5-1983

Cell-Mediated Immunity of the Dog Lung

Jennifer Baker Galvin

Utah State University

Follow this and additional works at: https://digitalcommons.usu.edu/etd

Part of the Chemistry Commons

Recommended Citation

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations by an authorized administrator of DigitalCommons@USU. For more information, please contact dylan.burns@usu.edu.
CELL-MEDIATED IMMUNITY OF THE DOG LUNG
AND EFFECTS OF INHALED 239PuO2 OR
PULMONARY TUMORS

by

Jennifer Baker Galvin

A Dissertation submitted
in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY
in
Toxicology

Approved:

Major Professor, USU Committee Member

Committee Member Committee Member

Committee Member Dean of Graduate Studies

Utah State University
Logan, Utah
1983
ACKNOWLEDGMENTS

There have been many people involved in helping make this research work possible; therefore, let me start at the beginning.

I thank Dr. S.G. Oberg for accepting me into the USU toxicology program and guiding the commencement of my scientific career. Your guidance has resulted in a good scientific foundation. A special thanks to Dr. D.E. Bice, who has patiently helped me build on that foundation. I appreciate the opportunity to have worked in your laboratory under your guidance.

My appreciation is extended to Associated Western Universities and Dr. R.O. McClellan for making my research at the Inhalation Toxicology Research Institute possible. This research was conducted at the Lovelace Inhalation Toxicology Research Institute under U.S. Department of Energy Contract No. DE-AC04-76EV01013 and in animal facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

My thanks are extended to Dr. D.E. Bice, Dr. A.L. Brooks, Mrs. D.L. Harris, Mr. Lynn Byers, Dr. R.O. McClellan, and Dr. B.A. Muggenburg for reviewing this document and contributing constructive comments to its improvement.

I want to thank my committee, Dr. M.C. Healey, Dr. S.G. Oberg, Dr. R.P. Sharma, and Dr. R.P. Warren, for
their advice and review of this document.

Each project tended to involve a different group of people at the Institute and I enjoyed the team effort during each project. In my work with dogs, I appreciated the technical help of the veterinary medicine hospital staff and the personnel of the clinical pathology laboratory. In particular, a special thanks is extended to Dr. Bruce Muggenburg for making all those hours lavaging dogs a joy. Also my thanks to Vicki White, who was always willing to help.

I would like to express my gratitude to the Inhalation Toxicology Research Institute aerosol science personnel and radiobiologists for exposing the dogs to plutonium and allowing me to use them in these experiments. A special thanks is extended to Dr. Ray Guilmette for providing me with exposure information about these dogs and helping me with dose calculations.

There are various people at the Institute who have provided me with scientific, technical, and clerical support, who deserve mention for their unselfish willingness to help me. Diane Fehrenbach deserves special recognition for her help, cooperation, and friendship. We made a nice team in the laboratory. Other people include Larry Martinez, Richard Jaramillo, Bob Smith, Susan Barnett, Kathy Epler, Bill Griffith, and Emerson Goff.

A special thanks goes to Kathy Sass who has given me a place to retreat and personal support during my stay in New Mexico. Mrs. D.L. Harris also deserves a special thanks.
for providing personal, as well as scientific support during my research work. A special thanks goes to Barbara Ahern in Utah who has accepted responsibility for me as a result of my absence from the university.

My greatest appreciation goes to my family who has always offered me support and encouragement, and, especially to my husband, Mark, who has and I know will continue to "support and encourage my ideas and accept me as the individual I am".
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>xii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
</tbody>
</table>

## CHAPTER

### I. LITERATURE REVIEW

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CELL-MEDIATED IMMUNITY</td>
<td>3</td>
</tr>
<tr>
<td>IMMUNITY AFTER LUNG IMMUNIZATION</td>
<td>9</td>
</tr>
<tr>
<td>COAGULATION AND IMMUNITY</td>
<td>13</td>
</tr>
<tr>
<td>COAGULATION AND TUMORS</td>
<td>18</td>
</tr>
<tr>
<td>ADJUVANT, MURAMYL DIPEPTIDE</td>
<td>21</td>
</tr>
<tr>
<td>PLUTONIUM</td>
<td>27</td>
</tr>
</tbody>
</table>

### II. CELL-MEDIATED IMMUNITY OF THE DOG LUNG: PRIMARY AND SECONDARY RESPONSES

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>32</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>33</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>35</td>
</tr>
<tr>
<td>RESULTS</td>
<td>41</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>52</td>
</tr>
</tbody>
</table>

### III. EFFECT OF INTRAVENOUS OR INTRAPULMONARY ADMINISTRATION OF MURAMYL DIPEPTIDE ON PULMONARY CELL-MEDIATED IMMUNITY

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>57</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>58</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>60</td>
</tr>
<tr>
<td>IV. PULMONARY IMMUNE RESPONSE OF DOGS AFTER EXPOSURE TO 239Pu02</td>
<td>Page</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>ABSTRACT.</td>
<td>64</td>
</tr>
<tr>
<td>INTRODUCTION.</td>
<td>74</td>
</tr>
<tr>
<td>MATERIALS AND METHODS.</td>
<td>77</td>
</tr>
<tr>
<td>RESULTS.</td>
<td>77</td>
</tr>
<tr>
<td>DISCUSSION.</td>
<td>78</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>V. PULMONARY PROCOAGULANT ACTIVITY OF DOGS WITH LUNG TUMORS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT.</td>
<td>99</td>
</tr>
<tr>
<td>INTRODUCTION.</td>
<td>99</td>
</tr>
<tr>
<td>MATERIALS AND METHODS.</td>
<td>100</td>
</tr>
<tr>
<td>RESULTS.</td>
<td>101</td>
</tr>
<tr>
<td>DISCUSSION.</td>
<td>106</td>
</tr>
</tbody>
</table>

| VI. SUMMARY.                                               | 116  |
| LITERATURE CITED.                                          | 119  |
| VITA.                                                      | 143  |
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Skin Test Data</td>
</tr>
<tr>
<td>3.1</td>
<td>MDP Dosing Regimen Relative to SRBC Immunization (0 time) in the Dog Lung</td>
</tr>
<tr>
<td>4.1</td>
<td>The Aerosol Data from the 239Pu02-exposed Dogs. Exposure to Monodisperse Aerosols Occurred from 77140-78006</td>
</tr>
<tr>
<td>5.1</td>
<td>Procoagulant Activity</td>
</tr>
<tr>
<td>5.2</td>
<td>Bronchoalveolar Cell Migration Areas</td>
</tr>
<tr>
<td>5.3</td>
<td>Percent Cell Types in the Different Lung Lobes</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>The effect of SRBC antigen on the procoagulant activity of cells from the control, and immunized lung lobes, and blood after primary and secondary immunizations. Each point represents the mean of four dogs ± SEM, with the exception of day 7, which had data from two dogs for the blood and control lung lobe.</td>
</tr>
<tr>
<td>2.2</td>
<td>The effect of SRBC antigen on the migration of cells washed from the control and immunized lung lobes after primary and secondary immunizations. The percent inhibition of migration was determined by comparing the mean size of four migration areas in culture medium with antigen to the mean size of four migration areas of cells from the same lobe in culture medium without antigen. Each point represents the mean of four dogs ± standard error of the mean (SEM).</td>
</tr>
<tr>
<td>2.3</td>
<td>The effect of primary and secondary immunizations on the spontaneous migration of alveolar macrophages. The difference between migration areas of the cells from the control and immunized lung lobes cultured in medium. Each point represents the mean of four dogs ± SEM.</td>
</tr>
<tr>
<td>2.4</td>
<td>Lymphocytes, neutrophils (PMNs), and alveolar macrophages in the lung lavage fluid from the immunized left cardiac lung lobe from the primary and secondary immunizations. Each point represents the mean of four dogs ± SEM.</td>
</tr>
<tr>
<td>2.5</td>
<td>The cell types lavaged from the control lung lobes after primary and secondary immunizations. Due to a technical error the cell types from the control lobe for day 21 are not shown. Each point represents the mean of four dogs ± SEM.</td>
</tr>
<tr>
<td>2.6</td>
<td>ELISA optical density units with the background subtracted are plotted for the antigen-specific IgG present in the lavage fluid of the immunized and control lung lobes. The control lobe</td>
</tr>
</tbody>
</table>
antibody after challenge was not significantly different from background and is not shown. Each point represents the mean of four dogs ± SEM. ........................................ 50

2.7 ELISA optical density (O.D.) units with the background subtracted are plotted for the antigen-specific IgM present in the lavage fluid of the immunized and control lung lobe. Each point represents the mean of four dogs ± SEM. 51

3.1 The effects on procoagulant activity of cells from the control and immunized lung lobes after instillation of SRBC antigen and muramyl dipeptide (MDP) administered by two different routes. The intravenous MDP treatment is represented by the broken line. Each point represents the mean of four dogs ± SEM. For clarity, the error bars were not added to the antigen only treatment group, but they can be found on the figures in Chapter 1................................. 65

3.2 The percent shortening of plasma clotting time after cells isolated from the blood of all three groups were cultured 20 hours with and without SRBC antigen. Each point represents the mean of four dogs ± SEM......................... 67

3.3 The effect of SRBC antigen on the migration of cells washed from the control and immunized lung lobes of Beagle dogs. The percent inhibition of migration was determined by comparing the mean size of four migration areas in culture medium with 200 ug/ml SRBC antigen to the mean size of four migration areas of cells from the same lobe in culture medium without antigen. Each point represents the mean of four dogs ± SEM..........68

3.4 Lymphocytes, neutrophils (PMNs) and alveolar macrophages in the lung lavage fluid from the immunized lung lobes of dogs that received MDP iv and a second group that received MDP intrabronchially with antigen are presented. Each point represents the mean of four dogs ± SEM. The antigen only cytologies are pictured on the next page......................... 70

3.5 Lymphocytes, neutrophils (PMN), and alveolar macrophages in the lung lavage fluid from the
3.6 The control lung lobe cell populations: Lymphocytes, PMN, and alveolar macrophages in the lung wash fluid from dogs that received MDP iv and a second group that received MDP intrabronchially with antigen. .......................... 73

4.1 The serum antigen-specific IgG antibody from 0 to 20 days after immunization with SRBC for the control age-matched control dogs. Each line represents the immune response of an individual dog. .......................... 85

4.2 The IgG antigen-specific antibody of the lavage fluid from the immunized lobe of the age-matched control dogs. Each point is the value for the ELISA optical density unit of an individual dog. .......................... 86

4.3 The serum antigen-specific IgG antibody from 0 to 20 days after immunization with SRBC for dogs exposed to plutonium. Each line represents the immune reponse of an individual dog. .......................... 88

4.4 The antigen-specific IgG antibody in the control (A) and immunized (B) lung lobe lavage fluid from the plutonium-exposed dogs. Each point represents the value of an individual dog. .......................... 90

4.5 The control lung lobe cell population: total number of PMN (A) and lymphocytes (B) in the lung wash fluid from dogs exposed to 239Pu02. Each point represents the total number of cells (10⁶) from an individual dog. .......................... 91

4.6 The immunized lung lobe cell population: total PMN (A) and lymphocytes (B) in the lung lavage fluid of dogs exposed to 239Pu02. Each point represents the value of the total cells (10⁶) from an individual dog. .......................... 92

4.7 The effect of SRBC antigen immunization on the procoagulant activity of cells from the
immunized lung lobe of plutonium-exposed dogs. Each point represents the value of an individual dog. 94

5.1 The means of the plasma clotting time and macrophage migration areas are plotted. Each point represents these values for one dog. The migration assay required many cells, and there was frequently not enough cells to do this assay. The r values represent the correlation coefficients for the lines. 109

5.2 Tumor cells lavaged from the tumor-bearing lung lobes. There are numerous PMN surrounding the agglutinated tumor cells, along with red blood cells (RBC). (Papanicolaou stain, 56X magnification).
## LIST OF ILLUSTRATIONS

### ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Illustration</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Coagulation Cascade Scheme</td>
<td>16</td>
</tr>
<tr>
<td>1.2</td>
<td>Cellular Interactions Resulting in Activation of the Extrinsic Coagulation System</td>
<td>18</td>
</tr>
</tbody>
</table>
ABSTRACT

Cell-mediated Immunity of the Dog Lung
and Effects of Inhaled 239Pu02 or
Pulmonary Tumors

by

Jennifer Baker Galvin, Doctor of Philosophy
Utah State University, 1983

Major Professor: Dr. S.G. Oberg
Department: Toxicology

Cell-mediated immunity (CMI) in the lung has not been well characterized due to the lack of applicable tests. A major objective was to define pulmonary CMI serially in immunized and control lung lobes using the leukocyte procoagulant activity (LPCA) assay in young adult dogs. This was compared to a standard, but less reproducible CMI assay, macrophage migration inhibition factor (MIF). The CMI response, as measured by the LPCA assay, peaked in the blood 7 days after lung immunization. The pulmonary CMI response measured with cells obtained by bronchial lavage from the immunized and control lung lobes peaked at 9 to 12 days after intrapulmonary immunization with 10xx sheep red blood cells (SRBC). This peak pulmonary immune response corresponded with the day of lymphocyte influx into the lung.

An attempt was made to increase the level of CMI in the lung by administration of muramyl dipeptide by two different routes. The administration of the adjuvant muramyl
dipeptide (MDP) into the lung or given intravenously suppressed the CMI response in the lung after instillation of antigen (10xx SRBC).

Four dogs were exposed to 239PuO2 when the dogs were one year old. When the dogs were 6 to 7 years old the pulmonary CMI was evaluated after lung immunization. Age-matched control dogs were immunized for comparisons. The noticeable difference between the control and plutonium-exposed dogs was the dramatic cellular changes produced in the control and immunized lung lobes of the plutonium-exposed dogs. Inhalation of plutonium in addition to sequential lavages produced high numbers of neutrophils to be recruited to the lung. However, the effects of inhaled 239PuO2 on pulmonary CMI of 6 to 7 year old dogs were obscured by the low pulmonary immune response in the age-matched control dogs. The age-matched control dogs showed no cellular changes in either the saline lung lobe or the immunized lung lobe. This result was attributed to the age of the dogs. The control dogs produced high amounts of immunoglobulins as measured in the serum, however, they could not recruit these serum immunoglobulins into the lung. The plutonium-exposed dogs showed a similar immunoglobulin immune response.

The effects of naturally occurring tumors or those produced by inhaled radioactive compounds were evaluated for their effects on the procoagulant activity assay and spontaneous macrophage migration. The procoagulant activity of the lung is significantly increased if a tumor is present.
in the lung. The migration area of the cell population lavaged from the tumor-bearing lung lobe was significantly increased over control lobe migration areas. (161)
INTRODUCTION

Pulmonary defense mechanisms are constantly challenged by inhalation of infectious agents and air pollutants. The immunological mechanisms responsible for defense of the lung are humoral immunity, cell-mediated immunity (CMI), and macrophage functions. Of these three, the cell-mediated immune response in the lung is the least characterized.

The primary objective of this research was to define the pulmonary CMI response after deposition of antigen and, hence, the pulmonary CMI response after inhalation of a toxic material. This research resulted in four distinct yet interrelated manuscripts that describe the characterization of the cell-mediated immune response of the dog lung. This research is unique in that a relatively new method for examining CMI was applied to the cell population lavaged from the lung.

Each manuscript in this dissertation was written as an independent manuscript within the constraints of the format required by Utah State University. Each manuscript includes an abstract, introduction, materials and methods sections, results, and discussion. All references are found at the end of the summary section (Chapter VI).
STUDY OBJECTIVES

The purpose of this investigation was to evaluate the cell-mediated immune response in the dog lung after immunization of a specific lung lobe with antigen. Of central importance to this evaluation was the development of an assay for CMI that would use the proportions and types of cells lavaged from the lung. After this evaluation was completed and the primary immune response had waned, a secondary response to a lower antigen dose was evaluated in the same animals. Little information is available to explain how the lung mounts a secondary response to antigen.

Adjuvants are used in many facets of immunology research as tools to enhance the immune response. The immune modulating effects of muramyl dipeptide given by two different routes of administration, intravenous and intrapulmonary were investigated. Since this adjuvant is water soluble, it is one of the first adjuvants that could be instilled into the lung to evaluate local immunity.

The third section of this study evaluated the effects of inhaled 239PuO2 on pulmonary cell-mediated immunity. The final section of this investigation examined the relationship of pulmonary tumors induced by inhaled radioactivity and procoagulant activity of cells from tumor-bearing and control lung lobes. A relationship between spontaneous macrophage migrations and procoagulant activity was also evaluated.
CHAPTER I

LITERATURE REVIEW

CELL-MEDIATED IMMUNITY

In the last decade there has been a heightened interest in cell-mediated immunity (CMI) as functions of CMI have become better understood. Some of the functional aspects associated with the CMI system are delayed hypersensitivity (David and David, 1972; Turk, 1980; Dutton and Swain, 1982), graft versus host reactions (Grebe and Streilein, 1976; Stein-Streilein et al., 1981), contact sensitivity (Claman and Miller, 1980; Polak, 1980), autoimmune diseases (Kruisbeek et al., 1981; Traugott et al., 1983; Holoshitz et al., 1983; Marx, 1983), tumor surveillance (Herberman, 1974; Civan et al., 1979; Oka, 1981; Pross et al., 1981) and recognition of virus and other infectious agents (Ennis et al., 1977; Clancy et al., 1977; Yap et al., 1978; Braciale and Yap, 1978). There have been many studies examining the natural Killer cell and the null cell, and although these cells have many lymphoid cell characteristics they are not true T lymphocytes (thymus derived) (Eremin et al., 1980). The following are accepted characteristics of cell-mediated immunity: a) it must be possible to transfer reactivity to an unsensitized recipient by injection of cells rather than serum alone, b) responses are delayed in onset by several hours, c) hypersensitivity responses exhibit a cellular infiltration with perivascular cuffing
that is predominantly mononuclear in nature, and d) it exhibits carrier protein specificity (Turk, 1980). There is an exception to the above profile; i.e., Jones-Mote hypersensitivity displays only some of the criteria listed above. The term CMI, however, is still applicable.

