.), Loma Linda, California

Key words: lymphokine, J774 macrophages, macrophage-activation, growth factor

MAILING ADDRESS
Dr. Jeffrey M. TOSK
Loma Linda University
School of Medicine
Psychiatry Service (116A)
Pettis Memorial Veterans' Hospital
LOMA LINDA, CA 92357

Tel: (714)-825-7084 Extn 2344
FAX: (714)-796-4508

Reprint requests: Jeffrey M. Tosk, Neuroimmunology Laboratory, Psychiatry Service (116A), Pettis Memorial Veterans' Hospital, Loma Linda, CA 92357.

RUNNING TITLE

IL-4 ENHANCES CHEMILUMINESCENCE IN MURINE MΦ

5.1. ABSTRACT

We evaluated the effects of murine recombinant interleukin-4 (rIL-4) on murine peritoneal macrophages. We showed a marked, dose-dependent stimulation of respiratory oxidative burst by IL-4 in peptone-elicited murine peritoneal macrophages. This effect was abolished by a neutralizing monoclonal antibody (mAb) to rIL-4 confirming that the enhanced chemiluminescence was due to IL-4. In contrast, rIL-4 depressed the respiratory oxidative burst of a transformed murine macrophage cell line, J774, in a dose-dependent mAb-reversible manner.

5.2. INTRODUCTION

Interleukin-4 (IL-4) was originally described as a murine B-cell growth factor [10] and has subsequently been shown to have pleiotropic effects on multiple hematological [reviewed in 14, 19] and nonhematological [15] cellular lineages. The effects of IL-4 on macrophages include the induction of class I and class II antigen expression as well as enhancement of their tumoricidal activity in vitro. This has led to the description of IL-4 as a macrophage-activating factor [3,21]. Recently, macrophages have been implicated in IL-4-mediated antitumor activity in vivo [22].

In this study, we have evaluated the macrophage-activating properties of murine IL-4 on the formation of reactive oxygen intermediates (ROI) measured by monitoring zymosan-triggered chemiluminescent oxidative burst. We report here that IL-4 induced a dose-dependent enhancement of chemiluminescence (CL) in murine peritoneal macrophages triggered with zymosan. In contrast, IL-4 inhibited the zymosan-triggered CL in the J774 murine macrophage cell line.

5.3. MATERIALS AND METHODS

Animals

Balb/c mice 8-12 weeks old, purchased from Charles River Laboratories, (Wilmington, MA) were screened and found to be negative for bacterial pathogens, parasites, and subclinical viral infections.

IL-4

Murine rIL-4 was kindly provided by Dr. Steven Gillis (Immunex Corp. Seattle, WA). This protein had a specific activity of 10^8 U/mg, 1 U being defined as half-maximal stimulation in a standard antiimmunoglobulin comitogenesis assay [8].

Anti-IL-4 Monoclonal Antibody (mAb)

Anti-IL-4 (TexStar Monoclonals, Dallas, TX) is an $IgG_1\kappa$ affinity-purified mAb from 11B115E3 (a high producer subclone of 11B11 [18]) hybridoma culture supernatant. This anti-IL-4 mAb blocked the biological activity of IL-4 as measured in HT-2 proliferation, B-cell differentiation factor-γ, and B-cell growth factor-IαIg-costimulation assays.

Cell Preparations

The macrophage cell line J774A.1 was obtained from the American Type Culture Collection (Rockville, MD). J774 macrophages were grown in DMEM (GIBCO, Grand Island, NY) supplemented with 10% heat inactivated defined fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT), penicillin (50 U/ml) and streptomycin (100 µg/ml), hereafter known as complete media (CM). Peritoneal macrophages from Balb/c mice treated 3 days previously with 3 ml sterile 3% proteose peptone (DIFCO, Detroit, MI) in PBS were collected after intraperitoneal injection of CM containing 10 U/ml heparin [3]. Macrophages were withdrawn through the anterior abdominal wall by peritoneal lavage. Harvested macrophages were washed three times in CM for 5 min at 400*g* at 4°C. The pellet was resuspended in CM and a sample taken for differential and total cell counts. The

remainder was centrifuged at 250*g* for 10 min at 4°C and resuspended to 1.3 x 10^6 macrophages/ml. Wright-stained cell smears were prepared by cytocentrifugation and counted on a light microscope. These smears showed >90% macrophages. The viability of cells used in all experiments was >90% as determined by Trypan blue dye exclusion.