The T cell plays the central role in cell-mediated immune reactions through its cytolytic activity and through the production of soluble mediators (Lawrence and Landy, 1969). For many years, it was thought that T cell reactions were only cytolytic. A limiting factor in studying the CMI system was developing a suitable in vitro model. A new model to study CMI was developed when George and Vaughan (1962) showed migration inhibition from capillary tubes of peritoneal exudate cells taken from tuberculin-sensitive guinea pigs when tuberculin was added to the cell migration chambers. In the same year, Willoughby et al., (1962) suggested that a soluble mediator was involved. David et al., (1964) showed that the lymphocyte reacting with the antigen was responsible for inhibition of the macrophage migration. Subsequently, David (1966), concurrently with Bloom and Bennett (1966), showed that sensitized lymphocytes released soluble mediators into culture medium. This was the first CMI mediator identified, and it was named for its function, macrophage migration inhibition factor (MIF). As different test systems evolved, different functions of the mediators were found; macrophage activating factor (Nathan et al., 1971; Adams et al., 1973), mitogenic factor (Mills, 1975), macrophage aggregation factor (Lolekha et al., 1970).
chemotactic factors for monocytes and neutrophils (Ward et al., 1970) and lymphotoxin (Remold, 1972), and many others that will not be named. Because the macrophage was thought to be the primary site of action for these mediators many were named to describe their action on macrophages. Since then, mediators have been found to act on a number of other cell types, including fibroblasts, osteoclasts, vascular endothelial cells, and other lymphocytes. In 1969, the general term "lymphokine" was adopted to describe the soluble mediators released from antigen-stimulated T lymphocytes (Dumonde et al., 1969).

During the ten years after the soluble mediators were classified under the name "lymphokine," other soluble mediators were found and studied. These have regulatory effects on other lymphocytes and are thought to be produced by monocytes/macrophages and by lymphocytes. Hence, these regulatory mediators, called monokines and lymphokines, were classified as Interleukin I (IL 1) and Interleukin II (IL 2), respectively (Aarden et al., 1979). A group at the Second International Lymphokine Workshop proposed that IL 1 was produced by macrophages, and IL 2 required T lymphocytes and macrophages for production. Since then, it has been shown that T cells produce IL 2 (Mizel and Ben-Zvi, 1980), and a monoclonal antibody has been used to identify both IL 2-producer and -responder cells (Steinmann et al., 1983).

The question may be asked, "Why do we need another lymphokine classification?" IL 1 and IL 2 are different from other lymphokines primarily because they regulate the
response of other lymphocytes. Under the old system lymphokines effected many different cell types, only interleukins effect other lymphocytes. IL 1 and IL 2 encompass various biological effects of two biochemical entities once thought to be many different lymphokines. IL 1 produces the effects previously named as lymphocyte activating factor (LAF), mitogenic protein, B cell activating factor, and B cell differentiating factor. IL 2 has previously been called T cell growth factor, factor-dependent mitogenic activity, antibody helper activity, and cytotoxic T cell helper activity (Hanson et al., 1982). T cell growth factor is best known for facilitating the establishment of T lymphocyte lines.

Regulation of lymphokine-mediated effects can be achieved at two levels, control at the level of lymphokine production (either suppression or augmentation) and at the level of lymphokine expression. IL 1 exerts its influence at the level of lymphokine production. Macrophages produce IL 1 in response to a variety of stimuli, many of which are known adjuvants (Simon and Willoughby, 1981). IL 1 formation may contribute to immune stimulation by adjuvants (Oppenheimer et al., 1979). At first it was thought that IL 1 augmented the action of mitogen or antigen, but more detailed studies indicate that IL 1 and IL 2 work in concert (Lachmann and Maizel, 1982).

Lymphocyte stimulation is thought to occur in two steps (Hanson et al., 1982). There is direct stimulation of the lymphocyte by the antigen or mitogen. This is independent
of the presence of macrophages and causes the lymphocyte to pass through the initial step of cell cycling and become susceptible to the effect of IL 2. The subsequent step is the production of IL 2 by T helper cells and is macrophage-dependent as well as dependent on IL 1. The monokine and the lymphokine, IL 1 and IL 2, therefore, synergistically augment lymphokine production after lymphocyte stimulation by an antigen or mitogen.

The metabolism of prostaglandins is important in the regulation of lymphokine expression. Prostaglandins are oxygenated derivatives of arachidonic acid and have long been known to stimulate adenylate cyclase and thus act as inhibitors of activation of many cells, including lymphocytes (Smith et al., 1971). In contrast, it was also shown that metabolites of arachidonic acid, thromboxanes, can markedly enhance lymphocyte activation. This phenomenon has been extensively studied, and results suggest that these two processes are due to a divergence of metabolism of arachidonic acid: thromboxanes are stimulatory and prostaglandins are inhibitory for effects on lymphocytes (Demenkoff et al., 1980). What signals one or the other metabolite to be formed is unknown.

It has been established that the alveolar macrophage can produce all the metabolites of arachidonic acid; prostaglandins (Morley, 1981), thromboxanes (Morley et al., 1979) and leukotrienes (Rouzer et al., 1982). It was just stated that prostaglandins and thromboxanes have opposite effects on the lymphocyte. A third group of metabolites,
leukotrienes are mediators of immediate hypersensitivity reactions and inflammation (Samuelson, 1983). It is well documented that macrophages can suppress immune responses (Demenkoff et al., 1980; Pennline et al., 1979; Holt and Batty, 1980). It has also been shown that macrophages can enhance the immune response (Simon and Willoughby, 1981). Alveolar macrophages, in some instances, are superior to other macrophage populations for enhancing (Gorenberg and Daniele, 1978) or suppressing the immune response (Holt, 1979). Again, the macrophage and the immune system are intricately linked.

MIF remains one of the most studied and well-characterized lymphokines. The biochemical properties of MIF have primarily been studied in the guinea pig (Yoshida, 1979); however, other species have been characterized (Rocklin et al., 1972; Schnizlein and Bice, 1980). The literature abounds with conflicting reports as to the nature, molecular weight, charge, and stability of MIF. This situation is due to the use of many different culture conditions, and the use of different antigens or mitogens for stimulating lymphocytes. Even the particular culture medium used seems to change the properties of MIF. Several articles are available that list the properties of the known lymphokines (David and David, 1972; Hanson et al., 1982). In brief, MIF, like many other lymphokines, is stable at 56°C for 30 minutes, but is rapidly destroyed at higher temperatures. Guinea pig MIF is stable for 3-7 days after storage in solution at 4°C and can be frozen for
several months; however, MIF is unstable to repeated freeze-thawing (Hanson et al., 1982).

The macrophage is considered the major target of MIF. At first it was thought that alveolar macrophages did not have receptors for MIF (Leu et al., 1974). Later, it was shown that all macrophages, including alveolar macrophages (Bice et al., 1977), are influenced by MIF. The lymphokine's interaction with the macrophage as measured in vitro results in inhibited macrophage movement and an increased stickiness. In time, the macrophages spread out more and become more mobile than normal. After 3 days in culture with MIF-rich Sephadex fractions, macrophages increase their phagocytic rate concomitantly with increased glucose oxidation (David and David, 1972). Ultrastructurally, the macrophage undergoes increased ruffling of microvilli, an increased number of phagocytic vacuoli, an increased cytolytic granule incidence, and an enlargement of the endoplasmic reticulum (Forteza-Vila et al., 1972). The initial decreased mobility of macrophages in vitro has been construed to be an in vivo mechanism for keeping macrophages at an inflammatory site.

IMMUNITY AFTER LUNG IMMUNIZATION

The lung is a major route of entry into the body for infectious agents, air pollutants, and carcinogens. Until recently, the lung was not studied extensively because of its relative inaccessibility to researchers. The advent of bronchoalveolar lavage has provided an invaluable tool for
evaluating responses of the lung to various substances and during disease processes (Hunninghake et al., 1979).

Because of the technique of bronchoalveolar lavage, the immune response of the lung is better understood now than it was a few years ago. The bronchoalveolar cell population that can be washed from the lungs consists chiefly of macrophages, but differs for each species. The rat has about 100% macrophages in fluid lavaged from its lungs, whereas guinea pigs have about 50% macrophages (Hunninghake and Fauci, 1976). The dog (Rebar et al., 1980), monkey (Kazmierowski et al., 1977), rabbit (Ford and Kuhn, 1973), and human nonsmoker (Reynolds and Newball, 1974) have from 85-90% macrophages in lavage fluid, with the remainder being primarily lymphocytes. Hunninghake et al. (1979) report that approximately 73% of the alveolar lymphocytes are T cells, and 7% are B cells. The remaining 19% of alveolar lymphocytes do not have surface markers that react with conventional reagents and are classified as "null" cells.

The macrophage has recently gained attention as an antigen-presenting cell (Lipscomb et al., 1981). In vitro studies have shown macrophages to be essential for induction of the following immune responses: stimulation of T lymphocytes to proliferate (Hersh and Harris, 1968), provision of T helper activity for antibody production (Pierce and Kapp, 1976), and production of mediators of cellular immunity (Epstein, 1977). The exact mechanism of this lymphocyte/macrophage interaction is unknown. Some studies indicate that alveolar macrophages that have
phagocytized antigen may leave the alveoli and migrate to lymphoid tissues as a form of antigen presentation (Stein-Streilein et al., 1981). Most data, however, suggest that alveolar macrophages do not move from the alveoli into the interstitial tissues of the lung (Brain et al., 1978). It would also seem possible that the phagocytized antigen is degraded and excreted in low molecular weight forms. Such excreted antigens may be the immunogenic material that enters the lymphatics and reaches the lung-associated lymph nodes.

The results of several studies with rodents (Bice et al., 1978), rabbits (Bice and Muggenburg, 1982), and dogs (Bice et al., 1980a) indicate that the lung-associated lymph nodes are a primary site for the induction of immunity to particulate antigen deposited in the lung. However, immune responses are also found in the spleen after exposure of the lung to higher concentrations of antigen (Stein-Streilein and Hart, 1980). In dogs, the responses in the lung-associated lymph nodes consisted of typical histological changes normally seen in immune responses, starting two days after immunization (Brownstein et al., 1980).

Antibody response to inhaled or instilled antigen has been the principal immune response studied (McDermott et al., 1982; Tomasi and Bienenstock, 1968; Bice and Muggenburg, 1984). The pulmonary cell-mediated immune response has received less attention. One of the first reports of T cells taking part in pulmonary defense was by
Henney and Waldman (1970), who showed that bronchoalveolar cells could inhibit the migration of peritoneal macrophages by elaborating a lymphokine, MIF. This suggested that T cells were present in the lung. Since that time, other studies have evaluated the presence of T cells in the lung by evaluating the presence of MIF (Jurgensen et al., 1973; Cantey and Hand, 1974). Lymphocyte stimulation assays have also been used, with limited success, to evaluate bronchoalveolar lymphocyte response to mitogens or antigens (Hill and Burrell, 1979; Clancy and Bienenstock, 1974). Few tests exist that allow direct investigation of pulmonary cell-mediated immunity.
COAGULATION AND IMMUNITY

A new function ascribed to lymphocyte/macrophage interaction through a lymphokine is procoagulant activity, i.e. the capacity to accelerate the clotting of normal plasma. Colvin et al. (1973) showed a decade ago that the coagulation system was implicated in the pathogenesis of cell-mediated hypersensitivity reactions. Edwards and Rickles (1978) suggested a correlation between the induration of delayed hypersensitivity skin lesions and intradermal fibrin deposition. After systemic anticoagulation, the skin test induration area was significantly decreased, suggesting that fibrin deposition was responsible. Since that time, fibrin deposition has been identified in the extravascular space of several immunologically mediated diseases: cutaneous, delayed-type hypersensitivity reactions (Colvin and Dvorak, 1975), allergic dermatitis (Dvorak and Mihm, 1972), rheumatoid joints (Zvaifler, 1973), rejected kidney allograft (Braun and Merrill, 1968), and the kidney of lupus nephritis (Kanyerezi et al., 1971).

Many experiments have been performed in an effort to define the immunological signals that initiate the procoagulant activity. As a result, procoagulant activity has been found to be increased by soluble immune complexes (Schwartz and Edgington, 1981; Rothberger et al., 1977), antigen-stimulated lymphocytes (van Ginkel et al., 1981), mitogens (Rich et al., 1980), and allogenic cells (Rothberger et al., 1978).
The exact mechanism of this increased procoagulant activity is unknown. Several researchers have tried, with mixed results, to elucidate the cell type or cell-cell interaction responsible for the procoagulant activity. The following types of macrophage/monocyte populations have been found to have basal procoagulant activity: peritoneal macrophages (Hopper et al., 1981), alveolar macrophages (Sitrin et al., in press), and blood monocytes (Edwards et al., 1979). This basal procoagulant activity is augmented by the interaction with stimulated lymphocytes (Levy and Edgington, 1980; Geczy and Hopper, 1981).

Lymphokines increase procoagulant activity of monocytes in two steps: 1) interaction of "triggered" lymphocyte with monocyte, and 2) monocyte generation and expression of procoagulant activity. The second step is thought to be due to the production of thromboplastin, since protein synthesis by the monocyte is necessary for expression of procoagulant activity (Schwartz et al., 1982). Preliminary studies by Geczy and Meyer (1982), however, show that lymphokine-induced leukocyte procoagulant activity (LPCA) of human monocytes may not be entirely due to an increased synthesis of thromboplastin, but may involve direct activation of clotting Factor X as well. Geczy and Meyer (1982) have also proposed increased procoagulant activity as the mechanism of macrophage migration inhibition. This is not a new idea; Meade et al. proposed the same mechanism in 1976. Geczy and Meyer (1982), however, showed procoagulant activity was positively correlated with the
increase in macrophage migration inhibition.

Endotoxin (Sitrin et al., in press), bacterial phagocytosis (van Ginkel et al., 1979), and complement activation (Prydz et al., 1977) increase basal macrophage procoagulant activity; therefore, it appears there is more than one mechanism for increasing the macrophage procoagulant activity. This mechanism may be synonymous with immunological versus non-specific activation (zymosan) of the macrophage.

An overview of how these different situations may affect the plasma coagulation system is necessary. Current evidence suggests that coagulation is initiated by the intrinsic and/or the extrinsic system. It was thought at one time that these two systems were independent; more recent information suggests the two systems are not as separate as once thought (Mann and Taylor, 1980).

The intrinsic system is initiated by activation of Hageman factor by collagen and other negatively charged surfaces, e.g., glass. A complete review of the intrinsic coagulation system is presented by Kaplan et al., (1982). The procoagulant activity induced by lymphokines is thought to activate the extrinsic coagulation system; hence, this brief overview will deal with the extrinsic system.

The extrinsic coagulation system is activated by the formation of a complex between tissue factor, i.e., thromboplastin, and clotting Factor VII. It is thought, however, as stated earlier that direct activation of Factor X could be involved in immune-enhanced procoagulant
activity. The scheme that follows shows how this activation could occur: (Nemerson and Pitlick, 1972).

Illustration 1.1 Coagulation Cascade Scheme
This cascade scheme shows that each "complex" formed acts as a cofactor for the formation of the next complex. This leads to the conversion of prothrombin to thrombin, which is essential for the proteolytic breakdown of fibrinogen to its fibrin monomer. Fibrin stabilization factor then causes the fibrin monomer to cross-link and form a clot. Notice that lipid is required for the activation of Factor X and the conversion of prothrombin to thrombin. It has been recognized for years that plasma clotting is facilitated by the lipid providing a catalytic surface on which various coagulation factors adsorb to acquire spatial interaction (Zwaal et al., 1980). The same group showed that the clot-promoting activity of phospholipids in vitro is not due to a certain phospholipid class, but to a specific negative charge of the phospholipid surface. In light of this evidence, Meade et al. (1976) and Geczy and Meyer (1982) may indeed be correct in suggesting that increased procoagulant activity is the mechanism by which the macrophages are inhibited in their migration. The macrophage produces thromboplastin which is thought to be activated on the lipid membrane of that cell.

Lung, brain, and placenta contain high amounts of thromboplastin, and no organ has been found completely devoid of it. All of the named organs have high blood flows, so it is logical that they would have high amounts of thromboplastin to help maintain hemostasis in case of injury.

As mentioned earlier, other situations have been found
to increase procoagulant activity, e.g., immune complexes, endotoxin, and complement activation. The scheme below shows how, through a dynamic system of cellular interactions, the extrinsic coagulation system can be activated (Zimmerman et al., 1977).

Illustration 1.2 Cellular Interactions Resulting in Activation of the Extrinsic Coagulation System.

COAGULATION AND TUMORS

O'Meara and Jackson (1958) were the first to observe that growing carcinomas had deposits of fibrils on the peripheral cells that were invading other tissues. In the same year, O'Meara described the "Coagulative Properties of Cancers" by using tissue slices from normal and malignant tissue in the plasma clotting (recalcification) assay. The primary finding in these studies was that normal tissue usually showed a baseline clotting time, followed by clot lysis. Malignant tissue, however, showed decreased plasma
clotting times with persistent clot formation. Since that time, many types of tumors, malignant, as well as solid tumors, have been shown to possess procoagulant activity (Hiramoto et al., 1960; Ogura et al., 1971; Pochedly et al., 1973; Dickersin et al., 1980; Kirchner and Reheis, 1982).

Reports about the nature of tumor procoagulant activity differ, with varying descriptions of its biochemical characteristics. Dvorak et al. (1981) reported that cultured carcinomas from guinea pigs and mice produced procoagulant activity neither dependent on new protein synthesis nor inhibited by diisopropyl fluorophosphate, a serine protease inhibitor. Detailed clotting studies by this group showed that clotting Factors II, V, X, and calcium were needed, unlike thromboplastin, which did not require Factor VII. Gordon et al. (1975) found that extracts from human carcinomas produced a procoagulant activity that also did not act through Factor VII and concluded, therefore, that it was not a tissue thromboplastin. This human tumor procoagulant activity, however, was inhibited by diisopropyl fluorophosphate. Both groups found that their respective tumor procoagulants had similarities to thromboplastin in that they were cell membrane-associated and heat-stable after 30 minutes at 70 C. It is reasonable to assume then that tumor procoagulant activity is different biochemically from the immunological procoagulant activity described earlier and probably activates the coagulation system in a different way.
Tumors release other mediators in conjunction with procoagulant activity. Snyderman and Pike (1976) showed that neoplasms release a macrophage migration inhibitor factor. Folkman et al. (1971) showed that tumors produce an angiogenesis factor which is responsible for the increased vascularity of tumors, an essential feature of tumor growth. Dvorak et al. (1979) reported the presence of four distinct mediators in supernatants of cultured carcinomas: a vascular permeability factor, a procoagulant factor, a plasminogen activator (enzyme that activates fibrinolysis), and macrophage migration inhibitory factor.

In a separate report, Dvorak et al. (1981) described how these mediators may function together to allow the establishment of a viable tumor. Tumors secrete biological mediators to create their own microenvironment. These mediators enhance vascular permeability, allowing many plasma proteins, i.e., fibrinogen, plasminogen and various clotting factors, to leak into the tissues. The procoagulant mediator invests the tumor cells in a protective "cocoon" of fibrin that allows for some degree of mechanical rigidity and the ingrowth of fibroblasts that lay down collagen. Fibrin/fibrinogen degradation products are thought to be partly responsible for angiogenesis, activation of complement, fibroplasia, and to be chemotactic for inflammatory cells. The fibrin cocoon would then restrict tumor growth were it not for the secretion of plasminogen activator. This enzyme is responsible for activation of plasmin, a fibrinolytic enzyme; therefore,
the tumor has the capacity to generate and modify the enveloping gel cocoon. The role of the fourth mediator, MIF, is somewhat undetermined. It differs slightly from the immunologically released lymphokine by losing its activity after dialysis. The MIF-like activity, however, is reported to be secreted in serum-free culture and not dependent on a component in serum, as was found with other tumor cells (Dvorak et al., 1981). Dvorak et al., (1981) proposed that tumor-produced MIF diffuses away from the site of the tumor and immobilizes the inflammatory cells, primarily the macrophage, before these cells can reach the tumor. These cells are cytotoxic for tumor cells, and keeping them away from the tumor would be necessary for the tumor's survival. In conclusion, Roth et al. (1982, 1983) have isolated factors from tumors that suppress lymphocyte proliferative and cytotoxic responses. The tumor, therefore, provides a microenvironment for its survival.

ADJUVANT, MURAMYL DIPEPTIDE

For the past two decades, many attempts have been made to treat cancer by enhancing patient immunity with immunological tools such as adjuvants. This section will describe adjuvant action in general and specifically the adjuvant used in this research, muramyl dipeptide (MDP).

An adjuvant can be described as any substance that when administered with antigen has the capacity to potentiate the immune response against various antigens, including infectious agents and tumor cells. Many compounds with
adjuvant activity have been described to date: Freund's complete adjuvant (Mycobacterium tuberculosis in an oil emulsion, Freund, 1956); Mycobacterium bovis (BCG); Corynebacterium parvum (Dvorak et al., 1981); chitin (Diamantstein et al., 1982); and Klebsiella pneumonia (Yokochi et al., 1980) are just a few. These are primarily bacterial cell wall constituents and polysaccharides, which present certain problems for their use as adjuvants. They are themselves immunogenic, many are also not water soluble, and some are associated with disease states. Freund's complete adjuvant, the best known, is considered unsafe for human use because of its potentiation of plasma cell tumors in mice, induction of autoimmune reactions, formation of disseminated focal granulomata (Siddiqui et al., 1978), polyarthritus (Parnham and Schoester, 1980), and emphysema-like reactions in the lung (Colombo et al., 1979).

Recently, adjuvants that are chemicals or that can be chemically synthesized were discovered: Al(OH)2 (Osebold, 1982); dimethyl dioctadecyl ammonium bromide (Snippe, 1982); Evans Blue dye (Arora and Crowle, 1978); and muramyl dipeptide (Adam et al., 1981). The most studied of these is the synthesized muramyl dipeptide (MDP). MDP is the minimal adjuvant-active structure that can substitute for M. tuberculosis in Freund's complete adjuvant.

Muramyl dipeptide (N-acetylmuramyl L-alanyl D-isoglutamine) is a subunit found in most bacterial cell walls. This molecule, however, is water soluble and is not antigenic or mitogenic (Chedid and Lederer, 1978). The
discovery of this simple, well-defined synthetic adjuvant stimulated studies of the relationship of its structure, biochemical activity, and mechanism of action.

The structural activity has been defined primarily by investigations in France and Japan. Kotani et al. (1975), Chedid et al. (1978), Parant (1979), and Adam et al. (1981) described the necessity of having the proper stereochemistry and proper amino acids in this structure to get adjuvant activity. The presence of the D-isoglutamine and L-alanine residues are necessary for potent adjuvant activity. There are various similar structures, e.g., D-glutamic acid, with some adjuvant activity, but the N-acetylmuramyl L-alanyl-D-isoglutamine structure is the most potent. Parant et al. (1979) reported that after intravenous or subcutaneous injections of 14C-MDP in mice, more than 50% of the intact compound is excreted in the urine after 30 minutes, and more than 90% after 2 hours. The action of this compound must, therefore, be immediate, or small amounts of the adjuvant are retained.

The biological activity of MDP is a subject of prolific, but contradictory investigations. MDP has been found to boost the humoral and cellular immune response after administration by various parenteral routes, and it is also active given orally (Chedid et al., 1976). MDP has also been shown to increase the tumoricidal activities of murine macrophages when the adjuvant is encapsulated in liposomes (Fidler and Poste, 1981; Sone and Tsuburo, 1982). The bulk of the MDP literature concerns its effect on the
humoral immune system; however, there is sufficient literature to be reviewed concerning the cell-mediated immune system.

An excellent review of MDP effects on the CMI system has been published (Matter, 1979). There are varying effects between in vivo and in vitro systems and with relative doses of adjuvant, of antigen and with the timing of adjuvant or antigen administration (Souvannavong and Adam, 1980). In vitro culturing conditions, i.e., low density (stimulation) or high density (suppression), have also been shown to affect the results of the adjuvant, MDP (Leclerc et al., 1979a).

Richerson et al. (1982) and Hadden et al. (1979) reported that MDP has no mitogenic effect on human peripheral blood lymphocytes. Specter et al. (1977), however, reported that MDP has a direct twofold-to-threefold stimulation on spleen cultures as assayed by 3H-thymidine incorporation. Brummer and Stevens (1981) reported that MDP depresses mitogenic effects of PHA, ConA and LPS on spleen cultures, but induces a cellular hyperplasia in lymph nodes. Matter (1979), who used a mixed lymphocyte culture of spleen cells, suggested that background proliferation produced by the MDP alone masked effects of any stimulation in the test cultures. These results could represent the effect of MDP on different populations of lymphocytes.

MDP has shown no adjuvant activity in the in vivo generation of cell-mediated cytotoxic effector cells in the spleens of mice after immunization with allogeneic (same
species, but genetically distinct) mastocytoma cells. MDP did show enhanced in vitro sensitization of mice spleen cells to allogeneic mice spleen cells (Azuma et al., 1976). Igarashi et al. (1977) also showed that MDP enhanced cell-mediated cytotoxicity in in vitro spleen cell systems to syngeneic (individuals with identical genotypes) mastocytoma cells.

MDP increases the delayed hypersensitivity skin test to ovalbumin in rats (Tanaka et al., 1977) and guinea pigs (Souvannavong et al., 1978). It is interesting that MDP enhanced the CMI response (Masek et al., 1978) and antibody formation (Heymer et al., 1978) to soluble protein antigens, but only weakly enhanced antibody production for particulate SRBC antigen. MDP also enhances MIF production to ovalbumin after footpad injections with a dose of 0.028 mg/kg (Souvannavong et al., 1978).

The mechanism of MDP's action upon the immune system has been the subject of many experiments. Activation of the macrophage by MDP is manifested by an increased phagocytic index (Tanaka et al., 1977), increased synthesis of collagenase (Wahl et al., 1979), increased production of superoxide anion (Pabst and Johnston, 1980), increased release of B-glucuronidase and secretion of lysozyme (Imai and Tanaka, 1981), and increased spreading and adherence of macrophages (Nagao et al., 1981).

The T lymphocyte is implicated as the target cell for MDP's action. Leclerc et al. (1979b) reported that MDP activity is mediated like a helper T-cell because nude mice
(deficient in T cells) treated with MDP gave a response to a T-dependent antigen. Sugimoto et al. (1978) drew the same conclusion from MDP's stimulating effect on the generation of carrier-specific T-helper cells. Leclerc et al. (1982) identified the T cell with anti-Thy-1.2 antiserum as the cell responsible for MDP-induced suppression after repeated injections of high doses of MDP. Because of the close interaction of macrophages and T lymphocytes, it has been difficult to discern action by only one cell without the help of the other.

All adjuvants are thought to function through a similar mechanism. The importance of the macrophage to adjuvant action has been demonstrated (Allison, 1979). Unanue et al. (1969) showed that adjuvants enhance antibody production only after uptake by macrophages of adjuvant and/or antigen. Adjuvant effects, however, are the most pronounced to T-cell dependent antigens (Osebold, 1982). The majority of antigens are T-cell dependent, which implies that T-cell help is required to evoke a primary immune response. An adjuvant effect, therefore, can result from substances that expand the population of helper T cells. This is how Sugimoto et al. (1978) hypothesized that MDP functions. Because immune responses are regulated by amplifying and suppressing systems, the adjuvant appears to alter the equilibrium between helper and suppressor T cells, which leads to enhancing the helper T cell effect.
PLUTONIUM

Plutonium has been proclaimed the most studied hazardous element (Bair and Thompson, 1974). Scores of books and reviews covering many aspects of plutonium have been written. Some of the more comprehensive reviews are Hodge et al. (1973), Stover and Jee (1972), and Thompson and Bair (1972); therefore, this presentation will highlight these reviews.

Plutonium, a daughter product of uranium, is a silvery white metal which melts at 639.5°C and oxidizes readily on warming in moist air (Taylor, 1973). Plutonium will form compounds in solution exhibiting valences of +3 to +6. In neutral solutions, the formation of Plutonium +4 is favored. Plutonium-239 dioxide (239PuO2) is an important compound of plutonium because of its use as a fuel in breeder reactors. 239Pu has a half-life of $2.44 \times 10^4$ years and a specific activity of $6.13 \times 10^4 \text{ mCi/gm}$. This compound's principle mode of decay is by emission of an alpha particle and an L shell x-ray. The mean alpha energy is 5.15 MeV and the x-ray has an approximate energy of 17 keV (Nenot and Stather, 1979).

The principle routes of exposure for persons employed in the plutonium industry are inhalation, ingestion or through lacerations and abrasions. Voelz (1975) reports that the majority of exposures are due to accidental inhalation of plutonium. The human data available regarding effects of plutonium inhalation are few and incomplete and have come from studies of plutonium workers and necropsy
tissue analysis. Voelz (1975) reported that no significant harmful effects have been noted in humans, other than a small foreign-body type nodule around dermal implantations of plutonium. These are usually readily removed by surgery.

Watts (1975) reviewed previously published cases of human inhalation exposure to insoluble plutonium to ascertain clearance rates from the lung. These data support the concept of a 3-phase clearance of insoluble plutonium, as noted by the Task Group on Lung Dynamics (1966). The early rapid phase in man is about one day, which appears to be clearance of material deposited in the upper respiratory tract, nasopharynx, and tracheobronchial regions. This material is cleared rapidly by ciliary mucus transport to the mouth where it is swallowed and is eventually excreted in the feces. The second intermediate clearance mechanism has a half-life of about 30 days. This is due to macrophage phagocytosis of small particles and clearance up the bronchi and trachea by ciliary action. The third and final mechanism of clearance is due to removal of particles deposited in the alveoli. The half-time of this phase is greater than 300 days. These particles are cleared by the blood or lymphatics or the phagocytic action of macrophages, as described above.

The experimental animal exposure data for plutonium far outweigh the human data. A good review of animal exposures to plutonium and other transuranium elements is found in the Proceedings of the Eleventh Hanford Biology Symposium (1972). The effects of inhalation and intravenous (iv)
exposure of animals to various chemical forms of plutonium are well documented. The intravenous exposure route is reviewed by Jee (1972). After iv injection $^{239}$Pu is rapidly deposited on the bone surface and in the liver. Only a small fraction deposits in other organs. Organ uptake is governed by the route of administration, physicochemical state of the plutonium and the age of the animal (Sikov et al., 1978). The principal effects of low doses of plutonium given iv is bone necrosis and the induction of osteogenic sarcomas; however, head sinus carcinomas, liver tumors, and eye melanomas have also been induced (Mays and Dougherty, 1972).

Inhalation is a more realistic route of exposure and will be considered in more detail. Several groups across the United States have been involved in a concerted effort to evaluate various aspects of the effects of inhaled plutonium. The groups involved primarily in research with dogs are Hanford Laboratories (West and Bair, 1964), University of Rochester (Morrow et al., 1967), Battelle Northwest Laboratories (Park et al., 1972), and Lovelace Inhalation Toxicology Research Institute (McClellan, 1972). Groups in Russia and France have also actively studied inhaled plutonium in dogs (Buldakov et al., 1972) and in baboons (Metivier et al., 1974). These investigations confirm what Langham (1972) said about plutonium: "It appears quite certain that the transuranium elements in general, and plutonium specifically, are or will be the most thoroughly studied of the valuable, but potentially harmful
substances to be introduced into man's ever-expanding industrialized society."

The primary effects seen in dogs (Park et al. 1972) and in other species (Sanders et al., 1976; Thomas and Smith, 1979) after inhalation of 239PuO2 are radiation pneumonitis, pulmonary fibrosis, and/or neoplasms. Most of the tumors found were bronchio-alveolar carcinomas; however, squamous cell and epidermoid carcinomas were observed.

The plutonium retention and distribution in animals is affected by chemical form (Stather and Howden, 1975) and particle size (Smith et al., 1977). The primary route of removal of insoluble plutonium from the lung is by clearance up the airways, by excretion through the gastrointestinal tract or to the lung-associated lymph nodes. Raabe et al. (1973) and Fleischer (1975) studied the dissolution rates of 238Pu and 239Pu in simulated lung fluid and found 239PuO2 to be highly insoluble. The dissolution rate is temperature-independent and varies as \( r^{-1} \) (radius of the particle). Thomas (1972) said that by 50 years after exposure to 239PuO2, the lymph nodes would have received about 300 times the dose originally given to the lung. There are small percentages of the plutonium translocated to liver and bone, but the relatively insoluble 239PuO2 tends to stay in the lung and in the tracheobronchial lymph nodes (Brooks et al., 1983).

Lymph nodes are the domain of T and B lymphocytes. These cells are extremely sensitive to ionizing radiation.
Lung-associated lymph nodes receive significant doses of radiation after inhalation of plutonium. The lung-associated lymph nodes are damaged and their resident cells are destroyed by exposure to plutonium. The immune reaction responsible for defense of the lung initially develops in the lung-associated lymph nodes. It is important to evaluate the immune response of plutonium-exposed dogs with damaged lung-associated lymph nodes to see if they can produce normal immune reactions to inhaled or instilled antigens.
CHAPTER II

CELL-MEDIATED IMMUNITY OF THE DOG LUNG:

PRIMARY AND SECONDARY RESPONSES

ABSTRACT

This study evaluated cell-mediated immunity sequentially in control and immunized lung lobes of dogs using the leukocyte procoagulant activity assay (LPCA) and antigen-specific inhibition of alveolar macrophage migration (MIF). Beagle dogs were immunized with $10^{10}$ sheep red blood cells in the left cardiac lung lobe, and the primary immune response was evaluated in blood and in cells obtained from bronchial washings. Four months later, a challenge dose of sheep red blood cells ($10^6$) was instilled into the same lung lobe, and the secondary immune response was examined. The primary MIF response for either the control or immunized lung lobe was highest 12 to 14 days after immunization, whereas the LPCA assay from either lobe had the highest response 9 to 12 days after immunization. The secondary response showed a significantly elevated immune reaction as measured by both the LPCA and MIF assays during all days of testing. The secondary immune response also occurred earlier than the primary response. The spontaneous (without antigen added to culture) macrophage migration patterns were significantly different between the primary and secondary immunizations. Challenge of the immunized lung lobe with a low dose of antigen resulted in small migration areas for the macrophages, in comparison to macrophages from the
control lobe. This suggested local production of MIF.