CL Assays

CL assays were performed at 37°C in an automated luminometer (Picolite model 6500, Packard Instrument Co., Downers Grove, IL) interfaced with a PC-compatible computer [23]. Experiments were performed in triplicate and repeated at least three times. Peritoneal macrophages and J774 macrophages were made up in DME/F-12 medium without indicator (Sigma) supplemented with 10% FBS and antibiotics. These suspensions were added to 55 X 12 mm plastic luminometry tubes (Los Alamos Diagnostics, Los Alamos, NM). Each tube contained 2 x 10^5 cells in a volume of one ml. Fifty microliters of IL-4 or media were added to cell suspensions and incubated in 5% CO_2 atmosphere at 37°C for 18-24 hr prior to the assay to allow for adherence of cells to the bottom of the luminometry tubes. After incubation the supernatant was removed and discarded. The viability of macrophages was consistently >90% for all incubation times reported in both the J774 and peritoneal macrophages. The CL assay was initiated by the automated addition of 200 µl of a zymosan A suspension. The zymosan A suspension was prepared according to a modified method reported previously [2], and contained per 100 ml complete veronal buffer pH 7.3, 250 mg zymosan A, 200 µl of a 20 mM solution of luminol in DMSO, and 100 µg globulin-free bovine serum albumin. Zymosan A suspensions were prepared in advance and stored frozen at -70°C in 10 ml aliquots. The photon emission from samples was

counted every 8 min for 72 min. The area under each of the curves representing light emission over time was reported as integrated photon emission.

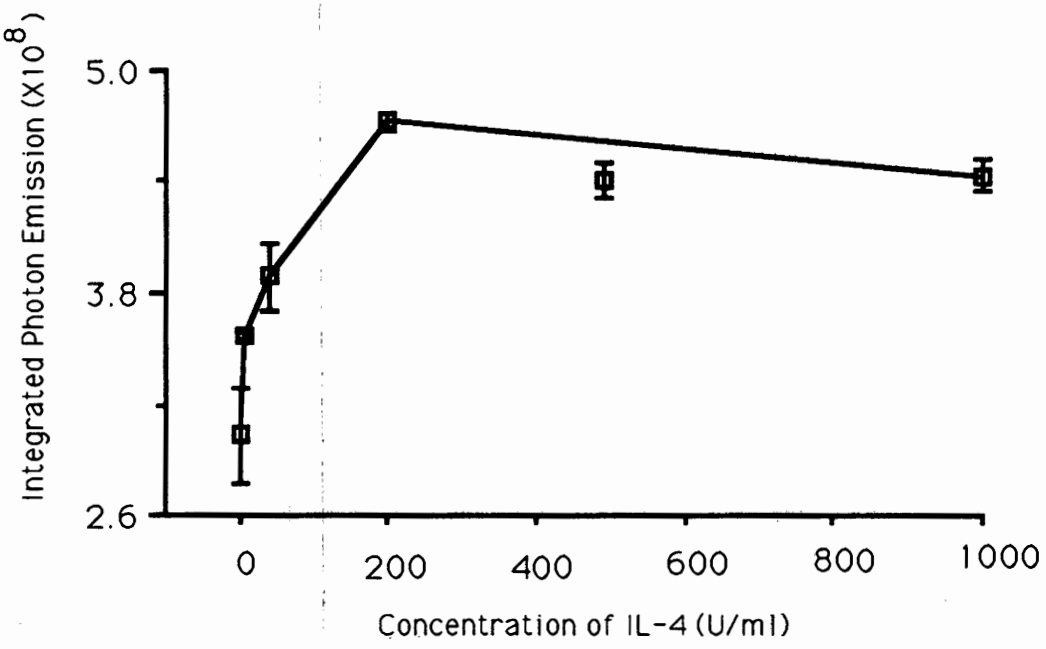
Statistical Analysis

Where appropriate, data were expressed as the mean ± SEM. For multiple group comparisons, the significance of overall difference between the group means was determined by one-way analysis of variance (ANOVA); and between individual groups by Tukey's test for honestly significant difference (HSD). Significance between experiments and controls were defined as $P < 0.05$. Statgraphics software version 3.0 (STSC, Inc., Rockville, MD) was used.