INTRODUCTION

Cell-mediated immunity (CMI) of the respiratory system has been evaluated using lung lavages in various species: the pig (Charley, 1977), rat (Bice and Schnizlein, 1980), guinea pig (Burrell and Hill, 1975; Waldman and Henney, 1971), rabbit (Cantey and Hand, 1974; Moore et al., 1978) and the dog (Reif et al., 1975). Few studies have examined CMI after secondary challenge to the lung (Nash and Holle, 1973; Gadol et al., 1974). No studies have examined development of the cell-mediated immune response sequentially after localized antigen deposition in the same animals. The canine model was used in this study to allow the immune response to be followed in immunized and control lung lobes of the same animals. This allowed each animal to serve as his own control.

The earlier studies mentioned above have used lymphocyte stimulation assays, macrophage migration inhibition factor (MIF) assays and skin tests to evaluate CMI in the lung. Lymphocyte stimulation assays using lung lavage cell populations provide varying results. Several studies with in vitro antigen stimulation suggest that only low levels of lymphocyte division occur in the lung because of the suppressive effects of alveolar macrophages (Anfield et al., 1979; Holt, 1979; Pennline et al., 1979). If only limited division occurs in lymphocyte populations in the lung, the use of this assay to study pulmonary CMI may
provide negative data, suggesting that lymphocyte populations in the alveoli are minimally involved in CMI.

The small number of lymphocytes and macrophages washed from the lungs of many species require addition of cell populations from different compartments of the body for the MIF assay. Pearsall and Weiser (1970) showed distinct metabolic differences among various macrophage populations. Clancy and Bienenstock (1974) showed lymphoid cell division rates to be different, depending on which lymphoid cell population is evaluated after various routes of immunization. Therefore, the mixing of cell populations may not be representative of the in vivo immune process. Past studies have shown that sufficient cells can be washed from the dog lung so that mixing of cell populations is not necessary.

A relatively new test of CMI, leukocyte procoagulant activity (LPCA), was used in this study. The LPCA assay has been compared with other indicators of CMI: the MIF assay, skin tests, and lymphocyte stimulation index. The response of the LPCA assay correlated to a high degree with these tests and was more sensitive and required fewer cells (Geczy and Meyer, 1982).

The purpose of this study was to examine the time course of development and the magnitude of both primary and secondary pulmonary CMI responses in the Beagle dog using two tests specific for the lung, MIF and LPCA.
MATERIALS AND METHODS

Animals. Four male Beagle dogs from the Inhalation Toxicology Research Institute's colony, 1 to 2 years old, were used in this study. The dogs were found to be healthy after physical examinations. The dogs were brought in from indoor-outdoor kennels 3 days before immunization to allow the dogs to acclimate to indoor cages. The dogs were housed one to a cage and fed once a day (Wayne Dog Food, Allied Mills, Chicago, IL) and given water ad libitum. Food was withheld from the dogs 24 hours before immunization and before each lavage which required general halothane anesthesia.

Immunizations. Intrabronchial immunizations were carried out as previously described (Bice et al., 1980a). Anesthesia was induced using a face mask with 5% halothane mixed with oxygen. An endotracheal tube was placed in the trachea. Light surgical anesthesia was maintained with 1.5% halothane in oxygen. A fiberoptic bronchoscope was then introduced into the airways by way of the endotracheal tube. The primary immunization involved intrabronchial deposition of $10^{10}$ sheep red blood cells (SRBC) in 1 ml of saline into the left cardiac lung lobe; 1 ml of physiological saline was instilled into the right cardiac lung lobe as a control. The secondary challenge, $10^6$ SRBC instilled into the left cardiac lung lobe and saline instilled into a control lung lobe, occurred 16 weeks after the primary immunization. The SRBC came from a single sheep and were never more than 2 days old when used for immunization.
The dogs were lavaged and a venous blood sample taken from the jugular on days 5, 7, 9, 12, 14, 16 and 21 after primary and secondary immunization. On designated days, the immunized and control lung lobes were re-entered with the bronchoscope, using the same anesthesia routine as outlined above, and washed five times with 10 ml (50 mls total) of sterile saline through the biopsy channel of the fiberoptic bronchoscope. The lung wash fluid recovered from the lavage procedure ranged from 85 to 95%. Recovered fluid was stored in centrifuge tubes in ice until the laboratory procedures were done.

Cell preparation. The cells obtained by segmental lavage were washed three times by centrifugation at 200 x g for 10 min in RPMI 1640 medium (Grand Island Biological Co., GIBCO, Grand Island, NY) supplemented with 25 mM Hepes buffer (N-2 Hydroxyethylpiperazine N'-2-ethanesulfonic acid) and 50 μg/ml Gentamycin (Sigma, St. Louis, MO). The cells were counted and resuspended in 1.5 ml of complete medium. The lavage fluid was poured off and retained for immunoglobulin determinations.

Venous blood was drawn into heparinized tubes and later diluted with an equal volume of Hank’s balanced salt solution (HBSS, GIBCO, Grand Island, NY). Lymphocytes were separated by centrifugation on Isolymp (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY), washed three times in medium, and suspended at a concentration of 4 x 10^6/ml in complete medium (RPMI 1640 supplemented as above.
plus 10% heat-inactivated newborn calf serum (GIBCO, Grand Island, NY)).

Evaluation of lavage and blood cells. Cytocentrifuge slides were prepared on all bronchoalveolar and blood samples. These slides were stained with a rapid Romanowsky-type stain (Diff-Quick; Harleco Co., Gibbstown, NJ). The percent of alveolar macrophages, lymphocytes, and polymorphonuclear leukocytes was determined by counting 300 total cells. The normal cell population from an unimmunized control dog is approximately 85-95% alveolar macrophages, 5% neutrophils, and 5% lymphocytes (Rebar et al., 1980).

Cytology results were statistically analyzed using a two-way analysis of variance (ANOVA) between the two groups over time. The total number of cells/ml of lavage fluid was determined using an electronic cell counter (Coulter Electronics, Hialeah, FL).

Leukocyte procoagulant activity assay. The leukocyte procoagulant activity assay was performed on washed lavage cells and leukocytes separated from blood. This assay examined the ability of cells to enhance the clotting of normal autologous plasma using a modified one-stage recalcification assay (Geczy and Meyer, 1982). Calcium is required for initiating the coagulation cascade. The name recalcification assay is because calcium must be added to the assay system to measure clotting time. The LPCA assay was performed on cells that were cultured with antigen (200µg/ml) and without antigen. SRBC ghosts, used as the antigen, were prepared by the hypotonic phosphate buffer.
method of Hanahan and Ekholm (1974). The protein concentration of the antigen preparation was measured according to the method of Lowry et al. (1951). Incubation of these cells was carried out in duplicate in Nunc Minisorp tubes (Vanguard International, Neptune, NJ) in a final volume of 1 ml (4 x 10⁶ cells/ml). After incubation for 20 hours in an atmosphere of 4% CO₂ in air at 37°C, cells were washed once with cold HBSS and resuspended in 0.5 ml HBSS.

The plasma for this assay was pooled from the dogs used in this study before immunization. Blood was drawn into evacuated tubes containing sodium citrate (3.8% v/v in 0.9% NaCl) and plasma prepared by centrifugation at 1800 x g for 30 min. This centrifugation procedure removed most of the platelets from the plasma. Plasma was aliquoted into appropriate volumes and stored at -70°C for not longer than one month.

The recalcification time was determined by incubation of 0.1 ml of each the cell suspension, plasma, and 0.9% NaCl for 1 min at 37°C. CaCl₂ (0.03 M prepared in 0.9% NaCl, 0.1 ml) was added, and the clotting time assessed visually. The percent shortening of plasma recalcification time was calculated using the clotting time (seconds) of the cultured cells from each of the immunized and control lung lobes. The calculation was made as follows for each lobe:

\[
\text{Percent Shortening of Recalcification Time} = \left( \frac{\text{Clotting time for cells with antigen}}{\text{Clotting time for cells without antigen}} \right) \times 100
\]

**Macrophage migration inhibition factor assay.** The
migration of lavage cell populations with and without antigen was evaluated as a measure of MIF production. Canine MIF characterization has shown that canine aveolar macrophages have receptors for MIF (Schnizlein and Bice, 1980). The bronchoalveolar cell pellets were centrifuged from the medium, then resuspended in 1 ml of complete medium. The cell suspensions were drawn into 75 µl nonheparinized capillary tubes, sealed by flaming, and packed by centrifugation as described by Bice et al. (1977). The capillary tubes were cut at the cell interface and placed into migration chambers (Superior Plastics Products, Cumberland, RI) containing complete medium or complete medium plus 200 µg/ml SRBC ghosts. The migration chambers containing the lung lavage cells were incubated 24 hours at 37°C in 4% CO₂. Migration areas of cells from each lung lobe were measured by planimetry, and percent inhibition calculated as follows:

\[
\text{Inhibition} = \left( \frac{\text{areas of cell migration without antigen}}{\text{areas of cell migration with antigen}} \right) \times 100
\]

A percent difference between the spontaneous migration areas of alveolar macrophages from the control and immunized lung lobes cultured without antigen was also calculated by the following equation:

\[
\text{Percent Difference in Migration Area} = \left( \frac{\text{Migration area of Control Lobe} - \text{Migration area of Immunized Lung Lobe}}{\text{Migration area of Control Lobe}} \right) \times 100
\]
The MIF and LPCA assays were statistically analyzed over time using a two-way analysis of variance (ANOVA) and Bonferroni's test for multiple comparisons to determine significant differences. Specific time points were tested using Student's t test.

**Enzyme-linked immunosorbent assay.** The enzyme-linked immunosorbent assay (ELISA) was performed to examine the antigen-specific antibody produced in the alveoli. This was accomplished by evaluating the wash fluid from the lavaged control and immunized lung lobes using the Gilford PR-50 automatic analyzer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). This assay was performed using a modified method of Voller et al. (1976). The modification was to attach solubilized antigen to the plates first. SRBC ghosts previously described were solubilized with 0.1% SDS. The solubilized SRBC antigen (5 μg/ml) in coating buffer (pH = 7.4) was incubated overnight in polystyrene test cuvettes. The cuvettes were washed with PBS-Tween, and the test lavage fluid sample containing the antigen-specific antibody was diluted with PBS-Tween and added to the cuvette. This assay using specific antisera conjugated with alkaline phosphatase (diluted 1:1000, Cappel Laboratories, Cochranville, Pa.) was used to determine the presence of IgG and IgM by a spectrophotometric method using a wavelength of 405 nm.

**Skin tests.** The dogs were skin tested with 150 and 75 μg of SRBC ghosts in 0.1 ml. Saline was also injected in the same volume as a negative control. The ventral side of the dogs was shaved, and a total of three intradermal
injections were made 22 days after immunization.

RESULTS

**Leukocyte procoagulant activity assay.** Four unimmunized control dogs were lavaged to evaluate the nonspecific effect of antigen on the procoagulant activity assay. The addition of antigen to cultured nonimmune cells had no effect on the percent shortening of plasma clotting time which was 0.0 to 4.4%.

The percent shortening of plasma clotting time in the cells washed from the lung lobes after primary and secondary antigen challenge and cultured *in vitro* is shown in Figure 2.1. The primary immunization resulted in a significantly (20% considered significant) shorter recalcification time of cells from the immunized lung lobe that were cultured with antigen, compared to cultured without antigen. The peak response was observed between 9 and 12 days after immunization. The cells from the control lung lobe followed a similar pattern, but the response was lower than for cells from the immunized lung lobe. The response in the blood peaked on day 7, and after that time showed a gradual decline. The secondary challenge showed an increased response at 5 days after challenge that was significantly higher than that at day 21 after primary immunization. This response was sustained throughout the experiment, but never reached the height seen in the primary immunization. The level of response in the blood was lower in both the primary
Figure 2.1. The effect of SRBC antigen on the procoagulant activity of cells from the control, and immunized lung lobes, and blood after primary and secondary immunizations. Each point represents the mean of four dogs ± SEM, with the exception of day 7, which had data from two dogs for the blood and control lung lobe.
and secondary responses, relative to that seen in the lungs.

**Inhibition of macrophage migration.** Unimmunized control dogs were lavaged to assess the basal level of nonspecific antigen inhibition on the migrating alveolar macrophage. The nonspecific percent inhibition ranged from -3.2 to 15.7% in 4 dogs. The level set for significant inhibition, therefore, was 20%.

The results of this study showed that the primary response reached significant inhibition (20% considered significant) in the immunized and control lobes on days 9 to 12, then peaked on day 14 and 12, respectively (Fig. 2.2). The two lobes were not significantly different from each other at any point. Macrophages from the immunized and control lobes had returned to normal migration patterns 21 days after immunization. The secondary challenge resulted in significantly increased MIF activity in the cells from the lobes examined on the first day of testing, which was 5 days after challenge with $10^6$ SRBC. This booster resulted in macrophage migration inhibition occurring 9 to 7 days earlier than the primary response in the immunized and control lobes, respectively. The degree and duration of the secondary response were greater and extended longer than the response with the primary immunization.

A comparison is made in the percent difference between the spontaneous migrations of the control and immunized lung lobe without antigen in Figure 2.3. This comparison approximates what the macrophage mobility should resemble in vivo at these times after immunization. The primary
Figure 2.2. The effect of SRBC antigen on the migration of cells washed from the control and immunized lung lobes after primary and secondary immunizations. The percent inhibition of migration was determined by comparing the mean size of four migration areas in culture medium with antigen to the mean size of four migration areas of cells from the same lobe in culture medium without antigen. Each point represents the mean of four dogs ± standard error of the mean (SEM).
Figure 2.3. The effect of primary and secondary immunizations on the spontaneous migration of alveolar macrophages. The difference between migration areas of the cells from the control and immunized lung lobes cultured in medium. Each point represents the mean of four dogs ± SEM.
immunization resulted in large migration areas from cells harvested from the immunized lung lobe; hence, the negative inhibition of migration. With time, this effect disappeared, and the migration areas of the control and immunized lobes were essentially the same by day 16. The secondary challenge showed an opposite effect, with the control lobe migration areas being larger than the immunized areas initially. There was reversal of this phenomenon by day 12, but by day 21 the areas were approximately the same again. The two-way ANOVA showed the primary and secondary spontaneous migrations to be significantly different over time (\( P = 0.0005 \)).

Cytology. The primary immunization of the left cardiac lung lobe with \( 10^{10} \) SRBC resulted in significant changes in the cell types present in that lung lobe (Fig. 2.4). The cell types primarily responsible for this change were an influx of neutrophils (PMN) and lymphocytes. The percentage of neutrophils increased dramatically on day 7, then showed a sharp drop and remained low for the rest of the experiment. Lymphocytes, however, showed an increase on day 9 and a gradual decline for the other times.

The challenge with \( 10^6 \) SRBC did not produce dramatic changes in cell types in the immunized lung lobe. This lung lobe showed minimal change in the percentage of lymphocytes after immunization. The neutrophils in the immunized lung lobe, however, showed only a slight increase on day 7 and another increase on day 16. When examining the difference between the primary and secondary cell type curves, only the
Figure 2.4. Lymphocytes, neutrophils (PMNs), and alveolar macrophages in the lung lavage fluid from the immunized left cardiac lung lobe from the primary and secondary immunizations. Each point represents the mean of four dogs ± SEM.
neutrophils (P = .068) and macrophages (P = .016) showed a significantly different trend with time.

The control lung lobe showed a relatively stable population of all three cell types for both the primary and secondary immunizations (Fig. 2.5). In both instances, a slight increase in neutrophils was noted on day 14. The lymphocyte population in the control lobe after primary immunization was greater than 20% on day 5, then fluctuated downward to around 10% thereafter. The secondary challenge showed the lymphocytes in the control lung lobe at day 5 to be approximately 14% with a peak on day 12 at 17%

**Enzyme-linked immunosorbent assay.** The results from the ELISA assay, which measured antigen-specific antibody present in the lavage fluid, are presented in Figures 2.6 and 2.7. In all cases, the antibody measured in the control lung lobe primarily fluctuated around the background measurement and was significantly lower than the antibody in the immunized lobe. IgM and IgG from the immunized lung lobe peaked on day 9 after primary immunization. IgG, however, was significantly higher than IgM. The secondary response shows little IgM or IgG in the immunized lobe. The time of appearance of detectable antibody, however, was 2 days earlier after challenge. The levels of both immunoglobulins were detectable on day 5 after challenge, but were not higher than that of day 21 after primary immunization.

**Skin test.** The primary skin test showed a positive dose-dependent indurated area 48 hours after intradermal
Figure 2.5. The cell types lavaged from the control lung lobes after primary and secondary immunizations. Due to a technical error the cell types from the control lobe for day 21 are not shown. Each point represents the mean of four dogs ± SEM.
Figure 2.6. ELISA optical density units with the background subtracted are plotted for the antigen-specific IgG present in the lavage fluid of the immunized and control lung lobes. The control lobe antibody after challenge was not significantly different from background and is not shown. Each point represents the mean of four doses ± SEM.
Figure 2.7. ELISA optical density (O.D.) units with the background subtracted are plotted for the antigen-specific IgM present in the lavage fluid of the immunized and control lung lobe. Each point represents the mean of four dogs ± SEM.
inoculation (Table 2.1). The area of the skin test after challenge was not palpable.

TABLE 2.1. Skin Test Data.

Primary Skin Test Data

<table>
<thead>
<tr>
<th>Dog Number</th>
<th>150 µg/0.1 ml</th>
<th>75 µg/0.1 ml</th>
<th>saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.38*</td>
<td>0.30</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
<td>0.35</td>
<td>0.30</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
<td>0.29</td>
<td>0.20</td>
</tr>
<tr>
<td>4</td>
<td>0.35</td>
<td>0.25</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* all areas measured in millimeters.

The SRBC doses listed were given intradermally in 0.1 ml and the indurated area measured 48 hours later.

DISCUSSION

Other investigators have shown recruitment into the lung of antigen-sensitive T cells after primary and secondary pulmonary or nasal immunization (Nash and Holle, 1973; Gadol et al., 1974). This study verifies their findings and evaluates a new test to specifically measure pulmonary CMI.

Coagulation is an integral part of the host's immune response (Marx, 1982; Kaplan et al., 1982). The LPCA assay involves cellular cooperation between the macrophage and lymphocyte (Levy and Edington, 1980). This test requires the presence of lymphocytes in the same proportion as found in vivo. This proportionality has been measured in blood (Edwards et al., 1979), spleen (Levy and Edington, 1980), and peritoneal exudate cells (Hopper et al., 1981).
The LPCA assay measures interaction between the lymphocyte and the macrophage. Hopper et al., (1981) reported that a lymphokine produced by antigen-stimulated lymphocytes induces macrophage procoagulant activity and that this occurs with a concomitant decrease in the ability of the cells to migrate from capillary tubes. The LPCA assay should, therefore, reflect the inability of macrophages to migrate. Figures 2.1 and 2.2 reveal the resemblance found in peak times between the two assays for both the immunized and control lung lobes after primary and secondary immunizations. However, the LPCA assay was found to be the more useful assay because as shown in Figure 2.2 the MIF assay showed a decreased sensitivity in the assay for the immunized lung lobe.

A significant immune response was seen on day 5 after challenge in the control and immunized lung lobes for both the MIF and LPCA assays. This suggests that T cells were residents of the alveoli at the time or soon after secondary immunization. Gowans and Knight (1964) and Sprent (1977) showed that T cells constantly recirculate throughout the body. This early response, therefore, is not unexpected, since some memory T cells would be in the lung at the time of antigen deposition. The recirculation of the T cell through the entire lung could be the reason why the immune response was so high in the control lung lobe. Eventhough few sensitized T lymphocytes would be present, David and David (1972) showed that only a few lymphokine-producing T lymphocytes were necessary to produce an effect on a large
number of macrophages.

In studies by Gadol et al. (1974), significant macrophage migration inhibition started as early as 6 to 7 days after nasal immunization with influenza virus in guinea pigs and peaked on day 21. This study, however, mixed pulmonary cell lavage suspensions with peritoneal exudate cells, which served as indicator cells, to examine macrophage migration inhibition. These differences in methods, species, and antigen could account for the difference in time of onset of MIF. Nash and Holle (1973) gave their soluble antigen intratracheally and used the method of measuring MIF as mentioned above. This study also showed significant inhibition on day 6, but the peak response occurred at day 10 in the primary immunization. The secondary response in bronchial washings that was measured after challenge with a low dose of antigen shows significant inhibition on day 4 (Nash and Holle, 1973). This corresponds with present data, which showed significant inhibition at day 9 after primary immunization and day 5 after challenge, the first day of testing. Their study also showed that on day 17 the percent inhibition of the secondary response showed a dramatic rise to approximately 60%, which is also similar to that in our work. This late peak possibly reflects the cyclic nature of the immune system.

Migration area patterns as represented by the percent difference figure (Fig. 2.2) revealed an initial activation of the alveolar macrophages after exposure to antigen in the
primary immunized lung lobe, as compared to the control. This was probably due to the large burden of antigen placed in the immunized lung lobe. The particulate nature of the antigen probably activated the macrophage, i.e., increased mobility, to clear the antigen from the alveoli. To avoid this nonspecific activation of the macrophage, a low amount ($10^6$) of antigen was instilled for study of the secondary response. This was successful, and the migration pattern of the immunized lobe was restricted initially in comparison to the control lobe, suggesting local production of migration inhibitory factor.

The increase in neutrophils in the primary immunized lung lobe was probably due to an inflammatory response after the instillation of the antigen into that lobe. The influx of lymphocytes into the immunized lung lobe during the primary response on day 9 corresponded with all of the tests performed, i.e., significant MIF's, a dramatic rise in the procoagulant activity, and the appearance of antigen-specific antibody as shown by the ELISA assay. This points to inflammation, indicated by increasing numbers of neutrophils as possibly aiding the influx of immune products and cells. Inflammation has been shown by Kaltreider and Salmon (1973) and North and Spitalny (1974) to aid preferentially in the influx of T cells into inflamed tissues. Bice et al., (1982) showed this to be true with antibody producing B cells.

The skin test showed systemic immunity after primary immunization. The skin test 22 days after secondary
challenge, however, showed no systemic immunity to the SRBC antigen. The amount of antigen-specific antibody in the lavage fluid was also minimal. Both of these results could be due to the low amount of antigen instilled into the lung. The antigen probably never reached the lymph nodes to trigger a B cell response as shown by virtually no LPCA response or antigen-specific antibody in the blood (data not shown). A small percentage of long-lived B cells recirculate; therefore, the low amount of antigen instilled was probably not sufficient stimulus to elicit a memory antibody response. The data also showed a huge difference between the antigen-specific antibody content of the control and that of the immunized lung lobe whereas the CMI response showed both lobes to be essentially equal. This suggests that there is an increased recruitment of T cells or it took fewer T cells to see measured effect in the assays. David and David (1972) showed by mixing stimulated lymphocytes with nonimmune indicator cells that few of the lymphocytes were necessary to produce significant inhibition of macrophages.

This study has shown that antigen-sensitive T cells enter the lung after primary pulmonary immunization and are recirculating through the lung as shown by the early responses seen in all the tests of the secondary challenge. This study has also shown that a secondary immune response can be elicited with a small amount of antigen, which is probably important in reducing lung infections after repeated exposures to antigen.
CHAPTER III
EFFECT OF INTRAVENOUS OR INTRAPULMONARY ADMINISTRATION OF MURAMYL DIPEPTIDE ON PULMONARY CELL-MEDIATED IMMUNITY

ABSTRACT

This study examined the effects on pulmonary cell-mediated immunity (CMI) of an adjuvant, muramyl dipeptide (MDP). The MDP was given by intrapulmonary instillation or by intravenous injection. Four Beagle dogs were given intrapulmonary immunizations with antigen only, $10^{10}$ sheep red blood cells (SRBC), in the left cardiac lung lobe and received saline in the right cardiac lung lobe as a control. Eight Beagle dogs were administered MDP one day before intrapulmonary SRBC immunization and again on the day of immunization. Four dogs were given MDP and antigen in the left cardiac lung lobe. Another four dogs received MDP iv and the antigen in the left cardiac lung lobe. The tests used to evaluate CMI in lung lavage fluids and blood were the leukocyte procoagulant activity (LPCA) assay and the macrophage migration inhibition factor (MIF) test. The LPCA of dogs exposed to SRBC only in the lung showed a peak response from 9 to 12 days in both the immunized and control lung lobes. The blood LPCA peaked on day 7 after immunization. The MIF response was highest at 12 to 14 days
after immunization in the immunized and control lobes. In comparison to results from dogs receiving antigen only, treatments with MDP given in saline into lung and by the intravenous route resulted in immune suppression, as measured by the MIF and LPCA assays. Lymphocytes were present in the lung, however, to produce an immune response as shown by the cytology. Much of the literature describing in vitro studies indicates that MDP enhances the immune response. Previously published in vivo data, however, are contradictory; therefore, more research is needed to resolve the question of MDP's adjuvant activity.

INTRODUCTION

The cell-mediated immune response after immunization has traditionally been studied in blood, lymph nodes, spleen, and peritoneal exudate cells. Pulmonary CMI has not been studied extensively because of the low number of immune lymphocytes recovered from lung lavages. The use of an adjuvant concomitant with exposure to antigen in the lung could increase the number of lymphocytes recovered.

An adjuvant is "any factor that enhances the immune response" (Freund, 1956). Freund's complete adjuvant (FCA) is probably the best known. However, it is a crude and complex mixture of mycobacteria emulsified in oil and an emulsifying agent (Arlacel A). Instillation of FCA into the lungs results in an unacceptable, intense inflammation. Recently, N-acetyl muramyl-L-alanyl-D-isoglutamine (muramyldipeptide, MDP), the minimal adjuvant-active structure that
can substitute for mycobacteria tuberculosis in FCA, has been synthesized and made available. Because MDP is water-soluble, instillation into the lung should be possible with minimal histologic changes.

The biological activity of MDP has been well documented both in vivo and in vitro (Chedid et al., 1978; Parant, 1979; Waksman, 1979) with different responses observed in the whole animal than were predicted from the cell culture systems. The work examined humoral immunity (Azuma et al., 1976; Specter et al., 1977; Leclerc et al., 1979a), as well as the adjuvant's mechanism of action (Lowy et al., 1977; Fevrier et al., 1978; Wahl et al., 1979). Its ability to stimulate CMI has also been investigated (Sugimoto et al., 1978; Matter, 1979; Leclerc et al. 1979b). Although most studies have reported enhanced immune response, there are scattered reports of the inhibitory effects of MDP (Souvannavong and Adam, 1980; Heymer et al., 1978; Leclerc et al., 1982). The objective of our study was to evaluate the in vivo effects of MDP on pulmonary cell-mediated immunity (CMI). MDP was given by two different routes whereas the antigen was always instilled into a specific lobe of the lung. The pulmonary immune response was then evaluated using two tests for CMI, macrophage migration inhibition (MIF), and leukocyte procoagulant activity assay (LPCA).
MATERIALS AND METHODS

Animals. Twelve male Beagle dogs from the Inhalation Toxicology Research Institute's colony, 1 to 2 years of age, 7-10 kg, were used in this study. All dogs were found to be healthy after physical examinations were performed. The dogs were brought in from indoor-outdoor kennels 3 days before immunization to allow them to acclimate to indoor cages. The dogs were housed one to a cage and fed once a day (Wayne Dog Food, Allied Mills, Chicago, Ill.) and given water ad libitum. Food was withheld from the dogs 24 hours before immunization and before each lavage. The dogs were randomly divided into three equal groups, each of which received sheep red blood cells (SRBC) antigen in the left cardiac lung lobe. The control group received only antigen in the left cardiac lung lobe. In addition to the antigen, the second group was given MDP iv, and the third group received MDP in the lung with the antigen.

Immunizations. Specific airways were selected for immunization with a fiberoptic bronchoscope (Bice et al., 1980a). The immunization of four dogs (antigen only) involved intrapulmonary deposition of \(10^{10}\) SRBC in the left cardiac lung lobe; 1 ml of physiological saline was instilled into the right cardiac lung lobe as a control. The second group of four dogs received MDP (N-acetyl muramyl-L-alanyl D-isoglutamine, Syntex Chemical Co., Palo Alto, Ca.) in saline (0.1 mg/kg) intravenously one day before the antigen. Twenty-four hours later, the dogs received another iv injection of MDP in saline and \(10^{10}\) SRBC.
intrabronchially in the left cardiac lung lobe. Saline (1 ml) was instilled into the right cardiac lung lobe as a control. The third group of four dogs was given, MDP intrabronchially. One day before the antigen was given each dog received 1 ml of MDP (0.1 mg/kg) in sterile saline in the left cardiac and in the left diaphragmatic lung lobes. One day later, the left cardiac lobe received the same dose of MDP together with \(10^{10}\) SRBC; the left diaphragmatic lobe received 1 ml of MDP in saline. The right cardiac lung lobe was given 1 ml of saline as a control. The dose and timing of the adjuvant were based on the results of Richerson et al. (1982) and Souvannavong and Adam (1980). The SRBC came from a single sheep and were never more than 2 days old when used for immunization. An immunization scheme is shown in Table 3.1.

**TABLE 3.1 MDP Dosing Regimen Relative to SRBC Immunization (0 time) in the Dog Lung.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>- 24 hours</th>
<th>0 time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>LC  LD  RC</td>
<td>BLOOD</td>
</tr>
<tr>
<td>SRBC in Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRBC and MDP in Lung</td>
<td>MDPC MDP</td>
<td></td>
</tr>
<tr>
<td>SRBC in Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDP I.V.</td>
<td></td>
<td>MDP</td>
</tr>
</tbody>
</table>

LC, left cardiac; LD, left diaphragmatic; RC, right cardiac lung lobes.
a) The SRBC was administered in 1 ml sterile saline.
b) The saline was given in 1 ml.
c) The MDP was administered in 1 ml of saline at a concentration of 0.1 mg/kg for each dog.
d) Left diaphragmatic data are not reported in the text because they were not significantly different from the saline control.
The dogs were lavaged and a venous blood sample taken from the jugular on days 5, 7, 9, 12, 14, 16, and 21 after immunization. On the day of each lavage, anesthesia (5% halothane mixed with oxygen) was given by face mask. An endotracheal tube was placed in the trachea. Light surgical anesthesia was maintained with 1.5% halothane in oxygen. A fiberoptic bronchoscope with a biopsy channel was then introduced into the airways through the endotracheal tube. The same immunized and control lung lobes were re-entered with the bronchoscope after immunization on designated days and washed five times with 10 ml of sterile saline through the biopsy channel of the fiberoptic bronchoscope. The wash fluid was 85-95% recovered. Recovered fluid was stored in centrifuge tubes in ice until the laboratory procedures were done.

Cell preparation. The cells obtained by segmental lavage were washed three times in RPMI 1640 medium (Grand Island Biological Co., GIBCO, Grand Island, NY) supplemented with 25 mM Hepes buffer (N-2 Hydroxyethylpiperazine N'-2-ethanesulfonic acid) and 50 μg/ml Gentamicin (Sigma, St. Louis, MO) by centrifugation at 200 x g for 10 min.

Venous blood was drawn into heparinized tubes and later diluted with an equal volume of Hank's balanced salt solution (HBSS, GIBCO, Grand Island, NY). Lymphocytes were separated by centrifugation on Isolymph (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY), washed three times in medium, and suspended at a concentration of 4 x 10^6 /ml in complete medium (RPMI 1640 supplemented as above plus 10%
heat-inactivated newborn calf serum (GIBCO, Grand Island, NY). The serum had been absorbed with SRBC.

**Evaluation of lavage and blood cells.** Cytocentrifuge slides were prepared on all bronchoalveolar fluid and blood samples. These slides were stained with a rapid Romanowsky-type stain (Diff-Quick, Harleco Co., Gibbstown, NJ). The percent of alveolar macrophages, lymphocytes and polymorphonuclear leukocytes was determined by counting 300 total cells by light microscopy. The normal cell population from an unimmunized control dog is approximately 85 to 95% alveolar macrophages, 5% neutrophils, and 10% lymphocytes (Rebar et al., 1980). Cytology results were statistically analyzed using a two-way analysis of variance (ANOVA) among the three groups over time. The total number of cells/ml of lavage fluid was determined using an electronic cell counter (Coulter Electronics, Hialeah, FL).

**Evaluation of immunity.** The LPCA assay as described by Geczy and Meyer (1982) was performed on washed lavage cells and mononuclear leukocytes separated from blood. The assay is completely detailed on pages 37 and 38 of this document.

Migration of lavage cell populations with and without antigen was evaluated as a measure of MIF production. The method describing how this assay was performed is outlined on pages 38 and 39 of this document.

The MIF and LPCA assays were statistically analyzed over time using a two-way ANOVA and Bonferroni's test for multiple comparisons to determine significant differences; specific time points after immunization were tested using
Student's t test.

RESULTS

All of the dogs were healthy throughout this experiment. No clinical signs of illness or adverse reactions to the anesthesia, immunization procedures, or the adjuvant were noted.

Leukocyte procoagulant activity assay. Four unimmune dogs showed a percent shortening of plasma clotting time for lavage cells and blood lymphocytes that were cultured with and without SRBC antigen to be 0 and 14%. Twenty percent shortening of plasma recalcification time is considered significantly different from background. The percent shortening of plasma recalcification time for both the immunized and control lung lobes is displayed in Figure 3.1. The LPCA assay measured a rise in percent shortening of plasma recalcification time after immunization with SRBC only, which was highest from 9 to 12 days after immunization, then declined to day 21. Intravenous introduction of MDP resulted initially in a prolonged increase in procoagulant activity over that seen with SRBC alone with a peak at 9 days. After administration of the adjuvant and antigen into the left cardiac lung lobe, a minimal difference from background in plasma clotting time was noted. The intrabronchial administration of MDP resulted in a gradual rise in the percent shortening of plasma clotting time to day 12, then a gradual decline to day 21. The results, however, are marginally above the
Figure 3.1. The effects on procoagulant activity of cells from the control and immunized lung lobes after instillation of SRBC antigen and muramyl dipeptide (MDP) administered by two different routes. The intravenous MDP treatment is represented by the broken line. Each point represents the mean of four dogs ± SEM. For clarity, the error bars were not added to the antigen only treatment group, but they can be found on the figures in Chapter 1.
background of unimmunized dogs.

The control lobe of the lungs in all three groups received saline. The immune response, measured by the LPCA assay, in the control lobe cells was similar to the response of cells from the immunized lobes. The results were generally lower in the control lobes than in the immunized lung lobe, but not significantly lower.

Percent shortening of plasma clotting time was similar for cells isolated from blood after the two different adjuvant treatments (Fig. 3.2). Cells from the dogs that received the antigen alone peaked on day 7 with a 27% increase in percent shortening of plasma recalcification time, with a gradual decline to day 21. The percent shortening of plasma recalcification time of blood cells from the group given MDP iv also peaked on day 7 and 12, but was significantly lower than when the antigen was given alone. The intrabronchial administration of MDP with antigen showed a late rise in procoagulant activity on day 9 to 12 after immunization. Either route of adjuvant administration produced a minimal increase in the procoagulant activity in the blood that was not significant from background.