5.4. RESULTS

Murine Peritoneal Macrophages

The production of ROI by activated macrophages is an important mechanism of cytotoxicity. Therefore, we evaluated the production of ROI by activating murine macrophages with IL-4. Macrophages from peritoneal exudates induced by proteose peptone were treated with various concentrations of IL-4 (4-1000 U/ml) for 24 hr (Fig. 1). The CL of these macrophages triggered with zymosan was on the order of 10^7-10^8, integrated over a period of 72 min. Macrophages treated with ≤ 200 U/ml of IL-4 showed a dose-dependent enhancement of oxidative burst as measured by CL emission ($P < 0.05$). In the presence of 200 U/ml of IL-4, macrophages consistently showed an optimal ROI producing capacity twice that of control macrophages. The enhancement of ROI production was also


Integrated Photon Emission (X10^8)
5.0
3.8
2.6
0
200
400
600
800
1000
Concentration of IL-4 (U/ml)

seen at 48, 18, 12 and even 6 hr incubation times [This data is reproduced in Appendix M]. Dosages ≥ 200 U/ml of IL-4 were associated with a plateau in activity.

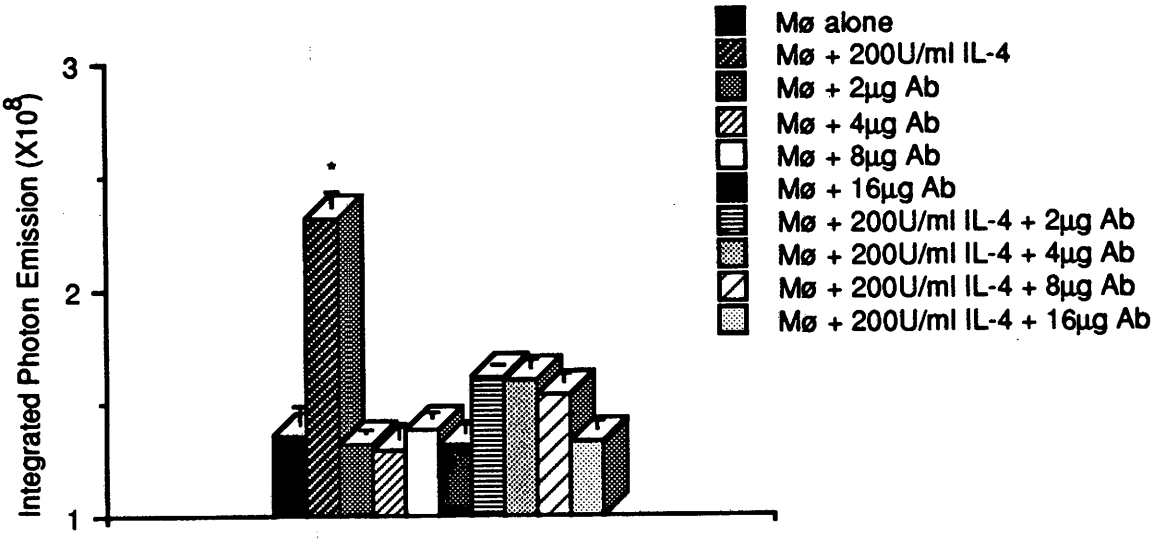
Monoclonal anti-IL-4 had no effect on macrophage CL (Fig. 2). We found that 16 µg/ml of IL-4 mAb completely abolished the CL oxidative burst of peritoneal macrophages activated with 200 U/ml IL-4.