Macrophage migration inhibition. Unimmunized control dogs were lavaged to assess the basal level of nonspecific antigen inhibition on the migrating macrophage. This ranged from -3 to 16% in 4 dogs. The level set for significant inhibition, therefore, was 20%.

The MIF assay was performed by adding antigen back to the
Figure 3.2. The percent shortening of plasma clotting time after cells isolated from the blood of all three groups were cultured 20 hours with and without SRBC antigen. Each point represents the mean of four dogs ± SEM.
Figure 3.3. The effect of SRBC antigen on the migration of cells washed from the control and immunized lung lobes of Beagle dogs. The percent inhibition of migration was determined by comparing the mean size of four migration areas in culture medium with 200 μg/ml SRBC antigen to the mean size of four migration areas of cells from the same lobe in culture medium without antigen. Each point represents the mean of four dogs ± SEM.
cultures; no adjuvant was used in any cell culture assay. The calculated percent inhibition for control and immunized lung lobes is displayed in Figure 3.3. Pulmonary macrophages from immunized lung lobes, after immunization with antigen alone, showed no difference in percent inhibition of migration when cultured with and without antigen on days 5 and 7. The percent inhibition increased until day 14 and declined rapidly to day 21. This time curve of macrophage migration inhibition had a distinct peak that was not seen in the time course from the cells collected from the dogs given adjuvant by either route. The iv administration of MDP with the antigen given in the lung resulted in a significant increase in macrophage migration inhibition on day 5, but was the same as background at later times. When MDP was given intrabronchially with antigen, macrophage migration inhibition reached a marginal level of significance on day 9, but fell thereafter.

The cells cultured from the control lobe of animals instilled with antigen only showed a peak MIF response on day 12 and a rapid fall in MIF production to day 21. The iv injected MDP resulted in an initial significant MIF response that was marginally sustained to day 14, after which the percent inhibition fell to day 21. The control lobe of animals that received MDP together with antigen intrabronchially never reached a significant level of inhibition at any time.

Cytology. The types of cells washed from the immunized lung lobe after different routes of adjuvant administration
Figure 3.4. Lymphocytes, neutrophils (PMNs) and alveolar macrophages in the lung lavage fluid from the immunized lung lobes of dogs that received MDP iv and a second group that received MDP intrabronchially with antigen are presented. Each point represents the mean of four dogs ± SEM. The antigen only cytologies are pictured on the next page.
Figure 3.5. Lymphocytes, neutrophils (PMN), and alveolar macrophages in the lung lavage fluid from the immunized left cardiac lung lobe. Each point represents the mean of four dogs ± SEM.
are presented in Figure 3.4. Figure 3.5 shows the cell types of the immunized lobe after antigen treatment alone. This figure was reproduced from Chapter 2. The macrophage population was never different between the antigen alone and either of the adjuvant iv studies. The percentage of macrophages generally dropped from the time zero normal of 85 to 90% as the immunization causes the influx of other cell types into the lung. As the immune response waned, the percent macrophage population rose as the lung cell population returned to normal. The polymorphonuclear leukocytes (PMN) were highest in number on day 5 after either of the MDP treatments, with a slow decline to day 21. Cells from the dogs given the antigen alone had a rise in neutrophil numbers 7 days after immunization, followed by a fall to baseline levels. The percentage of lymphocytes was generally lower in the dogs' lungs after iv administration of MDP, compared to that in dogs that received antigen alone. When the lung received MDP plus antigen, the percent lymphocyte population showed a discrete peak on day 12, followed by a sharp decline. The overall lymphocyte population curves from these two treatment groups were somewhat parallel.

The cell types from the control lung lobes after the two adjuvant treatments are displayed in Figure 3.6. The macrophage populations compared for the three treatments are never significantly different from each other. The lymphocyte population after iv MDP was slightly, but not significantly, depressed. The neutrophil population showed
Figure 3.6. The control lung lobe cell populations: Lymphocytes, PMN, and alveolar macrophages in the lung wash fluid from dogs that received MDP iv and a second group that received MDP intrabronchially with antigen.
a discrete peak on day 9 and was generally higher than the
treatment with antigen alone. The dogs given MDP and
antigen in the lung displayed no cytology differences from
when antigen alone was deposited in the lung.

DISCUSSION

This study reports on two different routes of
administration of MDP with antigen, both resulting in
suppression of the CMI response of the lung as compared to
when antigen alone was instilled in the lungs. Although MDP
is cleared rapidly from the body, as reported by Parant et al. (1979), it effects a change in the responsiveness of
the immune system during its short residence time.

The two CMI assays employed, LPCA and MIF, showed that
the immune response was depressed by either route of MDP
administration, even though the same amount of antigen was
given to each dog in the three groups. The LPCA assay
performed after iv administration of MDP was the exception.
The measurements made with this assay were high during the
entire experiment. The nonimmune control dogs also showed a
high baseline LPCA when this antigen preparation was used.
This could be due to the aggregated nature of this antigen
used in the cell cultures. The MIF assay performed at the
same time on nonimmune dogs was unaffected by this antigen.

The suppressed response indicated by the MIF and LPCA
assays was not due to a lack of immune effector cells, since
the cytology of the immunized lung lobes showed an increased
cell number of lymphocytes and neutrophils sufficient for
immune enhancement. The iv administration of MDP resulted initially in a high, prolonged neutrophil recruitment into the immunized lobe. Although cytolocy results were indicative of sufficient immune effector cells, the immune reaction after administration of MDP by either route resulted in suppression of the cell-mediated immune reaction to antigen instilled into the lung.

Matter (1979) reported that the antigen concentration used is just as important as the adjuvant concentration. He reported immune suppression at supraoptimal antigen doses and after single-dose treatments of adjuvant. The adjuvant was routinely given three or four times by various routes for the MDP to produce immune enhancement. Several of the cell-mediated immunity assays that Matter (1979) reported show MDP enhancing the assay when it was performed in vitro. The same assay showed suppression of the immune response in vivo after MDP treatment. Souvannaavong and Adam (1980) reported that the timing of the adjuvant administration in relation to the antigen is also important. They found that adjuvant given iv one day before or after antigen administration enhanced immunity. Adjuvants are hypothesized to exert their immunological enhancing action through the macrophage; however, MDP’s mechanism of action is different from other adjuvants. Haidden et al. (1979) compared four immunopotentiators, inoluding MDP, for their ability to enhance lymphocyte and macrophage functions. Two adjuvants were found to modulate macrophage and lymphocyte cytotoxicity and proliferation, respectively. MDP activated
the macrophage only by increasing phagocytosis. This implies that MDP has different mechanisms of action from other adjuvants. It is possible that MDP activation of the macrophage elicits both amplifying and suppressive immune effector cells that can play a facilitating or deleterious role in the resultant immune response. The manifestation of these immune effector cells will depend on many things: type of antigen, route of administration of antigen and adjuvant, concentration of antigen and adjuvant, and timing of MDP administration in relation to antigen. All these have led to some contradictory in vivo results; however, this is not new to adjuvant research. Studies with BCG have led to the same contradictions (Mathe et al. 1973).

Since most of the CMI systems are macrophage-dependent, more macrophage and T cell studies are needed to determine what combination of the above mentioned variables makes MDP function as an adjuvant in vivo. This study has raised some questions about the immune enhancement capabilities. In vivo data supporting MDP as an adjuvant are contradictory. MDP’s mechanism of action on an intact, dynamic system needs more study before conclusions can be drawn. Until then, this compound should be referred to as an immunomodulator rather than as an adjuvant.
CHAPTER IV

PULMONARY IMMUNE RESPONSE OF DOGS

AFTER EXPOSURE TO $^{239}$PuO$_2$

ABSTRACT

Insoluble plutonium dioxide particles are tenaciously retained in the lung after inhalation. One route of translocation of these particles from the lung is to the tracheobronchial lymph nodes, where the plutonium dioxide particles have a long retention time. The alpha radiation from plutonium can kill cells and exert a deleterious effect on lymphoid tissues that respond immunologically in defense of the lung. This study evaluated the influence of $^{239}$PuO$_2$ inhalation on the cell-mediated and humoral immune response of the lungs of Beagle dogs after local deposition of antigen. Four dogs had received a single brief exposure (5 to 6 years previous) by pernasal inhalation to one of three monodisperse PuO$_2$ aerosols having sizes of 0.72 μm, 0.75 μm and 1.4 μm activity median aerodynamic diameter. This resulted in initial lung burdens of 0.52 to 1.10 μCi and lymph node doses of 720-1440 Gy. Four non-exposed dogs served as age-matched controls. The cell-mediated immune (CMI) response in the lung was evaluated by the leukocyte procoagulant activity test. The results of this CMI test from both groups of dogs correlated with the number of lymphocytes recruited into the lung. The humoral immune
response was measured by the enzyme-linked immunosorbent assay, which showed that even though some dogs gave a high IgG antigen-specific antibody response in the serum, the same dogs could not recruit immune effector cells or products into the lung.

INTRODUCTION

In recent years, the alpha-emitting radionuclide, $^{239}$Pu, has aroused public concern because of its use in nuclear weapons and as a by-product and a potential fuel source in nuclear power reactors. Plutonium metal oxidizes readily to $\text{PuO}_2$. Particulates of $^{239}\text{PuO}_2$ may be inhaled after accidental release (Bair and Thompson, 1974).

After inhalation, the extremely insoluble $^{239}\text{PuO}_2$ particles are translocated to regional tracheobronchial lymph nodes (Park et al., 1972; McClellan, 1972). Once translocated to the lymph nodes, the resident T and B lymphocytes will continuously be irradiated because of the long physical and biological halflife of $^{239}\text{PuO}_2$. It has long been known that lymphocytes are one of the most radiosensitive cell types present in the mammalian body (Bloom and Bloom, 1954).

After challenge of the canine lung with particulate antigen, the immune response begins in the tracheobronchial lymph nodes; immune effector cells or products are released into the blood and recruited back to the lung to produce an immune response to the antigen (Bice et al., 1980a). Clearly, the translocation of plutonium to the lymph nodes
results in large radiation doses to them and may produce a toxic effect on the immune system. This study examined cell-mediated and humoral immune responses in the blood and lung lavage fluid of dogs that had been exposed to $^{239}$PuO$_2$ or were unexposed, age-matched controls that had been immunized with sheep red blood cells (SRBC).

MATERIALS AND METHODS

Animals. Eight Beagle dogs, 6 to 7 years of age, from the Inhalation Toxicology Research Institute’s colony were used in this study. Three days before immunization, the dogs were transferred from indoor-outdoor kennels to acclimatize them to indoor cages. The dogs were housed one to a cage and fed once a day (Wayne Dog Food, Allied Mills, Chicago, IL) and given water ad libitum during experimentation.

Exposures. Four dogs served as age-matched controls. The other four dogs underwent acute pernasal inhalation of a monodisperse aerosol of $^{239}$PuO$_2$. The dogs were approximately one year of age at the time of exposure. The $^{239}$PuO$_2$ aerosols were monodispersed with an activity median aerodynamic diameter (AMAD) of 0.72 or 1.4 μm. The exposure resulted in an initial lung burden of 0.52 to 1.10 μCi (see Table 4.1, Results). The method of preparation of monodisperse aerosols has been reported (Raabe et al., 1975; Guilmette et al., in press). Briefly, $^{239}$Pu (+4) was precipitated as a colloidal hydroxide. This colloid was then nebulized using a Lovelace nebulizer. The resultant
droplets were heated and passed through a furnace for oxidation to $239\text{PuO}_2$. The polydisperse aerosol was directed into a Lovelace Aerosol Particle Separator, and particles were separated according to aerodynamic diameter. The particle sizes to be used in each experiment were selected from the separator and subjected to ultrasonic agitation to resuspend the particles in distilled water. The resultant suspension was nebulized and dried before the exposure of Beagle dogs. The exposure apparatus has been described previously (Boecker et al., 1964; Mewhinney and Diel, 1983).

**Immunizations.** Food was withheld from the dogs 24 hours before immunization and before each lavage, which required anesthesia. Anesthesia, 5% halothane mixed with oxygen, was induced using a face mask. An endotracheal tube was placed in the trachea. Light surgical anesthesia was maintained with 1.5% halothane in oxygen. A fiberoptic bronchoscope was introduced into the airways by way of the endotracheal tube. Specific airways were selected for immunization with a fiberoptic bronchoscope as in previous studies (Bice et al., 1980b). The immunization of four control dogs and four plutonium-exposed dogs involved intrabronchial deposition of $10^{10}$ SRBC in the left cardiac lung lobe, and 1 ml of physiological saline was instilled into the right cardiac lung lobe so that each dog served as its own control. The SRBC came from a single sheep and were never more than 2 days old when used for immunization. Cells from only four dogs per day could be processed;
therefore, the eight dogs were separated into two groups. Each group contained two plutonium-exposed and two age-matched control dogs.

The dogs were lavaged and a venous blood sample taken on days 5, 7, 10, 12, 14, 17, and 20 after immunization. The immunized and control lung lobes were reentered with the bronchoscope on designated days and washed five times with 10 ml of sterile saline through the biopsy channel of the fiberoptic bronchoscope. The instilled wash fluid was 85 to 95% recovered and was stored in centrifuge tubes on ice until the laboratory procedures were done.

Cell preparation. The cells obtained by segmental lavage were washed three times in RPMI 1640 medium (Grand Island Biological Co., GIBCO, Grand Island, NY) supplemented with 25 mM Hepes buffer (N-2 Hydroxyethylpiperazine N'-2 ethanesulfonic acid) and 50 μg/ml Gentamycin (Sigma, St. Louis, MO) by centrifugation at 200 x g for 10 min. The cells were resuspended in 1 ml of supplemented medium.

Venous blood was drawn from the jugular into heparinized tubes and later diluted with an equal volume of Hank's balanced salt solution (HBSS, GIBCO, Grand Island, NY). Lymphocytes were separated by centrifugation on Isolymph (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY), washed three times in medium, and finally suspended at a concentration of $4 \times 10^6$ cells/ml in complete medium (RPMI 1640 supplemented as above, plus 10% heat-inactivated newborn calf serum (GIBCO, Grand Island, NY), which had been absorbed with SRBC.
Evaluation of lavage and blood cells. Cytocentrifuge slides were prepared on all bronchoalveolar and blood samples. These slides were stained with a rapid Romanowsky-type stain (Diff-Quick, Harleco Co., Gibbstown, NJ). The percent of alveolar macrophages, lymphocytes, and polymorphonuclear leukocytes (PMN) was determined by counting 300 total cells/slide. The normal cell population from an unimmunized control dog is approximately 85-95% alveolar macrophages, 5% neutrophils, and 10% lymphocytes (Rebar et al., 1980). The total number of cells/ml of lavage fluid was determined using an electronic cell counter (Coulter Electronics, Hialeah, FL). Clinical records of complete blood counts and blood chemistry were evaluated from birth to time of immunization to determine if the dogs had lymphopenia.

Evaluation of immunity. The leukocyte procoagulant activity (LPCA) assay measures the effect of a lymphokine on the macrophage to produce clotting factors; hence, an increased percent shortening of plasma recalcification time reflected an increased CMI response. The LPCA assay was performed on washed lavage cells and mononuclear leukocytes separated from blood, as described by Geczy and Meyer (1982). The procedure to perform the LPCA assay has been described on pages 37 and 38 of this document.

The enzyme-linked immunosorbent assay (ELISA) was performed to examine the antigen-specific antibody recovered in lavage fluids (diluted 1:50) and in the serum (diluted 1:200) using the Gilford PR-50 automatic analyzer (Gilford
Instrument Laboratories, Inc., Oberlin, Ohio). This assay was performed using a modified method of Voller et al. (1976). The procedure to perform the LPCA assay is described on page 40 of this document.

RESULTS

The deposited $^{239}\text{PuO}_2$ particles have been shown to be retained in the lung with an effective half-life of 680 and 1400 days for the 0.72 and 1.4 $\mu$m AMAD aerosols, respectively (Guilmette et al., in press). This would result in a dose rate of 0.86 and 13 Gy/day to the lung for the respective aerosol particles. Since the $^{239}\text{PuO}_2$ is translocated to the lung-associated lymph nodes at a rate with a positive exponential, the dose to the lung-associated lymph nodes would exceed that of the lung at the time of this evaluation.

The aerosol exposure data for each plutonium-exposed dog is presented in table 4.1. Three of the dogs, 996U, 905S, and 885B, were exposed to similar particle sizes. The dose to the lungs and the lung-associated lymph nodes is dependent on duration of exposure, activity deposited, and particle size. In this study, 908T had the highest dose to the lung-associated lymph nodes, with 996U and 905S having only about half as great a dose at the time of this experiment.
TABLE 4.1. The Aerosol Data from the 239PuO2-exposed Dogs. Exposure to Monodisperse Aerosols Occurred from 77140-78006.

<table>
<thead>
<tr>
<th>DOG NUMBER</th>
<th>INITIAL LUNG BURDEN (µCi)</th>
<th>AEROSOL SIZE (AMAD, µm)</th>
<th>LYMPH NODE DOSE (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>996U</td>
<td>0.52</td>
<td>0.75</td>
<td>770</td>
</tr>
<tr>
<td>905S</td>
<td>0.60</td>
<td>0.72</td>
<td>720</td>
</tr>
<tr>
<td>885B</td>
<td>0.90</td>
<td>0.72</td>
<td>1180</td>
</tr>
<tr>
<td>908T</td>
<td>1.10</td>
<td>1.40</td>
<td>1440</td>
</tr>
</tbody>
</table>

Two of the dogs, 905S and 908T, had a history of chronic lymphopenia after exposure to plutonium. The other two dogs, 885B and 996U, had transient lymphopenia after exposure.