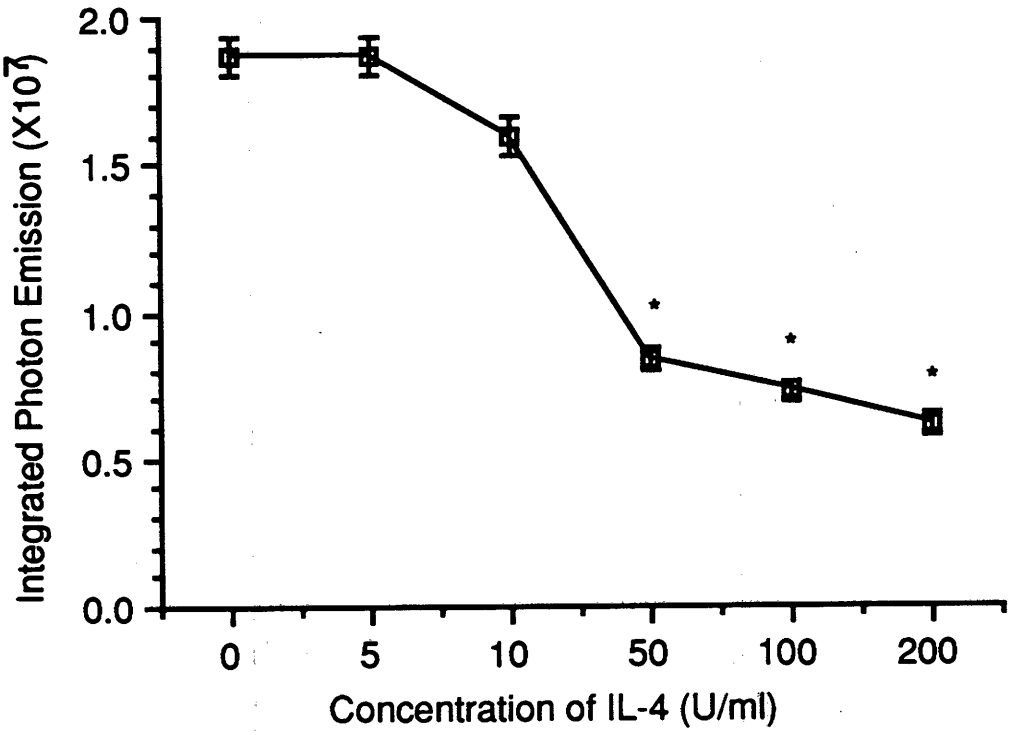
J774A.1 Macrophages

The effect of IL-4 on the oxidative burst of J774 cells is illustrated in Figure 3. In the presence of 200 U/ml of IL-4, ROI production was suppressed in J774 macrophages at 48 hr but no effect was evident at ≤ 12 hr incubation (data not shown). A dose-related suppression of CL emission is evident. This effect was abolished by monoclonal anti-IL-4 (Fig. 4). At a dosage of 8 µg/ml the mAb entirely abrogated the inhibitory activity of IL-4 (100 U/ml). Figure 4 also shows that the mAb had no effect of its own on the oxidative burst of J774 macrophages.

5.6. DISCUSSION

In the present study, we demonstrated that murine rIL-4 enhances CL during oxidative burst in murine peritoneal macrophages in a dose-dependent fashion. On the other hand, the CL exhibited during oxidative burst of the J774 macrophage cell line was significantly suppressed by IL-4. The activation of macrophages and their release of free ROI, including O_2^- and H_2O_2, have been correlated with an enhanced ability to kill bacteria [reviewed in 6], parasites [25], and tumor cells [reviewed in 24]. This is in contrast to results obtained with human mononuclear


Integrated Photon Emission (X10^8)
3
2
1
*
Mø alone
Mø + 200U/ml IL-4
Mø + 2µg Ab
Mø + 4µg Ab
Mø + 8µg Ab
Mø + 16µg Ab
Mø + 200U/ml IL-4 + 2µg Ab
Mø + 200U/ml IL-4 + 4µg Ab
Mø + 200U/ml IL-4 + 8µg Ab
Mø + 200U/ml IL-4 + 16µg Ab


Integrated Photon Emission (X10⁷)
2.0
1.5
1.0
0.5
0.0
0
5
10
50
100
200
Concentration of IL-4 (U/ml)