The humoral and CMI responses in these dogs was variable within each group, so that averaged data resulted in loss of important information. Therefore, data for individual dogs from each group are presented.

Age-matched control dogs. The age-matched control dogs, 6 to 7 years of age, were healthy. Three of these dogs had high levels of IgG immunoglobulins in their serum that peaked 10 to 12 days after immunization (Fig. 4.1). However, only one dog, 935U, had high amounts of IgG in the immunized lobe of the lung (Fig. 4.2). The IgG and IgM antibody response were evaluated; however, since IgG is the major class of immunoglobulins found in the dog, only the IgG will be presented.

The number of neutrophils and lymphocytes lavaged from
Figure 4.1. The serum antigen-specific IgG antibody from 0 to 20 days after immunization with SRBC for the control age-matched control dogs. Each line represents the immune response of an individual dog.
Figure 4.2. The IgG antigen-specific antibody of the lavage fluid from the immunized lobe of the age-matched control dogs. Each point is the value for the ELISA optical density unit of an individual dog.
the control lung lobe of the unexposed, control dogs over the course of the experiment was minimal, and all curves were flat (PMN and lymphocyte mean + SEM, $2.5 \times 10^6 + 1.1$ and $1.9 \times 10^6 + 0.6$, respectively). However, unlike lobes in younger dogs (Bice et al., 1980a), the immunized lung lobes in this group also had a constant low number of neutrophils and lymphocytes ($2.0 \times 10^6 + 1.0$, $2.1 \times 10^6 + 2.5$, respectively). The one dog that had a normal cellular response in the lung, 935U, was an exception. This dog had a normal influx of PMN and lymphocytes into the immunized lung lobe. The peak neutrophil response occurred at day 7, with a total of $8.3 \times 10^6$ neutrophils present in the immunized lung lobe. The total number of lymphocytes, $11.3 \times 10^6$, peaked on day 12. The immune competence of these cells recruited to the immunized lung lobe of dog 935U is shown in Figure 4.2.

LPDA data from the immunized lung lobe was similar to the ELISA and the cytology data in that 935U showed a good LPDA response in the immunized lung lobe, with 43% shortening of plasma recalcification time at 17 days after immunization.

Plutonium-exposed dogs. The antigen-specific antibody ELISA data from the serum of plutonium-exposed dogs are presented in Figure 4.3. Dogs 885B, 908T, and 996U, responded to immunization with an increased amount of antigen-specific IgG in blood. The times of peak response varied, but these three dogs eventually produced amounts of IgG that reached the maximal detection of our
Figure 4.3. The serum antigen-specific IgG antibody from 0 to 20 days after immunization with SRBC for dogs exposed to plutonium. Each line represents the immune response of an individual dog.
instrumentation at a serum dilution of 1:200. Dog 905S had a late, but measurable, response.

The antigen-specific IgG in lavage fluid of the control and immunized lung lobes of the plutonium-exposed dogs is shown in Figure 4.4 (A and B). Dog 908T had a high IgG response in both the immunized and control lung lobes. Dog 885B had an increased IgG response in the immunized lung lobe and a marginal response in the control lobe. Dogs 996U and 905S had the same low amount of IgG in the immunized as in the control lung lobe.

The total number of PMN and lymphocytes that were lavaged from the control lung lobe of the plutonium-exposed dogs over the course of the experiment are shown in Figure 4.5 (A and B). The effect of immunization and lavage resulted in marked changes in the number of neutrophils in the control lobe of these dogs. Even though the control lobe of the 239PuO₂ dogs received only saline, there was a dramatic rise in neutrophil numbers seen 7 days after saline instillation (Fig. 4.5A). The dog, 905S, had an unusually high neutrophil response on day 7. The total number of lymphocytes shown in figure 4.5B was marginally elevated above background, and only 908T showed a late lymphocyte increase, which was different from that in the other three dogs.

The number of neutrophils and lymphocytes lavaged from the immunized lung lobe is shown in Figure 4.6 (A and B). The neutrophil response of 905S and 996U was abnormally high in comparison to that of the age-matched control dogs or
Figure 4.4. The antigen-specific IgG antibody in the control (A) and immunized (B) lung lobe lavage fluid from the plutonium-exposed dogs. Each point represents the value of an individual dog.
Figure 4.5. The control lung lobe cell population: total number of PMN (A) and lymphocytes (B) in the lung wash fluid from dogs exposed to 239PuO₂. Each point represents the total number of cells ($10^6$) from an individual dog.
Figure 4.6. The immunized lung lobe cell population: total PMN (A) and lymphocytes (B) in the lung lavage fluid of dogs exposed to $^{239}$PuO$_2$. Each point represents the value of the total cells ($10^6$) from an individual dog.
compared to that of two year old dogs \((15 \times 10^6 + 5, 7\) days after immunization). 885B had a more normal neutrophil response compared to that of two year old dogs, with a late, distinct peak. 908T did not have an increase in neutrophil number. 885B had a gradual rise in the number of lymphocytes (Fig. 4.6B), whereas the other three dogs had lower numbers of lymphocytes that came into the lung.

The LPCA data for the immunized lung lobe of the plutonium-exposed dogs is shown in Figure 4.7. Three of the dogs, 885B, 908T, and 996U had positive LPCA responses, although the times of peak response were variable. Dog 905S generally had a lower response than did the other three dogs. The LPCA data for the control lobes were similar to those of the immunized lung lobes and are not shown.

DISCUSSION

The low immune response in three of the control dogs was an unexpected result that has made it difficult to evaluate the toxicity of inhaled plutonium in dogs of the same age. The individual variance within each group has also made it difficult to assess group responses. The low immune response in lungs of the control dogs was probably a result of the age of the dogs. By age-matching the control and plutonium-exposed dogs, we thought we could discount the effect of age and evaluate only the plutonium toxicity to the immune system. The data from the age-matched control dogs, as well as other published data (Adler, 1975; Yunis et al., 1976; Bice and Muggenburg, 1983), pointed out that
Figure 4.7. The effect of SRBC antigen immunization on the procoagulant activity of cells from the immunized lung lobe of plutonium-exposed dogs. Each point represents the value of an individual dog.
age alone can have a significant influence on the development of immune responses in the lung. Although some of the aged dogs had normal amounts of antigen-specific antibody in their blood after lung immunization, unless the appropriate cellular changes occur in the lung that result in increased vascular permeability, the immune cells and immunoglobulins in the blood are not recruited into the lung. This is exemplified by the antigen-specific antibody response of 935U. This dog had a normal serum IgG level, with a corresponding neutrophil influx into the lung followed by lymphocytes and immunoglobulins. This dog developed normal antibody and CMI responses in the lung. The other three control dogs did not have cellular changes in the lung after immunization, and consequently, a poor immune response in the lung.

The CMI and humoral responses of two year old dogs, as reported in the first chapter, showed a significantly different response from that of the 6 to 7 year old control dogs for this study. Yunis and Lane (1979) reported that although both the humoral and cellular immune system decline with age, the effect on the cell-mediated immune system is more pronounced.

Three dogs in the plutonium-exposed group had increased amounts of specific IgG immunoglobulins in their serum after immunization (Fig. 4.3). Two plutonium-exposed dogs had increased specific IgG immunoglobulins in lung lavage fluid (Fig. 4.4).

The most noticeable difference between exposed and
control dogs was the large number of neutrophils recovered in lavage fluid from both control and immunized lung lobes of the plutonium-exposed dogs. The high influx of neutrophils into both the saline control and immunized lung lobe of the plutonium-exposed dogs was not observed in the lungs of the age-matched controls or the two year old control dogs (Chapter 1). The neutrophil response in both lung lobes of the plutonium-exposed dogs was much higher than that seen in two year old dogs. The younger dogs' neutrophil count in the control lobe never exceeded 2.5 \( \times 10^6 \) cells and was 15 \( \times 10^6 \) neutrophils in their immunized lung lobe at 7 days after immunization. In contrast, the neutrophils in the saline control lobe of the plutonium-exposed dogs ranged from 6 to 35 \( \times 10^6 \) cells and 20-55 \( \times 10^6 \) cells in the immunized lung lobe (3 dogs only) on the day of peak immune response. It is possible that the plutonium caused a chronic low grade inflammation and the subsequent lavages resulted in an influx of neutrophils. An increase in neutrophils number is usually a result of an inflammatory response, with an extravasation of plasma proteins, immune products, and cells across the pulmonary epithelium. However, with the plutonium-exposed dogs this was not always the case. The dog, 905S had a tremendous number of PMN recruited into the lung, but this dog had severe chronic lymphopenia; therefore, few lymphocytes were present in this dog to be recruited to the lung or to produce immunoglobulins. The antibody level (Fig. 4.3) in the serum of this dog was also low, and, therefore, not
available for translocation into the lung.

As observed in control dogs, the exposed dogs with normal numbers of neutrophils and lymphocytes in the immunized lung lobe tended to give good pulmonary CMI and humoral responses, i.e., 885B and 908T. It is interesting that 996U had a delayed increase in antigen-specific IgG in the serum and lung, with an early positive LPCA response. This was probably due to what each assay measured. The LPCA assay is dependent upon the production of lymphokine, which can diffuse away from the antigen-stimulated lymphocyte and affect many macrophages. David (1966) showed that only a few stimulated T lymphocytes are necessary to produce a CMI response. Antibody production, on the other hand, has been shown to be directly related to the number of stimulated B lymphocytes in the lung or blood (Bice et al., 1980b).

In light of the data from dogs 885B (good responder) and 905S (poor responder), it is interesting that dog 885B received a greater dose of $^{239}$PuO$_2$ to the lung-associated lymph nodes, yet produced a good immune response. Since there was such a large dose to the lymph nodes of all the animals, this difference in dose may have little biological significance. Snipes et al., (1983) showed that each canine lung lobe has a specific pattern for lymphatic drainage. It is likely that exposure to plutonium obliterated the function of the lymph nodes that drain the lung for all these dogs. If true, the immune cells and immunoglobulins produced by 885B were probably produced in lymphoid tissues that were not exposed to $^{239}$PuO$_2$ particles. It is possible
that antigen drained from the lung may pass through damaged lymph nodes and be filtered in other lymphoid tissues, such as the mediastinals or even the spleen. A production of immune cells and antibody in these tissues could resume the immune function for protection of the lung from antigenic material and other infectious agents. It is also possible, but not likely considering the radiation dose to the lymph nodes, that there was enough residual functional tissue in the draining lymph node to produce the response. Immunization of dogs before sacrifice and evaluation of immune cells in individual lymphoid tissues could answer this question.
CHAPTER V

PULMONARY PROCOAGULANT ACTIVITY OF DOGS

WITH LUNG TUMORS.

ABSTRACT

Coagulation is an integral part of the host's immune and inflammatory response. It has recently been reported that cultured tumor cell lines release measurable procoagulant activity. This study evaluated the procoagulant activity and spontaneous macrophage migration of bronchial cells of: a) dog lung lobes that had radiographically-proven lung tumors, b) lobes in the same dogs without tumors and c) control dog lungs with no detectable lung masses. These dog lungs were lavaged, and the cell fraction and wash fluid were assayed for procoagulant activity by a one-stage recalcification assay. The spontaneous migration of bronchial wash cells was measured in vitro from capillary tubes. The procoagulant activity of the cells and fluid washed from the lung lobe with a tumor was significantly increased over that of the control lobe in the same dog. The control lung lobes examined in the tumor-bearing dogs had a procoagulant activity of the cell fraction that fell between that of tumor-bearing lung lobes and that of control dogs. In contrast, the lavage fluid from the control lobe of the
tumor-bearing dogs was essentially the same as the procoagulant activity of the lavage fluid from control dogs. The spontaneous migration area of cells from the tumor lobe was significantly greater than that of cells from both sets of control lung lobes. The migration area of cells from the control lobe of the tumor-bearing dogs was equal to the migration areas of the control lung lavage cells. In summary, the presence of the tumor changed the local environment in some way so that cells washed from the tumor-bearing lung lobes of dogs showed an increase in procoagulant activity and an increase in macrophage mobility.

INTRODUCTION

Systemic and pulmonary clotting abnormalities have been reported for patients with malignant neoplasms and after severe trauma (O'Meara, 1958; Saldeen, 1967; Ogura et al., 1971; Pochedly et al., 1973; Busch et al., 1975; Bone et al., 1976; Bachofen and Weibel, 1977; Luterman et al., 1977; Marx, 1982; Rinaldo and Rogers, 1982). Most of the pulmonary clotting abnormalities have been recognized during histological examination after necropsy. The mechanism of triggering the coagulation system and, hence, the deposition of fibrin are unknown. It is known, however, that the lung, brain, placenta, myocardium, liver, and kidney contain significant amounts of thromboplastin (Nemerson and Pitlick, 1972). The primary concentration of thromboplastin is in the intima of large and small blood vessels, thus placing it
in proximity to the plasma coagulation factors (Kirk, 1962; Astrup and Buluk, 1963).

Thromboplastin is a cell membrane-bound factor involved in the extrinsic coagulation cascade. Coagulation can occur through another system of factors called the intrinsic cascade. Either of these coagulation schemes can be triggered by a number of events: trauma to blood vessels, endotoxin, antigen-antibody complexes, complement activation, and other inflammatory mediators (Zimmerman et al., 1977). These interactions can cause fibrin to be deposited in the area of the traumatic reaction.

It has recently been reported that the supernatants of various cultured neoplasms contain procoagulant activity (Dvorak et al., 1981). It is thought that the neoplasm produces a factor that acts upon macrophages to increase their production of thromboplastin; hence, they can be involved in the clotting process once initiators have been presented either through the blood stream or by leaking into the lung because of the trauma of the neoplastic process. The present experiments evaluate the procoagulant activity of canine segmental lavage cells and fluid from animals with radiographically-proven lung tumors and compare results to nontumor-bearing control dog lung procoagulant activity. This was an effort to evaluate procoagulant activity produced in vivo in lung.

MATERIALS AND METHODS

Animals. Twenty Beagle dogs, 2 to 15 years old, from
the Inhalation Toxicology Research Institute's colony were used in this study. Seven dogs were used as nontumor-bearing control dogs. These ranged from 2 to 7 years old. The other thirteen dogs were diagnosed by radiography as having lung tumors at various stages of progression. These dogs ranged from 5.5 to 15 years old. Even though the ages of the two groups differ widely, two of the advanced neoplasms were found in younger (3 to 4 years old) dogs. Different types of neoplasm were identified by cytocentrifuge slides, and eventually, histologic evaluation of the neoplasm. The neoplasms were primary lung tumors with the exception of two dogs, which had primary mammary cancers which had metastasized to the lung. The primary lung tumors were caused by inhalation exposure to various inhaled radioactive compounds. The dogs were brought from indoor-outdoor kennels 3 days before lavage to allow them to acclimate to indoor cages. They were housed one to a cage and fed once a day (Wayne Dog Food, Allied Mills, Chicago, IL) and given water ad libitum.

Lung lavage. The tumors were located by radiography, and that specific area of the lung was lavaged. A venous blood sample taken from the jugular was drawn on the day of lavage. Food was withheld for 8 hours before lavage, which required anesthesia. Anesthesia, (5% halothane mixed with oxygen) was induced using a face mask. An endotracheal tube was placed in the trachea. Light surgical anesthesia was maintained with 1.5% halothane in oxygen. A fiberoptic bronchoscope was then introduced into the airways through
the endotracheal tube. The tumor-bearing lung lobe and areas of the same dog’s lung without radiographically visible tumors were lavaged. Two lung lobes in the control dogs were lavaged to harvest sufficient numbers of cells for the tests. These dogs had negative chest radiographs. The selected lobes in both groups of dogs were entered with the bronchoscope and washed five times with 10 ml of sterile saline through the biopsy channel of the bronchoscope. The lung wash fluid recovery from the lavage procedure ranged from 50 to 90% of amounts of fluid instilled. The airways to the tumor lobes were sometimes occluded, and the instilled wash fluid was hard to recover. This also presented a problem for recovery of sufficient cells from tumor dog lung lobes to perform the assays. Recovered fluids were stored in centrifuge tubes on ice until laboratory procedures were done.

**Cell preparation.** Cells obtained by segmental lavage were washed three times in RPMI 1640 medium (Grand Island Biological Co., GIBCO, Grand Island, NY) supplemented with 25 mM Hepes buffer (N-2 Hydroxyethylpiperazine N’-2 ethanesulfonic acid) and 50 μg/ml Gentamicin (Sigma, St. Louis, MO) by centrifugation at 200 x g for 10 min. The washing procedure was important because pulmonary surfactant and mucus have been reported to have procoagulant activity (Pineo et al., 1973).

**Procoagulant activity assay.** The procoagulant activity assay was performed on washed lavage cells and fluid as described by Geczy and Meyer (1982). This assay examined
the ability of cells and/or a substance in the lavage fluid to enhance the clotting of normal autologous plasma using a modified one-stage recalcification assay. The procoagulant activity assay was performed immediately on cells that were resuspended after centrifugation in Hank's balanced salt solution (HBSS, GIBCO, Grand Island, NY) at a concentration of $4 \times 10^6$ cells/ml. The lavage fluid was not concentrated before the assay.

The plasma for this assay was from dogs on study at the time. Initially, the plasma from the tumor-bearing and control dogs was kept separate. The procoagulant activity assay was performed with both lots of plasma. There was no difference in clotting times using the two lots; therefore, they were combined for the rest of the study. Blood was drawn into sodium citrate tubes (3.8% v/v in 0.9% NaCl), and plasma prepared by centrifugation at 1800 x g for 30 min. This centrifugation procedure removed virtually all the platelets from the plasma. Plasma was aliquoted into appropriate volumes and stored at -70 °C for no longer than one month.

The recalcification time was determined by incubation of 0.1 ml of each of the cell suspension or lavage fluid, plasma and 0.9% NaCl for 1 min at 37 °C. CaCl$_2$ (0.03 M prepared in 0.9% NaCl, 0.1 ml) was added and the clotting time assessed visually.

**Macrophage migration.** The spontaneous migration of lavage cells from capillary tubes was evaluated in all test animals. The bronchoalveolar cell pellets were resuspended
in 1 ml of complete RPMI 1640 medium (supplemented as stated above plus heat-inactivated fetal calf serum). The cell suspensions were drawn into 75 ul nonheparinized capillary tubes, sealed by flaming and packed by centrifugation as described by Bice et al. (1977). The capillary tubes were cut at the cell interface and placed in migration chambers (Superior Plastics Products, Cumberland, RI) containing complete medium. Migration areas were measured by planimetry. The macrophage migration assays and LPCA assays were statistically analyzed using Student's t test. The correlation coefficient was calculated after a line was fit to the graph by linear regression.

Evaluation of lavage cells. Cytocentrifuge slides of lung lavage cells were prepared on bronchoalveolar wash samples. These slides were stained with a rapid Romanowsky-type stain (Diff-Quick, Harleco Co., Gibbstown, NJ), and another set of slides was stained using a Papanicolaou stain (Sigma, St. Louis, MO). The percent of alveolar macrophages, lymphocytes, and polymorphonuclear leukocytes was determined by counting 300 cells/slide. The normal cell population from a nonimmunized control dog is approximately 85% alveolar macrophages, 5% neutrophils, and 10% lymphocytes (Rebar et al., 1980). Cytology results were statistically analyzed using Student's t test. The total number of cells/ml of lavage fluid was determined by an electronic cell counter (Coulter Electronics, Hialeah, FL.).
RESULTS

The types of lung tumors evaluated in this study were primarily carcinomas; however, there were a suspected alveolar adenocarcinoma and two primary mammary tumors, with metastasis to the lung. The two mammary tumors were in animals that were used for breeding and had never been exposed to radioactive compounds. It was obvious that the procoagulant activity was dependent on the progression of disease. The two dogs that had extensive neoplastic involvement of the lung had an increased procoagulant activity that was manifested by a decreased (shorter) plasma clotting time. The converse was also true. Dogs that had small tumors had more normal procoagulant activity (161 ± 3.2 seconds) in the lung.

The procoagulant activity data are presented in Table 5.1. The procoagulant activity of the cell fractions was significantly different between the control and tumoring-bearing dogs. The control lung lobe from the tumor-bearing dogs had procoagulant activity that was marginally different from the tumor lobe in the same dog. (P<0.10), but not significantly different from those in the control dogs.
TABLE 5.1. Procoagulant Activity

<table>
<thead>
<tr>
<th></th>
<th>Tumor Dogs</th>
<th>Control Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Lobe</td>
<td>Tumor Lobe</td>
</tr>
<tr>
<td>Cells</td>
<td>145 ± 11</td>
<td>123 ± 11</td>
</tr>
<tr>
<td>Lavage Fluid</td>
<td>198 ± 14</td>
<td>158 ± 18</td>
</tr>
</tbody>
</table>

Each number represents the mean ± SEM for the procoagulant activity assay done on cells and the lavage fluid of the two groups of dogs. The procoagulant activity assay is the measure of plasma clotting time (seconds). The tumor dog group, n = 13. Control dog group, n = 7.

The procoagulant activity in the lavage fluid from the tumor-bearing dogs was significantly greater (P<0.025) than that in the lavage fluid of the control dogs. Procoagulant activity is greater when plasma clotting time is less or shorter. Lavage fluid from the nontumor lung lobes of the tumor dogs was essentially the same as the control dog lung procoagulant activity and was significantly less (P<0.025) than that found in the same animal in a lung lobe with a tumor.

The area of spontaneous migration of cells from the tumor-bearing lung lobe was statistically greater than that from the control dogs (P>0.05, Table 5.2). The spontaneous migration area of cells from the control lobe of the tumor-bearing dogs was equal to that from control dogs.
TABLE 5.2. Bronchoalveolar Cell Migration Areas

<table>
<thead>
<tr>
<th></th>
<th>Tumor Dogs</th>
<th>Control Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Lobe</td>
<td>2.9 ± 0.3</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>Tumor Lobe</td>
<td>4.9 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

Each number represents the mean ± SEM for the spontaneous migrations (sq. in.) of cells lavaged from the lungs of both groups. There were sufficient cells harvested from 5 of the tumor-bearing dogs to perform this assay and six of the control dogs.

The correlation between plasma clotting time and cell migration areas is plotted in Figure 5.1. The control dogs showed a positive correlation between plasma clotting time and migration areas. The plasma clotting time increased as the migration areas increased. An inverse correlation was seen, however, between plasma clotting time and migration areas in the tumor-bearing lung lobes. The clotting time decreased as the cell migration areas increased. Not all of the animals had sufficient cells recovered from the lavaged lung lobes to perform the spontaneous migration assays.

The percentage of cell types found in each lung lobe of the two groups is shown in Table 5.3. The control dogs and the control lobe of the tumor dogs showed normal values for the percentage of PMN and lymphocytes. The tumor-bearing lung lobes showed essentially the same percentage of lymphocytes as in the control group, but a significantly greater percentage of neutrophils (PMN) and lymphocytes. The tumor-bearing lung lobes showed essentially the same
Figure 5.1. The means of the plasma clotting time and macrophage migration areas are plotted. Each point represents these values for one dog. The migration assay required many cells, and there was frequently not enough cells to do this assay. The r values represent the correlation coefficients for the lines.
percentage of lymphocytes as in the control group, but there was a significantly greater percentage of neutrophils \((P>0.05)\), and the percentage of macrophages was significantly lower \((P<0.05)\). The total number of cells recovered are not reported here because it would only reflect the variability of wash fluid recovery rather than actual numbers of cells present in the tumor-bearing lung lobes.

Table 5.3. PERCENT CELL TYPES IN THE DIFFERENT LUNG LOBES

<table>
<thead>
<tr>
<th>TUMOR DOGS</th>
<th>CONTROL DOGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Lobe</td>
</tr>
<tr>
<td>PMN</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>Macrophages</td>
<td>77 ± 4</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM of 300 total cells counted. These cell counts were made from cytocentrifuge slides stained with Wright-Giemsa or papanicolaou stain. There were seven control dogs and nine dogs with tumors. Dogs that had advanced suppurative neoplasms had red blood cells in their lavage fluid.

The stained cytocentrifuge slides of lavage fluid showed agglutinated tumor cells (Fig. 5.2). These cells were characterized by numerous dark staining nucleoli in single cells, altered nucleus/cytoplasm ratios, and an increased "stickiness", as shown by the clumps of cells. In the two
Figure 5.2. Tumor cells lavaged from the tumor-bearing lung lobes. There are numerous PMN surrounding the agglutinated tumor cells, along with red blood cells (RBC). (Papanicolaou stain, 56X magnification).
dogs that had advanced neoplasms, many neutrophils and some red blood cells were noted, as shown in these micrographs.

DISCUSSION

This study showed the plasma clotting activity to be greater for cells and lavage fluid from lung lobes that contained tumors than lung lobes without tumors, either in the same dog or control dogs. The cells from the control lobe of the tumor-bearing dogs showed procoagulant activity that fell between that of the cells from control and tumor-bearing dogs. It is possible that there was a neoplastic lesion in this group of lung lobes that was not radiographically visible at the time. It is also possible that once a neoplastic process is established in the lung it may cause a mild inflammation in the entire lung by some soluble mediator, causing increased procoagulant activity (Dvorak et al., 1979). Most studies of procoagulant activity point to a cell-associated tissue thromboplastin as the source of procoagulant activity (Zeldis et al., 1972; Levy and Edington, 1980; Hopper et al., 1981; Chapman et al., 1983). This study indicated extracellular release of the procoagulant activity by showing a significant decrease in the plasma clotting time after addition of lavage fluid to the assay from the tumor-bearing lung lobe. Dvorak et al. (1981), however, offered an explanation for this by presence of membrane vesicles with procoagulant activity that pelleted after ultracentrifugation of tumor cell culture supernatants. This indicated that the procoagulant activity
was still cell-associated in bits of membrane containing the procoagulant activity and that these bits were released into the surrounding medium.

Warren et al. (1975) and Dvorak et al. (1979) have demonstrated that certain neoplasms in culture release distinct biological mediators; one is macrophage migration inhibition factor (MIF). Our study did not evaluate the presence of MIF, but rather examined the spontaneous migration of cells from capillary tubes placed in chambers into complete medium. There was more than one type of lung neoplasm studied, but the migration areas were significantly larger from the tumor-bearing lung lobes than from either the control lung lobes from tumor dogs, or from cells in tumor-free dogs. This could represent an activation of the macrophage by the neoplasm. However, the literature is contradictory and reports both stimulation (Russell and McIntosh, 1977) and inhibition of the phagocytic cell population (Synderman et al., 1976) by neoplasms. It is possible that a soluble mediator liberated by the tumors activated the macrophages. The macrophages were more likely actively phagocytizing the debris resultant of the tumor, which could increase their mobility. Meltzer and Stevenson (1978) have shown that macrophages harvested from the same compartment as a tumor show a marked increase in phagocytosis. It is unknown whether it is the same factor causing the increased procoagulant activity and increased macrophage migration in the tumor-bearing lung lobes. Based on other studies with tumors, it is unlikely that the two
effects were mediated through only one factor (Roth et al., 1982).

The percentage of neutrophils in the tumor lung lobe was significantly elevated, indicating an inflammatory response in that lung lobe. The literature indicates that leukocytes can produce procoagulant activity (Niemetz and Fani, 1971; Lerner et al., 1971; Rothberger et al., 1978); however, there has been some recent controversy about contamination of leukocyte cultures by other cell types which aid in the production of procoagulant activity. To identify a cell source for procoagulant activity would be to overstate the results of this experiment; however, there were increased percentages of neutrophils and small numbers of identifiable tumor cells in the lavage preparation. It is possible that a factor was released from the tumor that acted upon another cell to make it express its procoagulant activity. This relationship has been shown with activated lymphocytes and macrophages (Geczy and Meyer, 1982).

The correlation data that showed clotting time decreased as migration area increased was contrary to published data. Geczy and Meyer (1982) and information reviewed earlier in this publication showed that decreased clotting time directly reflected decreased macrophage migration. The procoagulant activity manifested by an immune response has been shown to be due to T cell lymphokine production (Levy et al., 1981) and the lymphokine's effect on macrophages. The increased procoagulant activity is thought to be due to a tissue thromboplastin (Sitrin et al., in press). The
procoagulant activity from tumors has been found to a primary factor in addition to thromboplastin (Gordon et al., 1975); therefore, the mechanism by which tumors trigger the coagulation cascade may be different from the pathway followed by immunological stimulation.

There was a positive correlation between control dog plasma clotting time and migration area data. However, the variability in the migration areas was greater than that of the plasma clotting times. This is indicative of data not having a linear correlation and with an insignificant biological correlation (Steel and Torrie, 1980).

Researchers report other pathological pulmonary conditions that have increased fibrin content in the lung; however, little is known about the reason or consequence of its being there. Degradation products of fibrin/fibrinogen have been shown to produce an inflammatory response in the lung (Haynes et al., 1980). Neoplastic tissue, however, has been shown to produce mediators that inhibit the accumulation of inflammatory cells (Snyderman and Pike, 1976). More research, therefore, is necessary to work out the interactions between the coagulation system and pathological lung conditions.
CHAPTER VI

SUMMARY

The results of studies on cell-mediated immune (CMI) responses in the lung were presented in the preceding chapters. The procoagulant activity assay was used in all studies to evaluate the effects of lymphokine on the macrophage. The fourth manuscript described how the presence of lung tumors could effect the procoagulant activity of the lung cell population.

As presented in the first chapter, the results obtained with the procoagulant activity assay were compared with results from a standard CMI assay, the macrophage migration inhibition factor (MIF) assay. It was found that the two assays correlated well. The procoagulant activity assay was reproducible and easy to perform in comparison to the MIF assay. Therefore, this assay can be used as a good indicator of CMI in the lung.

This assay could be studied further by evaluating the biochemical characteristics of the lymphokine responsible for production of the procoagulant activity. This could be done by purifying lymphokine by column chromatography. The effects of column purified fractions could then be tested on an alveolar macrophage population and procoagulant activity evaluated. The use of certain plasmas deficient of clotting factors could help determine how the procoagulant activity
is manifested.

Augmentation of the immune system with adjuvants is a powerful tool to study immune reactions. However, to date there have been no satisfactory adjuvants found that can be instilled into the lung to enhance pulmonary CMI. Because MDP is water soluble and is not antigenic or mitogenic, we thought pulmonary instillation might result in augmentation of the immune response. However, instillation or intravenous injection resulted in suppression of the pulmonary immune response. If this project is to be pursued, different doses of adjuvant and/or antigen should be used to determine if this compound can enhance the pulmonary immune response. Perhaps MDP in mineral oil given iv and antigen instilled into the lung would result in enhanced pulmonary immunity.

The effect of inhaled 239PuO₂ on pulmonary immunity was difficult to determine because the age-matched control dogs had poor pulmonary immune responses. There seemed to be an overshadowing effect of age and some dogs from both groups produced high serum IgG responses to instilled antigen, although few of these dogs recruited immune cells or products from the serum into the lung. This was contrary to the younger dogs IgG lung response reported in the first manuscript. The plutonium with the addition of serial lavage seemed to act synergistically to result in inflammation of the plutonium-exposed dog lungs. The saline control lobe as well as the immunized lung lobe had high numbers of polymorphonuclear leukocytes present in lavage
fluid. If further immunological and toxicological studies are to be done on aged dogs, the immune competence of each animal needs to be determined before exposure and then followed after exposure to a toxicant to separate the effects of age and the toxicant.

It was shown that lung tumors affected the pulmonary cell population in a way that resulted in decreased plasma clotting time when these cells were added to the plasma recalcification assay. This is a relatively new area of study and there are several areas open for further investigation. What cell or soluble product is responsible for the procoagulant activity of the macrophage is unknown. It is not known whether this tumor-related phenomenon is similar to immunological activation of the macrophage. Plasmas deficient in clotting factors could help answer mechanistic questions. It is not known how changes in procoagulant activity can effect tumor growth.
REFERENCES


Allergy Appl. Immunol. 68, 377-381.


59.549-557.


New York.
CURRICULUM VITAE

JENNIFER BAKER GALVIN

DATE OF BIRTH: July 31, 1953
PLACE OF BIRTH: Lindsay, Oklahoma
MARITAL STATUS: Married (Mark Galvin, 1977), no children
MAILING ADDRESS: Jennifer Baker Galvin
Inhalation Toxicology Research Institute
P. O. Box 5890
Albuquerque, New Mexico 87185
BUSINESS TELEPHONE: (505) 844-1406

EDUCATION:
May 1973 Oklahoma State University, A.D. - Health Physics
May 1975 University of New Mexico, B.S. - Nuclear Medicine
1975-1976 Liverpool University, Rotary Fellowship - Pharmacology
May 1981 Utah State University, M.S. - Toxicology
1982-Present Utah State University, with research being performed at the Inhalation Toxicology Research Institute, Albuquerque, New Mexico, Ph.D. - Pulmonary Toxicology

PROFESSIONAL EXPERIENCE:
Government and Private Industry:
1977 National Center for Toxicological Research - Chemist/Health Physicist
1978 Enviro-Med Testing Laboratory - Chemist
1979 St. Francis Medical Center - Nuclear Medicine Technologist
1981 Inhalation Toxicology Research Institute - Laboratory Graduate Participant (Pulmonary Toxicology)
Academic Research:

1975 University of Liverpool - Warfarin binding and metabolism in liver microsomes

1980 Utah State University - Macrophage toxicity and activation after hexavalent chromium exposure

Teaching:

1980 Utah State University - Biology

1980 Utah State University - Radiation Health and Safety

MEMBERSHIP IN PROFESSIONAL SOCIETIES:

- Society of Nuclear Medicine
- Registry of Radiologic Technologists
- Health Physics Society (Salt Lake Chapter)
- Math-Science Network (Women in Science)

 AWARDS:

- President's Leadership Scholarship
- Wilham Scholarship, Rotary
- Fellowship, NIH Traineeship

AREAS OF RESEARCH:

- Development of specific tests to evaluate pulmonary cell-mediated immunity (CMI) and to use these tests to determine pulmonary immunotoxicity from various energy-related effluents. To date, the leukocyte procoagulant activity test has been developed for evaluation of CMI in the lung.

- Examination of the pulmonary procoagulant activity of dogs with various lung tumors and the correlation between macrophage activation and procoagulant activity.

- Alveolar macrophage - lymphocyte interactions: Macrophage activation and this cell's ability to modulate the pulmonary immune system through interactions with the lymphocyte.

- Evaluation of a new water soluble adjuvant, muramyl dipeptide, for pulmonary use.
Abstracts:


Publications:


7. J. B. Galvin, D. E. Bice and B. A. Muggenburg. The Effects of Age and Inhalation of $^{239}$PuO$_2$ on Pulmonary Cell-mediated Immunity. (in preparation)

Presentations:
