THE CLEARANCE KINETICS AND TISSUE DISTRIBUTION OF TUMOR NECROSIS FACTOR-α IN PARENTERALLY AND ENTERALLY FED RATS

By

Mary E. Keith

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Nutritional Sciences
University of Toronto

© Copyright by Mary E. Keith, 1997.
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-27975-8
The Clearance Kinetics and Tissue Distribution of Tumor Necrosis Factor-α (TNF) in Enterally and Parenterally Fed Rats.

Doctor of Philosophy, 1997
Mary E. Keith
Graduate Department of Nutritional Sciences
University of Toronto

ABSTRACT

We have developed an animal model in which the cachexia of chronic and acute disease is reproduced by the provision of a continuous infusion of tumor necrosis factor-α (TNF)-infused. Orally-fed animals developed anorexia and tissue-wasting, however there was no mortality. The provision of total parenteral nutrition (TPN) to TNF-infused animals resulted in a potentiation of the metabolic effects of TNF as well as in significant mortality. Since circulating levels of exogenous TNF were highest in the animals with the most severe metabolic abnormalities, we hypothesized that TPN affects TNF clearance.

Our initial study compared the plasma clearance of a bolus of labelled TNF in TPN-fed animals given sufficient nutrients to allow weight-gain (WGR) with those given 50% of the WGR nutrients called weight-losing rats (WLR) and also in orally fed rats (OFR). Data were analyzed using compartmental and linear systems analysis. In a second study, a mammillary model of compartmental analysis was used to compare the tissue distribution and uptake of TNF in rats sacrificed at 4, 10, 20, 60 and 180 minutes post-TNF injection.

Plasma data, from both studies, indicated that although metabolic clearance was similar, WGR had a slower fractional clearance rate as well as a larger volume of distribution than WLR or OFR. An increased proportion of the total mass of TNF resided in the plasma-
associated compartment in WGR suggesting that TNF is retained in this compartment in these animals. Organ studies suggested impaired uptake of TNF by the tissues of WGR, especially in the kidney, which is thought to be the major organ of TNF clearance. The low affinity for TNF in organs of WGR as well as the expanded volume of distribution suggest that TNF is retained in the plasma by soluble TNF receptors resulting in impaired clearance.

Malnutrition and tissue wasting observed in the clinical setting has resulted in the provision of aggressive nutrition support. Our studies suggest that aggressive nutrition support can affect TNF clearance, possibly enhancing the metabolic effects of TNF. Therefore, we suggest that treatment of the underlying cause of cachexia should have priority over the implementation of aggressive nutrition support.
ACKNOWLEDGEMENTS

I would like to acknowledge the many people, both friends and family, who have supported and encouraged me throughout these years. In particular, I thank my husband Brian, who has offered endless support through the ups and downs of my program as well as becoming an excellent lab assistant. I would also like to acknowledge the support of my parents as well as for their continued belief that I could accomplish anything that I put my mind to. I gratefully acknowledge the lab group. Annie, Regina and Nilima for their technical guidance and friendship throughout the years. I also wish to acknowledge the support of my supervisor. Dr. K.N. Jeejeebhoy, not only for his financial support and technical guidance of my program, but also for his interest in me as an individual through his continued support of my career goals. I would also like to acknowledge the contribution and patience of Dr. K.H. Norwich and Willy Wong, who were instrumental in the completion of the mathematical analysis of this work. Finally, I would like to thank Clintec Nutrition Company for their financial support.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 2 REVIEW OF THE LITERATURE</td>
<td>4</td>
</tr>
<tr>
<td>2.1 INTRODUCTION</td>
<td>4</td>
</tr>
<tr>
<td>2.2. THE CHRONIC EFFECTS OF TNF-α</td>
<td>4</td>
</tr>
<tr>
<td>2.2.1. The Basics of Tumor Necrosis Factor-α</td>
<td>4</td>
</tr>
<tr>
<td>2.2.2. Synthesis of Tumor Necrosis Factor</td>
<td>6</td>
</tr>
<tr>
<td>2.2.3. The Role of TNF in the Development of Cachexia</td>
<td>7</td>
</tr>
<tr>
<td>2.2.3.1. Development of Anorexia</td>
<td>7</td>
</tr>
<tr>
<td>2.2.3.2. Changes in Fat Metabolism Associated with TNF-α</td>
<td>9</td>
</tr>
<tr>
<td>2.2.3.3. Changes in Carbohydrate Metabolism</td>
<td>12</td>
</tr>
<tr>
<td>2.2.3.4. TNF Effects on Protein Metabolism</td>
<td>12</td>
</tr>
<tr>
<td>2.2.3.5. The Effect of TNF on Muscle Transmembrane Potential</td>
<td>14</td>
</tr>
<tr>
<td>2.3. CONTINUOUS TNF INFUSION AS A MODEL OF CHRONIC ILLNESS</td>
<td>15</td>
</tr>
<tr>
<td>2.4. NUTRITION SUPPORT AND TUMOR NECROSIS FACTOR-α</td>
<td>17</td>
</tr>
<tr>
<td>2.5. THE CLEARANCE OF TNF</td>
<td>20</td>
</tr>
<tr>
<td>2.5.1. TNF Receptors</td>
<td>20</td>
</tr>
<tr>
<td>2.5.1.1. Binding and Internalization</td>
<td>25</td>
</tr>
<tr>
<td>2.5.1.2. Factors Affecting TNF Receptor Expression</td>
<td>26</td>
</tr>
<tr>
<td>2.5.1.3. Regulation of TNF Receptors by other Cytokines</td>
<td>28</td>
</tr>
<tr>
<td>2.5.2. Studies of Tissue Binding and Clearance</td>
<td>29</td>
</tr>
<tr>
<td>2.5.3. Clearance of TNF by Alpha2-Macroglobulin</td>
<td>33</td>
</tr>
<tr>
<td>2.5.4. Regulation of TNF by Anti-TNF Antibodies</td>
<td>33</td>
</tr>
<tr>
<td>2.5.5. Regulation of TNF by Soluble TNF Receptors</td>
<td>35</td>
</tr>
<tr>
<td>2.5.5.1. Soluble TNF Receptor Production</td>
<td>35</td>
</tr>
<tr>
<td>2.5.5.2. Structure of Soluble TNF Receptors</td>
<td>37</td>
</tr>
<tr>
<td>2.5.5.3. Physiologic Roles of Soluble TNF Receptors</td>
<td>38</td>
</tr>
<tr>
<td>2.5.5.3.1. Soluble Receptors as TNF Receptor Binding Antagonists</td>
<td>38</td>
</tr>
<tr>
<td>2.5.5.3.2. Inhibition of TNF Activity Trials</td>
<td>39</td>
</tr>
<tr>
<td>2.5.5.3.3. Stabilization of TNF Activity</td>
<td>42</td>
</tr>
<tr>
<td>2.5.5.3.4. Soluble TNF Receptor Production in Disease</td>
<td>43</td>
</tr>
</tbody>
</table>
2.6. DEVELOPMENT OF A MODEL TO STUDY TNF CLEARANCE

2.7. THE IODINATION OF TNF

2.8. COMPARTMENTAL MODELLING

2.9. HYPOTHESIS DEVELOPMENT
   2.9.1. General Hypothesis
   2.9.2. Specific Hypotheses
   2.9.3. Objectives

CHAPTER 3.0 METHODS AND PRELIMINARY STUDIES

3.1. INTRODUCTION

3.2. SURGICAL PROCEDURES
   3.2.1. Animal Preparation and Central Venous Catheter Insertion
   3.2.2. Bile Duct Cannulation
   3.2.3. Bladder Cannulation

3.3. DIET FORMULATION

3.4. MEASUREMENT OF RADIOACTIVITY IN SAMPLES
   3.4.1. Plasma Radioactivity
   3.4.2. Radioactivity in Whole Blood, Bile and Urine
   3.4.3. Uptake of $^{125}$I-TNF by Organs

3.5. ANALYSIS OF CLEARANCE DATA
   3.5.1. Compartmental Modelling
   3.5.2. Linear Systems Analysis

3.6. ALBUMIN STUDY

3.7. THE STABILITY OF TUMOR NECROSIS FACTOR-α
   3.7.1. Denaturation of TNF
   3.7.2. Gel Filtration Chromatography of TNF
   3.7.3. Column Preparation
   3.7.4. Developing a Calibration Curve
   3.7.5. Molecular Weight Determination of Unknown Samples
   3.7.6. Results of Plasma Chromatography
3.8. GEL ELECTROPHORESIS OF BILE AND URINE
   3.8.1. Gel Preparation 67
   3.8.2. Sample Preparation 67
   3.8.3. Fixing, Staining and Destaining 69
   3.8.4. Results of Gel Electrophoresis 71

3.9. CHROMIUM LABELLING OF RED BLOOD CELLS 71
   3.9.1. Labelling Protocol 72
   3.9.2. Calculation of Blood Volume 73

3.10. HEMATOCRIT STABILITY 73
   3.10.1. Hematocrit Collection Protocol 73

3.11. TNF ELISA 74
   3.11.1. TNF ELISA Protocol 74
   3.11.2. TNF ELISA Results 76

3.12. STATISTICAL ANALYSIS 76

CHAPTER 4.0 INITIAL STUDY OF TUMOR NECROSIS FACTOR CLEARANCE
   IN PARENTERALLY AND ENTERALLY FED RATS 79

4.1 INTRODUCTION 79

4.2 MATERIALS AND METHODS 79
   4.2.1 Animal Preparation 79
   4.2.2. Cachectin/TNF 80
   4.2.3. Diet Formulation 80

4.3. EXPERIMENTAL PROTOCOL 81

4.4. MEASUREMENT OF RADIOACTIVITY IN SAMPLES 82
   4.4.1. Plasma 82
   4.4.2. Organs 83

4.5. GEL FILTRATION OF PLASMA AND 125I-TNF 83

4.6. ANALYSIS OF CLEARANCE DATA 83
   4.6.1. Compartmental Modelling 87
   4.6.2. Linear Systems Analysis 90

4.7. STATISTICAL ANALYSIS 93
### 4.8. RESULTS

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8.1</td>
<td>Weight Change of Experimental Animals</td>
</tr>
<tr>
<td>4.8.2.1</td>
<td>Plasma Clearance of $^{125}$I-Tumor Necrosis Factor</td>
</tr>
<tr>
<td>4.8.2.2.1</td>
<td>Compartmental Analysis</td>
</tr>
<tr>
<td>4.8.2.2.2</td>
<td>Linear Systems Analysis</td>
</tr>
<tr>
<td>4.8.2.3</td>
<td>Organ Uptake of TNF</td>
</tr>
<tr>
<td>4.8.2.4</td>
<td>Gel Filtration of Plasma and $^{125}$I-labelled TNF</td>
</tr>
</tbody>
</table>

### 4.9. DISCUSSION

### CHAPTER 5.0 TISSUE DISTRIBUTION AND CLEARANCE OF $^{125}$I-TUMOR NECROSIS FACTOR IN ENTERALLY AND PARENTERALLY FED RATS

#### 5.1 INTRODUCTION

#### 5.2 OVERALL HYPOTHESIS

5.2.1. Specific Hypotheses

#### 5.3 OBJECTIVES

#### 5.4. EXPERIMENTAL METHODS

5.4.1. Study Protocol

5.4.2. Linear Systems Analysis

5.4.3. Radioactivity in the Bile and Urine

5.4.4. Compartmental Modelling of Organ Uptake Data - A Mammillary System

5.4.4.1. Calculation of the Rate Constants For the Flow of TNF between Compartments

5.4.4.2. Determination of the Rate Constants for the Loss of TNF in the Bile and Urine

5.4.4.3. Interpretation of Rate Constants

5.5.1 Weight Change of Animals

5.5.2. Plasma Clearance Kinetics of $^{125}$I-Tumor Necrosis Factor

5.5.3. Organ Uptake and Distribution of $^{125}$I-Tumor Necrosis Factor

5.5.4 Compartmental Analysis of Rate of Uptake of Labelled TNF by Organs

#### 5.6. DISCUSSION AND CONCLUSION

### CHAPTER 6.0 SUMMARY AND CONCLUSIONS

#### 6.1 INTRODUCTION

#### 6.2 SUMMARY OF OVERALL CONCLUSIONS
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>Composition of TPN Solution</td>
<td>56</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Composition of Amino Acid Solution</td>
<td>57</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Composition of Lipid Emulsion</td>
<td>57</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>Albumin Clearance Kinetics</td>
<td>64</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Compartmental Analysis of the TNF Clearance Kinetics</td>
<td>89</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Clearance Kinetics of Orally and Parenterally Fed Animals Using Linear Systems Analysis (LSA)</td>
<td>92</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Clearance Data</td>
<td>119</td>
</tr>
<tr>
<td>Table 5.2</td>
<td>Area Under The Organ Uptake and Plasma Disappearance Curve</td>
<td>122</td>
</tr>
<tr>
<td>Table 5.3</td>
<td>Area Under The Organ Uptake and Plasma Disappearance Curve</td>
<td>123</td>
</tr>
<tr>
<td>Table 5.4</td>
<td>Rate Constants for the Movement of TNF</td>
<td>129</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>A Model for the Synthesis, Degradation and Clearance of TNF</td>
<td>21</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>The Open Compartmental Model</td>
<td>49</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>The Mammillary System</td>
<td>50</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Clearance of $^{125}$I-Albumin</td>
<td>58</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Chromatography of TNF</td>
<td>68</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>SDS Gel Electrophoresis of Bile Samples</td>
<td>70</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Autoradiogram of Bile Samples</td>
<td>70</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Hematocrit Test</td>
<td>75</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>TNF ELISA</td>
<td>77</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>TNF Clearance in TPN fed Weight-Gain Animals</td>
<td>84</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>TNF Clearance in TPN Fed Weight-Loss Animals</td>
<td>85</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>TNF Clearance in Orally Fed Animals</td>
<td>86</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Compartmental Analysis of Clearance Data</td>
<td>88</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Rat Body Weights</td>
<td>94</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Organ Uptake of TNF</td>
<td>96</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>TNF in the Bile</td>
<td>109</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>TNF in the Urine</td>
<td>109</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>Single Compartmental Model</td>
<td>111</td>
</tr>
<tr>
<td>Figure 5.4</td>
<td>Complex Compartmental Model</td>
<td>111</td>
</tr>
<tr>
<td>Figure 5.5</td>
<td>Rat Body Weights</td>
<td>117</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>5.6</td>
<td>Plasma Clearance of TNF</td>
<td>118</td>
</tr>
<tr>
<td>5.7</td>
<td>TNF Uptake by the Liver</td>
<td>125</td>
</tr>
<tr>
<td>5.8</td>
<td>TNF Uptake per Gram Liver</td>
<td>125</td>
</tr>
<tr>
<td>5.9</td>
<td>TNF in the Bile</td>
<td>126</td>
</tr>
<tr>
<td>5.10</td>
<td>TNF in the Bile - Precipitated</td>
<td>126</td>
</tr>
<tr>
<td>5.11</td>
<td>TNF Uptake by the Kidney</td>
<td>127</td>
</tr>
<tr>
<td>5.12</td>
<td>TNF Uptake per Gram Kidney</td>
<td>127</td>
</tr>
<tr>
<td>5.13</td>
<td>TNF in the Urine</td>
<td>128</td>
</tr>
</tbody>
</table>
Chapter 4 is a replication of the following material, published with permission.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumen</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>D</td>
<td>Dose</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapetaenoic Acid</td>
</tr>
<tr>
<td>FCR</td>
<td>Fractional Clearance Rate</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>G</td>
<td>Guage</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoproteins</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>im</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>Kcal</td>
<td>Kilocalorie</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoproteins</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor related protein</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MCR</td>
<td>Metabolic Clearance Rate</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>Mr</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OFR</td>
<td>Orally Fed Rats</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>pg</td>
<td>Picograms</td>
</tr>
<tr>
<td>PIA</td>
<td>2-phenylisopropyladenosine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PKA</td>
<td>Phosphokinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Phosphokinase C</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TPN</td>
<td>Total Parenteral Nutrition</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor-α</td>
</tr>
<tr>
<td>TNF-RI</td>
<td>TNF receptor I</td>
</tr>
<tr>
<td>TNF-RII</td>
<td>TNF receptor II</td>
</tr>
<tr>
<td>TVD</td>
<td>Total Volume of Distribution</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>μCi</td>
<td>Microcurie</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low Density Lipoproteins</td>
</tr>
<tr>
<td>WGR</td>
<td>Weight-Gain Rats</td>
</tr>
<tr>
<td>WGR</td>
<td>Weight-Lose Rats</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

The clinical management of inflammation, trauma, cancer, infection, sepsis, as well as many inflammatory and non-inflammatory diseases is complicated by the development of profound anorexia, tissue-wasting and weight-loss. Chronic and critical illness results in a metabolic shift from one of energy and protein conservation to one of enhanced substrate utilization characterized by fever, hypermetabolism and altered fuel mobilization resulting in exaggerated losses of muscle protein with a relative sparing of visceral protein. This accelerated metabolism continues despite the development of anorexia and decreased food intake leading to profound weight-loss and malnutrition. This wasting and severe malnutrition can continue despite aggressive medical therapy and can become the primary determinant of patient outcome. Originally, it was believed that the invading organism or cancer was responsible for the wasting observed during chronic disease. However, sustained low levels of circulating tumor necrosis factor-α (TNF), are now widely accepted to be responsible for the development of the characteristic wasting syndrome commonly observed in the clinical setting.

The recognition of this wasting syndrome, or cachexia, as a unique syndrome has led to the widespread implementation of nutrition support in the clinical setting. The aim of this nutrition support is to provide sufficient nutrients to prevent the continued loss of lean body mass as well as energy to meet the increased metabolic needs associated with wound healing. This aggressive approach to nutrition support has often resulted in the delivery of nutrients up to 140% of estimated resting energy requirements. Unfortunately, despite advances in the provision of nutrition therapy, there has been little to change the poor prognosis associated with the severe loss of lean body mass.

The natural response to chronic illness and inflammation is anorexia and reduced food intake. The forced provision of excessive nutrients by either enteral or parenteral nutrition support may therefore, upset this normal adaptive response. In addition, the preferred route of nutrition support has also been extensively debated. Enteral nutrition support has been
advocated in the clinical setting as it maintains gastrointestinal tract stimulation and preserves gut barrier function. The provision of nutrients by total parenteral nutrition has been associated with an increased risk of infectious complications as well as with decreased survival. Therefore, there are still many questions regarding what constitutes optimal or ideal nutrition support in the clinical setting. Questions such as how much nutrition support to provide and what is the ideal route to use remain unanswered.

Recently, there has been recognition of a potential interaction between the provision of nutrition support and the action of TNF. Since TNF is pivotal in the development of the wasting syndrome, an interaction between nutrients and TNF would be an important consideration when implementing nutrition support in the clinical setting. Therefore, this laboratory has set up a model that mimics the metabolic abnormalities associated with chronic human disease by the provision of a continuous infusion of TNF to rats. Such a model will not only allow the separation of the effects of TNF from those of its associated anorexia and malnutrition, but will also allow the study of the interaction between TNF and the provision of nutrition support. This thesis represents an extension of the continuing work in this laboratory and will contribute to our understanding of what constitutes the optimal nutrition support during chronic illness. We will strive to answer one question: does nutrition support affect the distribution and clearance kinetics of TNF? The results of this work has important implications for the feeding of critically ill patients who are likely to have increased production and circulating levels of TNF.

This thesis is organized into seven sections. The following section provides an insight into the structure, function and metabolic actions of TNF and examines the potential methods for regulating these biological functions. It provides the rationale for undertaking these experiments as well as the background knowledge required to understand the results and discussion. The third section outlines the majority of the experimental procedures and presents some preliminary studies which were undertaken to enhance the main experiments. The fourth and fifth chapters describe the experiments conducted and includes the results and preliminary discussion. The fourth chapter has been previously published. The final chapter of this thesis provides an in-depth discussion of the effect of nutrition on TNF production and
clearance and represents a summation of all of the experimental work. This thesis continues the study of the interaction of nutrition support with TNF metabolism by examining the interaction between TNF clearance and the provision of nutrition support. A clinical study investigating the effects of nutrition support on the circulating levels of TNF-α and soluble TNF receptors in ICU patients was undertaken but is not presented as part of this thesis. The abstract is found in Appendix 5.
2.0 REVIEW OF THE LITERATURE

2.1 INTRODUCTION

This chapter provides an overview of the metabolic effects of TNF and describes how TNF action affects nutrient utilization. It concludes by examining the major routes by which TNF is cleared from the plasma as well as factors that can affect this clearance. The following sections will review the work done previously in this laboratory leading to the development of the studies presented in chapters 4 and 5.

2.2. THE CHRONIC EFFECTS OF TNF-α

It is well established that acute over-production of TNF-α results in the release of a cascade of endogenous humoral mediators that mediate the metabolic activities characteristic of septic shock [1]. On the other hand, the chronic production of low levels of TNF during critical illness may result in host deterioration as a result of anorexia, tissue wasting and anemia. Weight-loss and the associated wasting of muscle protein may be the primary determinants of clinical outcome. This syndrome, called "cachexia", is not exclusively the end result of trauma or injury, but is also associated with many chronic inflammatory and non-inflammatory states such as AIDS, cancer and heart disease that result in continuous release of low levels of TNF [1]. TNF synthesis as well as evidence for the role of TNF as a mediator of this chronic wasting syndrome will be examined in the following sections.

2.2.1. The Basics of Tumor Necrosis Factor-α

Tumor necrosis factor is a polypeptide of known amino acid composition [4]. TNF is produced as a propeptide of 238 amino acids which is cleaved at several sites to yield the mature hormone [1]. The human propeptide contains 76 amino acids at the amino terminal end of the molecule. Tumor necrosis factor-α contains two cysteine residues connected by a disulfide bridge which differentiates it from lymphotoxin (TNF-β) [4]. TNF, together with its pro-piece, is inactive, indicating that this segment can mask the active center or maintain the
molecule in an inactive form. The protease(s) responsible for pro-hormone cleavage represents a possible target for anti-TNF-α therapy but remains poorly characterized.

Human TNF-α is translated as a transmembrane 26 Kd protein that is thought to be cleaved by metalloproteinase to give rise to the 17 Kd soluble form of TNF-α. There is interest in the potential regulation of TNF activity through modulation of this metalloproteinase.

Many species of TNF have been identified which have a high degree of interspecies homology for both nucleotide and amino acid sequences. Human tumor necrosis factor-alpha shares about 69% sequence identity and 85% homology with murine TNF. The reported molecular weight of human TNF is highly variable depending upon the method employed to determine it. TNF aggregates in vitro to yield a protein of variable size with molecular weight ranging from 34,000 to 140,000 daltons. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) done under reducing conditions shows a single major protein band at an apparent molecular weight of 17,000 kDa. However, using gel permeation chromatography, the molecular weight has been determined to be 45,000 ± 6000 kDa. Under non-denaturing conditions, SDS PAGE indicated dimers and higher oligomers of human TNF with Mr values of 45,000 and 70,000.

The folding of the TNF monomer predisposes it for non-covalent linkage and subsequently the formation of oligomers and polymers. It is clear that the bioactivity of TNF is associated exclusively with TNF oligomers. However, there is still some controversy over whether the oligomers are dimers or trimers. Although there has been some dispute as to whether the active form of TNF is dimeric or trimeric, analytical centrifugation, cross-linking, small angle x-ray scattering and gel electrophoretic studies have all shown TNF to be a trimer in solution. Crystal work has revealed that native TNF is a compact trimer with a stokes radius similar to that of a dimer. However, Petersen and others still favour a dimeric structure. Isolated monomers are only a small part of TNF and are only 12% as active as trimers in binding assays. Isolated TNF monomers were found to have a low binding affinity (Kd 70pm) whereas trimers showed high affinity binding (Kd 90nm). The ability of TNF to circulate in a variety of forms of varying molecular weights and bioactivities adds complexity to the study of this molecule. In this thesis, TNF will always refer to TNF-α.
2.2.2. Synthesis of Tumor Necrosis Factor

TNF is produced primarily by blood monocytes and tissue macrophages. However, there is recent evidence that natural killer cells, epithelial cells, adipocytes as well as cytotoxic cells and bone-marrow derived mast cells are capable of synthesizing TNF. Nevertheless, macrophages are the main producers of TNF since most other cells produce minute quantities in comparison with the production by macrophages. Macrophages produce TNF in response to chronic infection, parasites or malignancy. Studies of the control of TNF biosynthesis indicate that hormone production is tightly regulated. Both transcriptional and post transcriptional activation must occur to allow its production, making it unlikely that TNF will be inadvertently released.

Gram negative bacterial lipopolysaccharide (LPS) is the most potent inducer of TNF-α production. TNF gene transcription increases approximately three fold in response to stimulation of the macrophage by LPS whereas intracellular mRNA levels may increase 100 fold and TNF protein production may increase 1000-fold or more. Therefore, transcriptional activation by endotoxin appears to account for the elevated levels of TNF mRNA after induction. After stimulation, the TNF gene is downregulated and macrophages become refractory to LPS for 12-24 hours after which LPS will again induce a burst of TNF biosynthesis. Under normal circumstances, the macrophage contains small amounts of TNF mRNA in a pool which is not translated. The presence of endotoxin mobilizes the message for translation and stimulates the synthesis of additional messenger RNA.

TNF release occurs in a stereotypical pattern during acute endotoxemia. Initially, there is a burst of TNF within minutes into the circulation which reaches peak levels approximately 90-120 minutes post-stimulation. Subsequently, TNF-α is cleared rapidly from the circulation becoming undetectable after four to six hours. Even though TNF is present in the circulation for only a brief period of time, that appears to be long enough for TNF to stimulate the release of a cascade of secondary mediators that lead to both systemic and tissue responses. It is this phasic nature of TNF production which has led to substantial difficulties in the measurement and interpretation of circulating levels of TNF in clinical
studies. However, it may be that the circulating level of TNF itself is not that important but it is TNF's pivotal role in the stimulation of the myriad of endogenous mediators and cytokines which is of critical importance. In addition, there is evidence that TNF can also exist as a kDa cell-associated protein which is bioactive. This cell-associated TNF most likely represents the precursor of the secreted protein. The presence of a cell-associated TNF suggests that TNF may have important paracrine functions and that local production of TNF may be important for the initiation of the complete spectrum of TNF effects. For example, local TNF production at the liver might be responsible for the induction of the hepatic acute-phase response, including decreased synthesis of albumin. Therefore, considering the difficulty in measuring circulating TNF levels, tissue TNF levels or levels of cell-associated TNF may also be of greater biologic significance than levels of TNF in the circulation.

2.2.3. The Role of TNF in the Development of Cachexia

The cachexia of sepsis and chronic disease can be profound, resulting in significant tissue wasting and weight loss. This pattern of wasting involves alterations in fuel use, an increase in metabolic rate, profound anorexia, weight-loss and an exaggerated loss of peripheral muscle mass while retaining or even enhancing visceral organ mass. This is a unique scenario, quite different from the effects of starvation or hypocaloric feeding alone, which result in a depressed metabolic rate, a relative sparing of peripheral tissue and muscle and a subsequent loss of visceral organ mass. TNF has been implicated as one of the key mediators in the development of cachexia. The following sections provide evidence for TNF as the key mediator of the wasting syndrome seen frequently in the clinical setting.

2.2.3.1. Development of Anorexia

Tracey et al. demonstrated that chronic administration of sublethal doses of TNF results in a syndrome strikingly similar to disease-induced cachexia. The provision of a sub-lethal dose of TNF to rats in two-daily injections i.p. for 7-10 days resulted in a significant reduction of food intake in comparison with albumin treated controls. Animals subjected to chronic TNF exposure developed cachexia with depletion of total body protein and lipid which was similar to that observed in chronic disease.
in humans. These animals manifested a metabolic shift from energy conservation to one of energy mobilization resulting in the accelerated loss of vital proteins such as those required for respiration or circulation. This pattern of wasting is in stark contrast to the protein conserving adaptations observed during starvation. Metabolic alterations resulting in leukocytosis, anemia as well as altered metabolism of carbohydrates and lipids were observed.

Acute administration of TNF invariably leads to decreased food intake. However, while animals given bolus injections of recombinant TNF show decreased food intake acutely, adaptation occurs after 4-5 days of TNF treatment and food intake improves. This adaptation has been referred to as tachyphylaxis. However, anorexia can be maintained by escalating the injected dose of TNF. The mechanism for this adaptation is not understood but it is hypothesized that it relates to the clearance of TNF. In addition, TNF suppression of food intake has been shown to be blunted by the provision of anti-TNF antibodies indicating that TNF has a direct effect on appetite control.

TNF has also been provided to animals as a continuous infusion in order to avoid the tolerance effect observed during multiple dosing with TNF. Continuous infusion of TNF would most accurately reflect the chronic production of TNF hypothesized to occur during chronic illness. Continuous infusion of TNF into animals resulted in a significant and sustained anorexia which resulted in weight loss. Human cancer patients provided with a continuous infusion of TNF, as part of a phase I trial, also developed anorexia and subsequently lost weight. TNF infusion was also associated with marked hypoferremia and increased release of the acute phase protein, C reactive protein. These alterations occurred at circulating TNF levels which were undetectable by their assay system illustrating the difficulties associated with the measurement of circulating TNF.

The mechanism by which TNF mediates a decrease in food intake is not yet understood. TNF has been shown to decrease gastric motility leading to a retention of food in the stomach and small intestine which would depress appetite. Animal studies have suggested that the effect of TNF on food intake is mediated peripherally and not centrally since either intravenous or intracerebroventricularly-administered TNF resulted in reduced feeding. The intracerebroventricular injection of TNF resulted in 100-fold higher
concentrations in the brain than intravenous injection at the same dose. However, both routes of TNF administration resulted in equal blood concentrations in the trunk and an equal anorectic effect 49.

Studies utilizing a continuous infusion of TNF have shown that brain tryptophan levels as well as 5-hydroxyindole acetic acid (5HIAA) levels were elevated in cachectic rats in comparison with well nourished control rats 43. Increased 5-HIAA and tryptophan levels suggest altered metabolism of serotonin in these animals as tryptophan is a precursor of serotonin which is in turn metabolized to 5-HIAA 57. In addition, there is evidence that a disturbance of serotonin metabolism is associated with anorexia 58. Increased levels of brain tryptophan are associated with somnolence which has also been observed in animals treated with TNF 59. These results suggest that an increased breakdown of serotonin in the brain of cachectic animals results in a decreased drive to eat and subsequently in weight-loss. There is also evidence that TNF may cause anorexia by rendering the central nervous system incapable of responding to hyperphagic signals 42. The administration of insulin has resulted in an attenuation of the hypophagia with modest increases in food consumption, supporting the inability of animals to respond to hyperphagic signals 42. However, this is unlikely to be the sole answer as administration of insulin did not return food intake to the level of control animals.

These studies provide consistent evidence that TNF is key in the development of anorexia resulting in decreased food intake and subsequent weight loss. In addition to the effects of TNF on food intake, TNF is also thought to mediate other metabolic changes resulting in altered metabolism of macronutrients. Alterations in the metabolism of proteins, carbohydrates and fat contribute to the development of the hyperglycemia, hypertriglyceridemia and altered fuel use observed during chronic illness and infection. These metabolic alterations in fuel use result in a response that is unique from that observed during simple starvation and results in the profound wasting observed in critical and chronic illness.

### 2.2.3.2. Changes in Fat Metabolism Associated with TNF-α

The mobilization of fat reserves for the provision of energy is one of the key adaptive mechanisms of the body in
times of food deprivation. Stored body lipid can be used to spare the catabolism of protein for energy. TNF administration has been shown to interfere with the production of ketone bodies by decreasing their synthesis\textsuperscript{60}. In addition, TNF infusion results in a rapid rise in serum triglyceride levels resulting in a relative lipemia which was originally thought to be the result of decreased lipid clearance \textsuperscript{61}. Early work by Kawakami and Cerami showed that lipoprotein lipase activity was markedly decreased in the adipose tissue of mice exposed to endotoxin \textsuperscript{62}. The factor that was responsible for lipoprotein lipase suppression was found to be transferable in the serum and led to the isolation and identification of this serum factor as cachectin \textsuperscript{62}.

Semb et al. later demonstrated that LPL activity was decreased only in the adipose tissue and not in other tissues \textsuperscript{63,64}. Streptozotocin-treated rats (diabetic) have markedly decreased levels of LPL activity in adipose tissue secondary to their diabetes. Treatment of these animals with TNF resulted in a significant increase in serum triglycerides with no further decrease in LPL activity. Therefore, TNF does not decrease the clearance of triglyceride-rich lipoproteins from the circulation \textsuperscript{52,65}.

Studies by Krauss et al. used radiolabeled water to look at hepatic de novo fat synthesis in rats injected with endotoxin \textsuperscript{66}. They demonstrated an early increase in VLDL which was accompanied by an increase in LDL. There were no changes in HDL or chylomicrons. These results indicate an increase in hepatic lipid synthesis coupled with a decrease in the metabolism of circulating lipids. The stimulating effect on fatty acid synthesis is confined to the liver since TNF was shown not to affect this process in the small intestine, adipose tissue or muscle in rats. It is now generally accepted that TNF increases triglyceride production by increasing de novo fatty acid synthesis and by increasing reesterification of fatty acids from peripheral lipolysis \textsuperscript{67}. TNF has been shown to cause a net loss of triglycerides from the adipocytes via several mechanisms including an inhibition of fatty acid synthesis and an increase in cellular lipolysis \textsuperscript{68}. Consequently, increased hepatic triglyceride production is primarily responsible for the observed hyperlipemia following TNF infusion.

The overlapping roles of TNF and interleukin-1 have led to speculation that observed lipemia after TNF treatment is a result of the production of interleukin-1 (IL-1). Feingold
was able to identify several differences in the effects of TNF and IL-1 on lipid synthesis. TNF produces an increase in serum-free fatty acids and glycerol which are transported to the liver for triglyceride synthesis. This is in marked contrast to IL-1 which does not affect serum-free fatty acid or glycerol levels. Therefore, IL-1 is only able to stimulate increased liver lipogenesis with no subsequent effect on peripheral lipolysis. In addition, TNF leads to an increase in serum cholesterol levels several hours after exposure which is reflected in increased low-density lipoprotein levels while IL-1 is not able to affect serum cholesterol levels. The mechanism by which TNF can stimulate this rise in cholesterol levels is unknown. The fact that both IL-1 and TNF can increase serum triglyceride levels but only TNF can cause increased circulating cholesterol levels suggests that there is some mechanism by which TNF affects cholesterol levels other than simply by the processing of triglyceride-rich lipoproteins to cholesterol-enriched proteins.

Finally, when lipolysis is blocked by the drug R-2-phenylisopropyl adenosine (PIA), TNF stimulated animals show a blunted response in the production of triglycerides whereas IL-1 treated animals are not affected. These data suggest that, in TNF animals, peripheral lipolysis plays an important role in the increase in triglyceride synthesis whereas in IL-1-treated animals it does not play a role. Although downregulation of lipoprotein lipase activity was one of the first recognized properties of TNF, it now appears to play a relatively minor role in the development of hypertriglyceridemia. The fact that both IL-1 and TNF cause an increase in circulating triglycerides suggests that these changes in lipid metabolism are an important part of the host response to infection or inflammation. The role of hyperlipidemia in illness is not well understood but triglyceride-rich lipoproteins may bind endotoxin and protect animals from the mortality that normally occurs with endotoxin administration. There is also the possibility that alterations in hepatic lipid metabolism are a component of the host defense response.

In 1988, Herz cloned an encoding protein thought to be responsible for the clearance of chylomicron remnants and called this the LDL receptor-related protein (LRP). This receptor has subsequently been hypothesized to be the alpha-macroglobulin receptor. Current evidence indicates that LRP may be a dual receptor for both ligands.
Kristensen have determined the partial amino acid sequence of a molecule of the alpha₂-macroglobulin receptor and found it to be identical to LRP\textsuperscript{70,71}. If this receptor does serve both functions, lipids could compete with alpha₂-macroglobulin/TNF complexes for clearance at this site.

### 2.2.3.3. Changes in Carbohydrate Metabolism

TNF also affects carbohydrate metabolism resulting in increased cellular membrane transport of glucose, depletion of cellular glycogen and increased cellular efflux of lactate. Therefore, TNF appears to represent an early signal for anaerobic glycolysis\textsuperscript{36}. In addition, infusion of TNF results in increased gluconeogenesis at the liver which is one of the hallmarks of sepsis resulting in hyperglycemia\textsuperscript{67}. Catecholamines have been shown to play a crucial role in this increased gluconeogenesis as beta-adrenergic blockade was found to normalize this over production of glucose by the liver\textsuperscript{67}. Prolonged exposure to TNF-\(\alpha\) also is also characterized by the development of resistance to exogenous or endogenous insulin. Insulin resistance coupled with increased hepatic synthesis of glucose and enhanced degradation of glycogen by skeletal muscle results in the development of hyperglycemia which is one of the hallmarks of chronic wasting and critical illness.

### 2.2.3.4. TNF Effects on Protein Metabolism

While the effects of TNF on the development of anorexia are clear, it is significantly more difficult to determine the discrete effects of TNF on protein wasting during sepsis and chronic illness due to the confounding effect of TNF induced anorexia and malnutrition. Studies of tissue wasting during sepsis, in particular the role of TNF, show conflicting results. \textit{In vitro} and \textit{in vivo} studies have failed to delineate one distinct effect of TNF on protein metabolism. \textit{In vitro}, Goldberg et al. have failed to show an effect of recombinant TNF on protein synthesis or prostaglandin E\(_2\) production in skeletal muscle\textsuperscript{72}. They were also unable to find any effects with purified IL-1, TNF and several interferons and growth factors. They concluded that there was a substance that was as yet unidentifiable that was responsible for muscle protein breakdown\textsuperscript{72}.

\textit{In vivo} studies of protein metabolism have also resulted in conflicting results. Flores et al.
looked at the effect of increasing doses of intravenous interleukin-1, interleukin-1/TNF and TNF alone on rat protein metabolism *in vivo*. Infused $^{14}$C-leucine was used to estimate protein synthesis and metabolism through its appearance in tissue proteins and its dilution in the free amino acid pool. Infusion of recombinant cytokines was not associated with any differences in the rates of fractional protein synthesis. In contrast, animals injected with TNF alone or in combination with interleukin-1 were found to have higher protein breakdown rates than control animals. This effect was most pronounced when both cytokines were given together. The liver showed decreased protein breakdown and increased mass at a dose of 100/ug/kg of recombinant human TNF alone or with IL-1. They also demonstrated a 10-25% increase in total urinary nitrogen excretion during systemic infusion of TNF in comparison with control animals. The authors concluded that TNF has a catabolic effect on muscle proteins but they were unable to say whether this was a direct effect of TNF alone. It is possible that TNF may interact with other hormones or cytokines to elicit these effects on muscle.

In a similar study, *in vivo* administration of IL-1, TNF and LPS was compared in a whole-body study using pair-fed and ad-lib fed control animals. TNF infused animals had a larger absolute loss of gastrocnemius muscle in comparison with pair-fed animals even though each group had a similar amount of weight loss. In contrast, the liver weights in LPS, TNF and IL-1 treated animals were significantly higher than in pair-fed animals. These increased liver weights were associated with increased RNA and DNA suggesting an increased synthetic ability of the liver, probably for the production of acute phase proteins. The increase in DNA suggests an accumulation of new cells, supporting previous findings of mononuclear cell accumulation and bile duct proliferation in livers of animals chronically treated with TNF. The pair-fed animals demonstrated sparing of peripheral mass with increased loss of visceral mass which is the characteristic pattern of muscle loss during starvation. However, cytokine treatment resulted in a different pattern with redistribution of peripheral proteins with a relative sparing of visceral proteins. This pattern mimics that seen in injury.

The site of TNF synthesis also seems to play a role in the mediation of the metabolic consequences of TNF. TNF-producing tumors implanted in the brain resulted in the
development of profound anorexia and gross weight loss with severe metabolic abnormalities whereas TNF produced from tumors implanted in the hind leg took much longer to act and had much milder systemic effects. This response was seen, even though circulating levels of TNF were equal in both groups of rats \(^{42}\). This study suggests that the effects of TNF may depend on paracrine effects of the cytokine and/or tissue concentration locally and that organ-specific concentration or cell associated TNF may outweigh the effects of circulating levels \(^{42}\).

2.2.3.5. **The Effect of TNF on Muscle Transmembrane Potential**  

TNF has been shown to have direct effects on muscle-resting transmembrane potential which mimic the observed changes in sepsis \(^{74}\). Critical illness is associated with decreased skeletal transmembrane potential difference (Em), increased cellular sodium and water and decreased potassium stores \(^{75}\). Rennie et al. hypothesized that the muscle cellular membrane is affected early in sepsis, resulting in an efflux of glutamine and an influx of sodium \(^{76}\). This efflux of glutamine would result in a depleted cellular pool of this amino acid which might contribute to impaired protein synthesis and increased breakdown. Exposure of the extensor digitorum longus (EDL) and soleus muscle to TNF resulted in a significant decrease in muscle Em which was inhibited by the addition of TNF monoclonal neutralizing antibodies \(^{76}\). The TNF-mediated decrease in skeletal muscle Em *in vivo* may represent the primary signal that initiates the abnormalities of cellular metabolism following TNF infusion \(^{76}\). The glutamine:sodium co-transport is the driving force for glutamine transport and will depend on the net electrochemical potential of the amino acid plus the sodium and the glutamine distribution ratio across the membrane will depend on the reciprocal of the sodium gradient. Therefore, when intracellular sodium increases glutamine is lost \(^{75}\). No other amino acid shows such a close Na\(^+\) dependence for transport. The muscle glutamine system also differs from other muscle systems due to its sensitivity to hormones \(^{77,78}\). Therefore, it is regulated by nutritional factors, stress, starvation, disease and injury. The transporter is also sensitive to bacteria and bacterial endotoxins which accelerate the efflux of intracellular glutamine \(^{79}\). Interestingly, the synthetic rate of skeletal muscle is strongly related to the intramuscular concentration of glutamine. Cancer patients given an infusion of TNF demonstrated significantly increased
amino acid efflux from the forearm, two thirds of which was alanine and glutamine.

In conclusion, low circulating levels of TNF result in alterations in inter-organ fuel utilization resulting in impaired production of ketones, enhanced mobilization of lipids, hyperglycemia, hypertriglyceridemia and accelerated gluconeogenesis. These metabolic alterations lead to the accelerated breakdown of peripheral muscle for the provision of amino acids for gluconeogenesis. In addition, muscle glycogen is also released and the resulting carbon skeletons used for gluconeogenesis. This inefficient use of fuels results in the profound weight-loss and muscle wasting observed during acute and chronic illness.

2.3. CONTINUOUS TNF INFUSION AS A MODEL OF CHRONIC ILLNESS

Until recently, all studies of the metabolic responses to TNF involved the bolus injection of either bacterial endotoxin or TNF itself. While these studies clearly demonstrated the importance of TNF in the development of anorexia and malnutrition, they failed to clearly demonstrate a consistent effect of TNF on protein catabolism beyond that associated with the TNF-induced anorexia and malnutrition. In addition, bolus injection resulted in an inflammatory reaction at the site of injection which complicated the interpretation of findings. There was concern that bolus administration of TNF did not mimic in vivo production and therefore would not accurately replicate the expected physiological responses associated with chronic exposure to a low concentration of TNF. Therefore, our laboratory has developed a model which mimics chronic human disease in rats by the provision of a continuous infusion of TNF-α. This model has subsequently been used to separate the effects of TNF on muscle wasting from that attributed to the TNF induced anorexia, decreased food intake and weight-loss. In addition, this model has also been used to study the effect of the provision of nutrition support during chronic wasting.

In order to separate the effects on peripheral muscle of TNF-induced anorexia and malnutrition from those effects directly mediated by TNF, animals given a continuous infusion of approximately 100µg/kg/day of human recombinant TNF or saline via a central venous cannula were compared with animals who were pair-fed the same amount of food as consumed by the TNF infused group. The rats receiving the continuous infusion of TNF
were hypermetabolic in comparison with pair-fed animals. Pair-fed animals demonstrated the characteristic decline in metabolic rate associated with the metabolic adaptation to starvation, whereas TNF infused animals had a 51% increase in metabolic rate. Despite the increase in metabolic rate, TNF infused animals developed anorexia with a reduction in food intake of 50 to 65% that of control animals. Subsequently, these animals lost weight, however, the drop in weight did not differ between pair-fed animals and TNF infused animals suggesting that TNF induced anorexia was primarily responsible for the weight lost. In addition, the loss of muscle mass was similar in pair-fed and TNF-infused animals leading these authors to conclude that TNF does not increase muscle catabolism beyond that which is a result of reduced food intake. These data are in contrast to the work of Flores et al. who showed that TNF increased muscle catabolism 72. The conclusion that TNF is not primarily responsible for muscle catabolism is supported by studies showing a reversal of tissue wasting, weight loss, histopathologic abnormalities and decreased appetite with concurrent insulin therapy 80.

The lack of difference in protein loss with TNF infusion in peripheral muscle differs from the changes in the composition observed in the viscera. TNF infusion resulted in a redistribution of body protein with a loss of peripheral muscle, carcass mass and protein content on the one hand and enhanced protein content and mass of the visceral organs on the other. The liver, heart and lungs were shown to increase in protein and DNA, indicating cell proliferation 43. The kidneys, unlike the other viscera, lost protein. The authors concluded that their data were not consistent with the suggestion that TNF causes specific muscle catabolism. Instead, they suggest that TNF wasting is due to malnutrition and that the specific effect of TNF is on the viscera. In infection and trauma, there is often an increase in total liver protein, RNA and DNA which is consistent with a sustained or increased capacity for acute phase protein synthesis and for gluconeogenesis. In hepatocytes, TNF enhances the expression of certain acute phase proteins and decreases the production of albumin via changes in transcription rates for these proteins. These changes are also mediated in part by IL-6 81-83. This model of continuous TNF infusion resulted in the development of anorexia, hypermetabolism, anemia and tissue wasting with a redistribution of body proteins from the peripheral muscle to the viscera. In addition, liver function tests and creatinine levels were
comparable to those of control animals. The blood urea nitrogen was significantly higher in TNF infused animals than in pair-fed or control animals reflecting overall increased protein catabolism. Blood glucose levels in TNF infused animals were also significantly higher than those in the pair-fed animals suggesting that the expected reduction in blood glucose levels due to reduced food intake was counter-balanced by increased gluconeogenesis and/or insulin resistance in these animals. In summary, we have developed a model which replicates the metabolic alterations seen during chronic illness by the continuous infusion of a low dose of TNF. Although these animals were severely wasted, there was no mortality associated with TNF infusion in this model.

2.4. NUTRITION SUPPORT AND TUMOR NECROSIS FACTOR-α

Continuous infusion of TNF into rats resulted in anorexia and weight loss with a redistribution of protein from peripheral muscle to the liver, heart and lungs. Our initial study using this rat model of chronic disease suggested that the observed muscle wasting during chronic and critical illness was most likely due to the associated decreased food intake and malnutrition. Subsequently, it was hypothesized that if the decreased food intake and subsequent malnutrition were prevented, by the provision of nutrition support, that there would be a preservation of peripheral muscle mass during TNF infusion. A second study was designed whereby rats were provided not only with a continuous infusion of TNF but also with a continuous infusion of nutrients through a central venous catheter. If the addition of nutrition support as total parenteral nutrition prevented the wasting of peripheral muscle, there would be solid evidence that muscle wasting was simply a result of malnutrition and not due to any specific action of TNF on the muscle.

Animals given nutrients via total parenteral nutrition were shown to gain weight during TNF infusion. In fact, these TPN infused animals gained significantly more weight than control animals. However, after six days of TNF and TPN infusion, some rats (designated CACH-HOD) developed a marked hyperglycemic, hyperosmolar diuresis which was associated with a significant drop in weight and subsequent death. Biochemical analyses indicated that these animals were hyperglycemic with glycosuria and possibly insulin
These rats had cholestasis with increased levels of bilirubin as well as alkaline phosphatase (ALP) and alanine transaminase (ALT) \(^44\). Nitrogen excretion was highest in these CACH-HOD animals resulting in the lowest nitrogen balance despite the provision of nutrition support. TPN and TNF infused rats which survived the ten days of nutrient infusion (CACH) had significantly elevated levels of insulin, potassium, blood urea nitrogen and triglycerides in comparison with TPN fed control animals. Despite the provision of nutrients, nitrogen balance was significantly lower than that of control animals after 7 days of TNF infusion \(^44\). The total protein content of the lung, heart, kidney and liver were higher in TNF infused animals than in control animals.

Previous studies by our group and others had suggested that tissue wasting was entirely the result of reduced nutrient intake \(^43\). Therefore, the provision of nutrients by TPN was expected to maintain body and muscle mass. However, this was not observed. TPN fed /TNF infused rats developed significant biochemical abnormalities reflecting the development of insulin resistance as well as renal and hepatic failure characteristic of multi-system organ failure \(^44\). Surprisingly, the provision of nutrients also resulted in a significant increase in mortality over control animals. Several animals developed a large hyperosmolar diuresis and subsequently died. These animals had significantly reduced carcass weights from control animals and significantly heavier livers, lungs and hearts. The weight-gain observed in the liver was found to be associated with a proliferation of the biliary endothelium, whereas in the heart weight-gain was associated with endocardial proliferation.

In summary, administration of nutritional support as TPN to rats in this model of human chronic illness resulted in significant mortality and morbidity as well as in the manifestation of the typical syndrome of multi-system organ failure not seen previously in orally-fed wasted animals \(^43,44\). Orally-fed animals receiving TNF became severely wasted. However, there was no mortality or morbidity associated with TNF infusion in orally-fed animals nor were there any signs of organ failure. TNF was seen to not only reduce nitrogen retention in peripheral tissues, but to divert protein to the viscera, especially the liver, where, on histological exam, there was proliferation of biliary epithelium. These data suggest that when
the confounding effects of anorexia are prevented. TNF infusion also has specific catabolic effects not overcome by nutritional support.

The results of this study have raised many questions regarding the interaction of nutritional support with tumor necrosis factor metabolism. Of critical importance is the question of why the provision of nutrients by TPN resulted in such severe alterations in metabolism which were not seen previously in orally-fed wasted animals. We must consider that the development of anorexia and weight loss associated naturally with TNF exposure is an adaptive mechanism which allows the host to shut down some metabolic activities while enhancing others in order to mount a more effective defense system. If this is true, then the forced provision of nutrients by total parenteral nutrition could upset this adaptive process and result in a potentiation of the metabolic effects of TNF. Alternatively, there may be specific physiological effects associated with the provision of nutrients by TPN such as bowel rest and bacterial translocation which might enhance the metabolic effects of TNF. Finally, specific nutrients might interact with TNF production or its metabolism, resulting in a potentiation of the metabolic effects of TNF.

Circulating TNF levels have provided one clue to the cause of the potentiation of the metabolic abnormalities observed during TPN and TNF infusion. In our model, exogenous human recombinant TNF was infused into rats with the subsequent measurement of circulating levels by the WEHI 164 cytotoxicity bioassay, which is specific for human TNF. Circulating levels of TNF were significantly higher in the TPN animals that experienced the most severe metabolic abnormalities. Therefore, sustained elevated levels of circulating TNF provides one explanation for the observed potentiation of the metabolic effects of TNF. Elevated levels of circulating TNF could be the result of increased TNF production, enhanced effects of TNF on target tissues or impaired clearance of TNF. Since elevations in circulating TNF reflect the exogenously administered TNF, it is unlikely that an increase in endogenous rat TNF synthesis would contribute to the elevated levels of human TNF. Likewise, it also seems unlikely that changes in TNF action on target tissues could completely explain the observed potentiation of metabolic abnormalities associated with TNF. Therefore, impaired TNF clearance in TPN infused animals represents the most reasonable
cause of the elevated levels of circulating TNF. This thesis represents the results of two mechanistic studies designed to determine the effect of TPN on TNF clearance and distribution. The next sections will examine how TNF is cleared from the circulation as well as what factors can affect its clearance. There will also be some discussion of previous animal studies of TNF clearance.

2.5. THE CLEARANCE OF TNF

There are three potential fates for TNF in the circulation which are depicted in Figure 2.1. TNF is cleared from the circulation primarily through the binding of TNF to specific TNF receptors on tissues with subsequent internalization and degradation. The biologic effects associated with TNF are thought to be mediated by TNF binding to these receptors. Secondly, TNF can bind to activated alpha2-macroglobulin and subsequently be cleared through its receptors at the liver. Finally, circulating inhibitors of TNF such as anti-TNF monoclonal antibodies or soluble TNF receptors can bind TNF and prevent it from interacting with cell surface TNF receptors, retaining it in the plasma in an inactive state. In the following sections, background information, including previous studies on TNF clearance, will be presented as well as an examination of each of the above mentioned clearance pathways.

2.5.1. TNF Receptors

The biologic effects of TNF-α are believed to be mediated primarily by specific cell surface receptors. In addition, the binding of TNF to its receptors may lead to its subsequent internalization and degradation, thereby providing a mechanism of plasma clearance. These receptors are present on most tissues and cell lines with the exception of red blood cells, resting lymphocytes and a number of transformed beta cells. There are two distinct receptor proteins which have been identified, cloned and expressed from both human and mouse cell lines. These receptors have been designated as TNF-R1 (p-55-60 kDa) or TNF-R2, (p-75-80kDa). These receptors coexist on most cells. Receptor binding is generally believed to signal biofunctions, however, it is not known whether all or only some
Figure 2.1 THE REGULATION AND CLEARANCE OF TNF - TNF is produced by a variety of cells, primarily monocytes and macrophages in response to a stimuli. TNF (O) is released locally and into the circulation where it acts on target tissues through binding to cell-surface receptors. The biologic effects of TNF are associated with the binding of TNF to its receptors. There are two TNF receptors designated TNFR-I (□) or TNFR-II (△). These receptors also exist in a soluble form (sTNF-RI and sTNF-RII) and may affect TNF activity and clearance by competing for TNF binding with cell-surface receptors. In addition, soluble TNF receptors may retain TNF in the circulation in an active form, thereby acting as a reservoir of bioactive TNF. The kidney is the primary organ associated with TNF clearance. However, the liver may also play a role as activated alpha-macroglobulin:TNF complexes may bind and be cleared through the low density lipoprotein receptor related protein (LRP ●). TNF synthesis can be down regulated by the anti-inflammatory cytokines IL-4 and IL-10 as well as by prostaglandin E2 (PGE2) and by glucocorticoids. TNF cell-surface receptors can be up regulated by interferon gamma (IFN-gamma) while interleukin-1 (IL-1) and TNF itself result in receptor down regulation. TPN may result in increased synthesis of TNF due to prior activation of the inflammatory response as a result of bacterial translocation and changes in gut permeability. TPN may also affect TNF clearance by altering the number or affinity of cell-surface TNF receptors. Changes in cell surface receptor number may be reflected in an increased number of soluble TNF receptors.
activities are mediated by receptors 19. In addition, the presence of TNF receptors does not necessarily indicate TNF activity as several cell lines have TNF receptors yet are resistant to the effects of TNF 25. Therefore, while TNF receptors seem to be required for TNF activity, the number does not directly determine the cellular sensitivity to the cytotoxic or cytostatic effects of TNF 25. The nature of the signalling pathways utilized by TNF has begun to be understood, however, the molecular mechanisms by which the receptor proteins couple to and activate the various intracellular second messenger systems are not completely understood 88.

The human TNF-RII is a single membrane spanning receptor of 439 amino acids with an extracellular domain of 235 amino acids and an intracellular domain of 174 amino acids 89. Saturation isotherms for the binding of TNF-alpha to TNF-RII indicate that there is a single class of binding sites with a Kd of 0.18nM. The affinity of TNF-RII for TNF-β was lower at 1.05 Kd 90. Cross linking experiments with TNF-RII indicate that one high molecular mass cross-linked product of 211 kDa was formed, possibly representing two TNF-RII molecules complexed with one TNF-α trimer. Smaller complexes of 101,000 and 134,000 were also observed which would be consistent with the cross linking of one TNF-RII to one TNF monomer or trimer 90. No labelled complexes were observed when cross linking was performed in the presence of excess unlabelled TNF-α or TNF-β indicating that the observed binding and complex formation is specific.

The TNF-RI receptor has a predicted amino acid sequence of 426 amino acids with a single membrane span, an extracellular domain of 182 amino acids and an intracellular domain of 221 amino acids 89. The discrepancy between predicted and observed mass (47.5 vs 55) is probably due to N-linked glycosylation. Human TNF-RI and TNF-RII extracellular domains share only 28% identity 89. There is a complete absence of homology between the intracellular domains of the two TNF-α receptors which has led researchers to speculate that each receptor has a distinct signalling pathway 89. At present, the specific function of each receptor is still under debate, however, the development of specific receptor antibodies has substantially improved our knowledge of the function of each receptor. Initially, it appeared as though TNF-RI and TNF-RII had overlapping signals as monoclonal antibodies directed against human TNF-RII could partially antagonize the same TNF responses that could be
signalled through RI, resulting in receptor signalling redundancy. Thoma et al. did not support the idea of receptor redundancy as they believed that TNF-RI alone was the biologically relevant receptor and that the binding of TNF to TNF-RII was not sufficient to initiate TNF responses. Tartaglia and Goeddel have demonstrated that each receptor has a specific role in signal transduction, thereby arguing against a redundancy in receptor signalling. In their model, only TNF-RI can signal the majority of TNF activities such as cytotoxicity while only TNF-RII is responsible for signalling proliferation of primary thymocytes and T cells. Support for the critical importance of TNF-RI in signalling the cytotoxic effects of TNF has also come from the development of a strain of mice in which TNF-RI has been knocked out. The loss of this receptor in mice results in resistance to a lethal dose of lipopolysaccharide indicating a dramatic loss of response to TNF which is not replaced by the TNF-RII. In addition, these mice were very susceptible to Listeria infection in the absence of the TNF-RI, indicating that TNF-RI is an important mediator in both the harmful and beneficial effects of TNF in vivo.

Tartaglia and Goeddel set out to clarify the role of TNF-RII in signalling the effects associated with TNF-RI. In a series of elegant experiments using monoclonal antibodies to each receptor, they were able to disprove redundancy in receptor signalling and also to clarify a distinct role for each receptor. Initially, anti-TNF receptor antibodies to TNF-RI were shown to inhibit the killing of L929 cells by murine TNF. Monoclonal antibodies to TNF-RI completely eliminated TNF associated cytotoxicity at all doses of TNF as seen in the knock out model supporting the critical role of TNF-RI in signalling cytotoxicity. Surprisingly, treatment of L929 cells with anti-TNF-receptor RII monoclonal antibodies also partially inhibited the cytotoxicity of TNF, shifting the dose response curve to the right in comparison with the response to TNF when TNF-RII was available for binding. Since human TNF is unable to bind to murine TNF-RII, the provision of human TNF to this murine cell line also resulted in a shifting of the TNF dose response curve to the right in relation to murine TNF. Taken together, these two studies suggest a role for TNF-RII in inducing cytotoxicity.

These authors hypothesized two possible mechanisms by which TNF-RII could be involved in the signalling of cytotoxicity. The first possibility is that both receptors can
activate signal pathways that lead to cell death. However, at high concentrations of TNF, only TNF-RI signals cell killing whereas at low concentrations of TNF, signals from TNF-RII can synergize with TNF-RI resulting in increased sensitivity to TNF. In this case, TNF would form heterocomplexes with TNF-RI and TNF-RII. An alternative mechanism is that only TNF-RI is responsible for signalling cell killing but that TNF-RII can facilitate binding of TNF to TNF-RI resulting in the triggering of TNF-RI at lower concentrations. In order to determine which mechanism was correct, antibodies that activate TNF-RII were given in a cytotoxicity assay in order to determine whether they inhibited or promoted cell killing in human U937 cell line. Interestingly, these antibodies did not result in a shift of the dose response curve to the left which would be expected if TNF-RII was involved in signalling cytotoxicity but shifted the curve to the right, indicating that TNF-RII acted as an antagonist to cell killing. This finding argues against a distinct role of TNF-RII in signalling cytotoxicity.

Next they examined the association of \(^{125}\text{I}-\text{TNF}\) with TNF-RI or TNF-RII alone and with both receptors together using U937 cells. Rate constants for the association of TNF with each receptor alone were compared to those with both receptors by using blocking antibodies to examine the binding of labelled TNF to each receptor individually. The data indicated that when TNF-RII is available to bind TNF, it increases the rate of association of TNF to TNF-RI tenfold. Goeddel et al. and others using chemical cross-linking and immunoprecipitation studies have shown that TNF can form homocomplexes with both TNF-RI and RII (2-3 receptors + TNF) whereas no one to date has been able to demonstrate the existence of heterocomplexes on the cell surface. Using cells that expressed predominantly TNF-RI or RII Goeddel et al. were able to determine that TNF associated with TNF-RII was released rapidly. Therefore, this rapid association and dissociation of TNF to TNF-RII could explain how TNF-RI binding is facilitated by TNF-RII. On the other hand, dissociation of TNF from TNF-RI was found to be quite slow, indicating that low levels of TNF-RII expression can facilitate the triggering of TNF-RI but that high levels of expression of TNF-RII are detrimental to TNF-RII triggering at low TNF concentration. These effects are seen only at low TNF concentration (below Kd of TNF-RI) as it is at these concentrations that TNF preferentially binds to TNF-RII since it has
increased affinity for TNF. These authors concluded that the two TNF receptors do not appear to be redundant in their signalling activities. The rapid dissociation of TNF from TNF-RII would result in an increased concentration of TNF in the area of TNF-RI thereby facilitating the association of TNF with TNF-RI. The facilitation of the responses associated with TNF-RI signalling by TNF-RII binding seen at low TNF concentration has great significance since physiologic levels of TNF in vivo are often low or undetectable due to rapid TNF clearance. Therefore, exposure to TNF under these conditions may require TNF-RII for the proper induction of TNF-RI responses. In addition, the ability of TNF-RII to rapidly associate and dissociate from TNF provides a mechanism for cells to change sensitivity to a flux of TNF. Therefore, an overexpression of TNF-RII might dramatically reduce the sensitivity of a cell for TNF.

2.5.1.1. Binding and Internalization The fate of TNF can be followed by examining the disappearance of labelled TNF in cells in vitro. At various points in time, samples have been analyzed for cell-surface-bound plus released TNF, internalized TNF and degraded TNF-α after binding of 125I-TNF to 293 cells. After 2 hours at 37°C, approximately 40% of cell-surface-bound TNF was accumulated intracellularly in the 293 cells. Degraded TNF was detected at approximately 2 hours and continued to slowly increase to about 5% of the total bound cpm, whereas the surface-bound plus released TNF slowly decreased to about 50%. Shedding of the extracellular portion of the TNF-RII was observed. It appears that after binding, TNF undergoes receptor mediated endocytosis and intracellular degradation. It is not clear however, whether these steps are required for the biological activity of TNF.

Binding, internalization and degradation of TNF in cells is supported in work by Yoshie et al. who looked at the binding and internalization of 125I-TNF to human Hela S3 cells. Cells were incubated at 4°C for four hours to allow maximal binding. Subsequently, the cells were rinsed, placed in fresh culture medium and the spontaneous release of radioactivity into the medium was observed. Measurements were also made of cell surface radioactivity (released by an acid wash) and internalized cell radioactivity. The cell surface radioactivity decreased rapidly with a half-life of twenty minutes. The cell-associated (internalized) radioactivity
increased for approximately 60 minutes then decreased. TNF released spontaneously into the culture medium showed two phases of increase, an initial increase then a second after approximately 60 minutes. The authors concluded that the first phase of TNF release represented the loss of TNF bound to receptors while the second phase represented degradation of TNF intracellularly. This second phase of released radioactivity would correspond to the decrease in internalized TNF noted. The degradation of TNF inside the cell is supported by evidence that 92% of the radioactivity initially released by the acid wash was precipitable whereas only 22% of the radioactivity was precipitable at 60 minutes and 24% was precipitable at 120 minutes. These results strongly suggest that TNF is quickly internalized and degraded in the cell.

2.5.1.2. Factors Affecting TNF Receptor Expression Since TNF works through the activation of specific receptors on the cell surface, modulation of the expression of these receptors represents one possible level of control of TNF responsiveness. Several cytokines themselves have been shown to regulate TNF production. Scheurich found cAMP, butyrate and retinal to cause a significant up-regulation of TNF receptors\textsuperscript{98}. To verify the central role of phosphokinase A (PKA) in up-regulation of TNF binding capacity, they evaluated the effect of additional modulators of the PKA signal pathway. The two PKA activators 8-Br-cAMP and IBMX were found to significantly enhance the TNF binding capacity of HL-60 cells, whereas the protein kinase G activator DB GMP proved ineffective\textsuperscript{98}. In addition, the half-life of TNF receptors was identical in treated and untreated cells, indicating that the degradation of TNF receptors increases in parallel with increased receptor numbers triggered by PKA stimulation. Therefore, PKA treatment must increase receptor synthesis. The simultaneous addition of H-8, a potent inhibitor of protein kinases, in particular PKA, completely abolished cAMP mediated up-regulation of TNF binding capacity. The sensitivity to kinase inhibitors and the selectivity of cAMP for PKA point to a central role of PKA in the control of TNF binding capacity\textsuperscript{98}.

Up-regulation of TNF binding capacity could either be due to a change in TNF-receptor synthesis or alternatively might reflect changes in the kinetics of receptor degradation or
export to the cellular membrane. Scheurich et al. looked at the rate of receptor turnover by blocking TNF receptor synthesis with cyclohexamide. They observed that the decrease in TNF binding followed first order kinetics in both control and DBcAMP treated cells. These data suggest that a proportional enhancement of receptor synthesis rather than changes in turnover rate might explain up-regulation. Their data indicate that activators of PKA can selectively up-regulate TNF-receptor expression especially in normal and malignant cells of the myeloid lineage, whereas protein kinase inhibitors interfere with TNF-receptor expression. The kinetics of TNF receptor degradation after blocking receptor synthesis were found to be first order, suggesting TNF-receptor degradation might simply reflect normal cellular membrane turnover. At present, due to a lack of appropriate probes, TNF-receptor gene transcription and/or translation can not be directly analyzed.

Modulation of several receptor systems is dependent on the activation of protein kinase C. Unglaub et al. found that, in activated T cells, TNF receptors could be rapidly modulated by activators of protein kinase C resulting in the loss of TNF binding capacity. They felt that the loss of TNF binding capacity was most likely due to a change in affinity rather than to the internalization or shedding of TNF receptors. They looked at the effect of PMA on TNF binding capacity on tumor cell lines and found decreased binding on all cell lines after incubation with PMA but no associated change in binding capacity. When they gave membrane-permeable protein kinase C activator OAG, the effect was fully reversible and they observed a loss of high-affinity TNF receptors with no evidence of appearance of low-affinity TNF receptors. They concluded that the protein kinase C-mediated down-regulation of TNF binding capacity was neither due to internalization nor to shedding of the receptor molecule. Their results suggest that phosphorylation of the receptor proteins at a cytoplasmic site is responsible for the regulation of TNF binding capacity. Unglaub et al. concluded that there are several examples which show that the magnitude of a particular biological response to a given cytokine is proportional to the quantity of ligand receptor interactions, suggesting that the number of expressed membrane receptors is critical in the determination of cellular sensitivity to the cytokine in question.

On the other hand, Scheurich and others have provided evidence that the production of
TNF itself is also regulated by protein kinases. Activation of PKC leads to the induction of TNF mRNA and/or secretion of the protein while PKA has been shown to act as a negative regulator of TNF production. These effects are directly opposed to those of the respective protein kinase effects on TNF receptor synthesis. As both protein kinases apparently participate in TNF signal transduction, Scheurich suggests that a regulatory circuit could be effective in the following way: activation of PKA results in an enhancement of TNF receptor expression accompanied by an inhibition of TNF production. On the other hand, activation of PKC induces production and secretion of mature TNF and simultaneously inactivates the expression of TNF receptors, thereby inducing TNF resistance.

2.5.1.3. Regulation of TNF Receptors by other Cytokines The cytokine interleukin-1 (IL-1) is capable of modulating TNF binding. In order to determine if IL-1 regulation of TNF receptor binding worked through protein kinase C, staurosporine, a protein kinase C inhibitor, was utilized. Staurosporin did not suppress but rather slightly enhanced the effect of IL-1. This indicated that the effect of IL-1 is not mediated through the activation of PKC. IL-1 also has the ability to increase cAMP in some cells. The IL-1-induced increase in cAMP could subsequently activate protein kinase A, resulting in the modulation of TNF receptors. However, treatment with H8, a selective inhibitor of protein kinase A, did not inhibit IL-1-induced down-regulation of TNF binding so it seems unlikely that the effect of IL-1 is due to increased cAMP.

TNF itself and IL-1 increase cell surface expression of TNF receptors as well as mRNA levels of the 75kDa TNF-R1. Treatment of the fibroblastoid cell line SV-80 with IL-1 or TNF resulted in an immediate decrease in TNF binding. However, after further incubation, there was a recovery of binding activity. Treatment of these cells with mAb to TNF-R1 abolished almost all TNF binding. When cells were treated with IL-1 or TNF for several hours, there was a significant increase in binding which was not blocked by the mAb, suggesting that TNF-R1 production was enhanced. The expression of TNF-R1 gradually recovered but remained below the levels of untreated cells. Using cross-linking of labelled TNF to cell surface receptors, this group found two complexes of 75kDa and 95 kDa
corresponding to TNF monomers bound to TNF-RI and RII. Using this technique, they were able to demonstrate a significant increase in the amount of TNF-RII complex, while TNF-RI complex formation was decreased. Blocking antibodies to IL-1 indicated that TNF could produce these effects alone, without any induction of IL-1. Northern analysis of mRNA for two receptors indicated that incubation of the cells with the two cytokines resulted in a decrease in mRNA for TNFR-I and an increase in mRNA for TNF-RII. Therefore, downregulation of TNF receptors by these two cytokines reflects a rapid decrease in receptor number. Downregulation of TNF receptors by TNF might be physiologically beneficial in inducing the cells to become tolerant to persistent stimulation of TNF or lymphotoxin.

Interferon-γ has been shown to enhance the expression of TNF receptors on several lines which was thought to be the result of an increase in the synthesis of TNF receptors. Tsujimoto et al. hypothesized that interferon-γ resulted in increased receptor expression which was independent of TNF-induced down regulation of receptors. Aggarwal et al. conducted binding studies using labelled TNF bound to human tumor cells in culture in order to observe the effect of pre-treatment of cells with interferon-γ. Scatchard analysis of the binding curves indicated no change in the affinity of receptors for TNF, however, a 2-3 fold increase in the number of receptors was noted in those cells treated with interferon-γ in comparison with untreated cells. Since TNF receptors are central to the action of TNF, these findings may explain the observed synergism between interferon-γ and TNF-α.

2.5.2. Studies of Tissue Binding and Clearance

Tumor necrosis factor once secreted by macrophages travels to the various receptor sites via the circulatory system. TNF receptor sites are found in various organs including the liver, kidney, skin, lungs, gastrointestinal tract, muscle and adipose tissue. Red blood cells do not possess receptors for TNF. TNF has a very short half-life once released into the circulation lasting only minutes until it is cleared. The main sites of clearance appear to be the liver and the kidney. The clearance of TNF has been investigated in animals as well as in humans.

In classic work by Beutler and Cerami, TNF clearance and distribution were investigated.
in mice 15. Labelled TNF was injected by tail vein and blood was collected from the orbital plexus at various times post injection. The iodinated TNF was found to be cleared with a half-life of 6 minutes. Urine was also collected and although there was significant radioactivity in the urine, electrophoresis demonstrated that none of it was present as $^{125}$I-TNF. They found that 31% of the dose was in the liver and another 30% in the skin. The kidneys and gastrointestinal tract took up approximately 9% of the dose. Whole brain showed the lowest activity of any tissue suggesting that little TNF is capable of penetrating the central nervous system. SDS gel electrophoresis was done on tissue samples 8 minutes after injection and revealed little TNF, indicating that TNF is rapidly metabolized within tissues.

The tissue distribution of TNF was similar in a study by Ferraiolo et al. in which the kidney, liver, spleen and small intestine were found to be able to concentrate label in comparison with plasma 108. This group found that the disappearance kinetic of an intravenous bolus of TNF could be described by a bi-exponential function or a two compartment model. The half-life of TNF reported in the Beutler study, was six minutes for the decay of $^{125}$I-TNF in whole blood, whereas, in the study by Ferraiolo, the half-life was reported to be 19 minutes for human recombinant TNF in mice based on the decay of ELISA reactive protein in serum 15,108. It is interesting that, in the Beutler study they report that in the plateau phase of clearance, the residual radioactivity noted was not associated with TNF when subjected to electrophoresis in SDS gels. Ferraiolo et al. also noted a large difference in clearance curves when looking at the disappearance of label using radioactive TNF vs non-radioactive TNF and ELISA. They found that radiolabelled serum plateaued at about 50 minutes whereas the ELISA assay continued to show decay over 700 minutes. Organ studies supported earlier work which suggests that the liver and the kidneys are the most important sites of degradation. In addition, SDS page electrophoresis of tissues supports previous findings of rapid degradation of TNF in tissues. They felt that reincorporation of label into other serum proteins might contribute to the plateau seen. Our work with column chromatography indicates that, at least until 60 minutes post injection, $^{125}$I-TNF remains intact suggesting that the label has not been transferred to other plasma proteins.

Bemelmans et al. conducted elegant studies to demonstrate the importance of the kidney in
the clearance of TNF. Sham-operated or nephrectomized mice were administered either murine TNF, human TNF or lipopolysaccharide. TNF levels were determined in serum using ELISA. Biologic TNF activity was also determined using a WEHI 164 cytotoxicity bioassay. LPS injection resulted in peak TNF levels two hours post-injection. The sham-operated mice had a much lower peak TNF concentration than the nephrectomized animals which showed impaired clearance of TNF with detectable levels being present at 6 hours in nephrectomized mice but not in sham-operated mice. Biological activity of TNF was similar between groups with only minimal bioactivity present in both groups at 4 or 6 hours post-injection. When only one kidney was affected, all mice showed similar TNF clearance kinetics and bioactivity patterns indicating that one functioning kidney is sufficient for normal TNF clearance. Therefore, bilateral nephrectomy (BN) prolonged the detection of immunologically active but not biologically active TNF. Chromatographic data indicated that the serum of BN mice contained two fractions: the native TNF molecule (35 kDa) and a high molecular weight fraction 95 kDa with immunologically detectable TNF activity. This high molecular weight fraction was absent in the serum of sham-operated mice. This molecular weight corresponds well to a mixture of sTNF-R and TNF. Therefore, the presence of TNF bound to sTNF-R would account for differences observed between immunologically active TNF and biologically active TNF.

The kinetics of a bolus on murine TNF followed a similar pattern to that of endogenous TNF produced by injection of LPS with BN, resulting in impaired clearance of murine TNF in comparison with sham-operated mice. However, TNF bioactivity decayed equally between groups. At 60 minutes post-injection, there were substantial levels of immunologically active TNF present but almost no bioactive TNF was detectable. Therefore, nephrectomy did not result in increased TNF production, only differences in clearance. These results indicate that there are two distinct mechanisms in the clearance of TNF, the inactivation of bioactivity and the actual clearance of the protein. TNF inactivation and therefore loss of cytotoxicity is most likely a reflection of binding to sTNF-R which forms a complex with TNF.

The clearance of human TNF in mice provides a unique opportunity to examine the role of TNF-RII in the clearance of TNF since murine TNF-RII does not have affinity for human
TNF.$^9$ Injection of human TNF resulted in a pattern of TNF clearance similar to that of murine TNF with nephrectomized mice clearing human recombinant TNF more slowly than sham-operated mice. However, in this case, the data obtained by ELISA were similar to those obtained by bioassay. Therefore, TNF bioactivity was not inactivated in this model. These data strongly suggest that no soluble TNF-R-TNF complexes were formed and point to s-TNF-RII as being important in the inactivation of the bioactivity of circulating murine TNF in vivo. Unpublished pilot studies by this group, have revealed that nephrectomy resulted in a significantly increased rise in sTNF-RII release (10 fold vs >40 fold for BN mice) with little differences in sTNF-RI release between nephrectomized and sham-operated mice.$^{109}$ Therefore, it appears that sTNF-RII must be shed rapidly and in large quantities to neutralize TNF. This study also highlights the importance of the kidney in the clearance of TNF. Clearance of both native and complexed TNF may occur via peritubular binding to the renal ligand uromodulin.$^{110,111}$ Renal dysfunction, as is seen with multi-organ failure, may lead to reduced clearance of TNF resulting in prolonged circulation of sTNF-R-TNF complexes.$^{109}$ The importance of the kidney in the clearance of TNF is supported by studies using perfused rabbit and monkey organs.$^{112}$ The clearance of TNF from the perfusing blood by the organ was examined. The results suggest that both rabbit and monkey livers play a small catabolic role for TNF whereas the disappearance of TNF from the blood perfusing rabbit kidneys is monoexponential for TNF. They observed peak levels of radioactivity in their organs at about 30 minutes post-injection. The authors conclude that the kidney is the main site of TNF catabolism as the fractional clearance rate at the kidney is similar to the value observed in vivo$^{112}$. The tissue distribution and role of the kidney in the metabolism of TNF has also been studied in rats. Pang et al. conducted dual isotope clearance studies of the kinetics of TNF using$^{125}$I-albumin to correct for plasma bound counts.$^{113}$ Circulating TNF in rats had a short half-life of approximately 5 minutes. They reported a large difference in the beta phase (second exponential) between unlabelled and labelled TNF. They suggest that this may be due to the inactivation of biological activity but not TCA precipitable activity. Tissue distribution of TNF indicated that the liver, kidney and spleen took up a large part of the dose...
with little uptake by the central nervous system.

2.5.3. Clearance of TNF by Alpha₂-Macroglobulin

Alpha₂-macroglobulin is a plasma protease inhibitor which is synthesized by the liver. However, it can also be secreted by macrophages at the site of inflammation or immune response. The native form of the molecule is a tetramer of four identical subunits formed by the non-covalent association of two disulfide-bonded pairs. Alpha₂-macroglobulin has been shown to bind several cytokines including interleukin-1-β and interleukin-6. Radiolabeled TNF has been shown to bind to alpha₂-macroglobulin in human plasma or serum but only after reaction with methylamine or plasmin. After TNF is bound to activated alpha₂-macroglobulin, its cytotoxicity is not altered, suggesting that alpha₂-macroglobulin regulates the activity of tumor necrosis factor by clearing it rather than by competing with TNF receptors. Human plasmin has been shown to react with alpha₂-macroglobulin in the binding of TNF. It has also been shown that cachectin enhances plasminogen activating factor. Therefore, TNF may enhance its own clearance through the provision of the activating factor. This is hypothesized to be the explanation for the adaptation to repeated doses of TNF.

Alpha₂-macroglobulin/TNF complexes can be cleared by the newly identified alpha₂-macroglobulin receptor on hepatocytes. There is also evidence that the low density-lipoprotein receptor-related protein (LRP) is identical to the alpha₂-macroglobulin receptor. If LDL are cleared by this route, then there is a potential mechanism whereby nutrition support might interfere with the clearance of TNF. Low density lipoproteins might compete for clearance at the liver with alpha₂-macroglobulin/TNF complexes.

2.5.4. Regulation of TNF by Anti-TNF Antibodies

There has been speculation regarding the potential ability of anti-TNF antibodies to regulate TNF activity. Polyclonal anti-serum, purified immunoglobulin, monoclonal antibodies and Fab antibody fragments have all been developed against cytokines. Beutler et al. were the first to show that anti-TNF antibodies could protect mice from the lethal effects
of endotoxemia. Later, studies with baboons confirmed that pretreatment with TNF antibodies protected baboons from a lethal injection of *E. coli* and also protected them against the development of shock and multiorgan failure that occurred in unprotected animals. In addition, the sequential release of cytokines IL-1-beta, IL-6 and IL-8 was attenuated by pre-treatment indicating that this critical cascade was shut down by antibodies. Therefore, blocking TNF resulted in a blocking of the cytokine cascade and resulted in improved survival. In contrast to many of the previous studies showing a survival advantage with antibody treatment, there are also studies which fail to demonstrate any advantage of antibody therapy. For example, Eskandari et al. showed that anti-TNF antibodies failed to reduce mortality after cecal ligation and puncture in mice.

There are a few studies evaluating the safety and efficacy of anti-TNF antibodies in human sepsis. Fisher et al. conducted a phase II multi-center trial of 80 patients with severe sepsis. The patients received escalating doses of antibody from 0.1 to 1.0 mg/kg. These doses were found to be safe but not efficacious except in seven subjects who had very high TNF levels upon entrance to the study. In this study, 98% of the subjects developed an immunologic reaction to the antibodies. Several other human studies have failed to demonstrate decreased mortality or time-to-death after the administration of TNF antibodies. Recently, CB0006 murine monoclonal antibody against human TNF was tested in a multicenter prospective trial of 80 patients with severe sepsis and septic shock. The twenty-eight day all-cause mortality rate was similar between two groups suggesting no survival advantage with the provision of TNF antibodies. Finally, a large multicenter double-blind randomized trial of monoclonal antibodies in patients with sepsis (994 enrolled) found no improvement in mortality. There did however, appear to be some benefit of antibody to those in septic shock in the first three days but the effect was not significant at 28 days. Therefore, there may be specific groups of patients more likely to benefit from this therapy than others. However, one major drawback of these antibodies may be that an effect is observed only when TNF inhibitors are administered prior to induction of the septic response. Therefore it has been argued that they would have no useful therapeutic effect in clinical situations in which TNF is already present.
2.5.5. Regulation of TNF by Soluble TNF Receptors

Novick et al. were able to isolate and purify receptors for interleukin-6 and interferon gamma in the urine of healthy humans, indicating that release of soluble cytokine receptors into body fluids is a general phenomenon that occurs under physiologic conditions. Recently, the same group has identified tumor necrosis factor inhibitory proteins in urine from healthy subjects and has shown them to bind TNF specifically. Subsequently, two distinct TNF soluble receptors have been identified, an epithelial cell-type receptor and a myeloid cell-type receptor which correspond to the cell-surface receptors (p55-60 and p75-80kDa). Receptor shedding has the potential to compete with TNF cell surface binding thereby modulating the activity of TNF as well as its clearance from plasma (Figure 2.1). Therefore, it is important to understand the factors that result in the production of soluble TNF receptors as well as their roles as inhibitors and/or stabilizers of TNF bioactivity.

2.5.5.1. Soluble TNF Receptor Production Soluble TNF receptors may result from the proteolytic cleavage of membrane receptors, the shedding of cell surface receptors, the production through alternatively spliced mRNA or even through production from a distinct gene. It has also been suggested that there is an intracellular pool of preformed s-TNF-RI within polymorphonuclear leukocytes which may be available for rapid exocytosis. Comparison of the N terminal sequences of these TNF-binding proteins with those deduced from cDNA cloning of TNF receptors demonstrates that these binding proteins are shed forms of the two cell-surface TNF receptors. Loetscher et al., using genomic southern blot analysis of the TNF-RI, found no evidence for the existence of a distinct second gene that would encode the soluble receptor. In addition, they found no evidence of a shorter mRNA species which might encode for the soluble receptor by northern blot on various cell lines. They felt it was more likely to be the result of proteolytic cleavage of the receptor molecule than a spliced gene transcript. C terminal truncation of the intracellular domain of the human TNF-RI by 53% abolished its signalling ability. However, C terminal truncation, even up to 96%, did not abolish shedding of the receptor, indicating that signalling activity
resides in the cytoplasmic domain of the receptor. The ability to shed receptor molecules which are deficient in signalling activity indicates that the shedding and signalling mechanisms are distinct.

Lipopolysaccharide injection into mice results in a rapid rise in levels of soluble TNF receptors with peak levels of sTNF-RI after 30 minutes and sTNF-RII after 8 hours. The levels of both soluble receptors declined slowly with detectable levels still being present at 16 hours after injection. Levels of the two soluble receptors can be measured in human plasma by using ELISA, which measures soluble receptors that are free as well as those that are bound to TNF. Unlike TNF, detectable levels of soluble receptors are present in healthy humans. Levels of soluble TNF-RI and soluble TNF-RII were reported to be 0.79 ± 0.19 and 3.2 ± 0.6 ng/ml respectively in healthy subjects. However, a study by Lantz found that levels of soluble TNF receptors can differ significantly from one healthy individual to another by as much as four times. Aderka et al. also found significant individual variability in soluble TNF receptor levels. They noted that differences in soluble TNF receptor levels reflected stable changes unlike the transient changes seen in levels during disease. In humans, infusion of TNF into 5 patients with cancer resulted in rapid increases in soluble TNF receptor levels which tended to peak between 30 and 60 minutes post injection. Peak levels of soluble TNF receptors corresponded to peak TNF levels. Therefore, infusion of TNF results in a rapid increase in the shedding of cell surface receptors into the circulation. In addition, soluble TNF receptors remained elevated for up to six hours after TNF infusion, suggesting that these are cleared more slowly than TNF, which dropped off quickly. Soluble TNF production was also examined in five healthy male volunteers who were given endotoxin. Soluble TNF receptor levels increased rapidly with peak levels being attained after those of TNF. Elevated levels of soluble TNF receptors persisted for several hours beyond the time when circulating TNF levels were undetectable. Studies by Van Zee and Spinas have suggested that increased levels of soluble TNF-RII reflect exposure to TNF in the past, whereas levels of sTNF-RI do not. In addition, patterns of soluble TNF-R production suggest that different mechanisms are involved in the regulation of production of these two soluble receptors.
Agents that activate protein kinase C, such as phorbol esters, down-regulate TNF receptors as well as stimulate the production of soluble TNF receptors. In addition, exposure of monocytes to lipopolysacharide resulted in the release of soluble TNF-RII and, to a minor extent, soluble TNF-RI \textsuperscript{144}. Following exposure to lipopolysaccharide, the membrane expression of both types of receptors was rapidly downregulated, which is supported by the work of Ding et al., leading to a refractory state \textsuperscript{145}. Internalization was followed by \textit{de novo} synthesis of receptors within 18 hours as measured by binding experiments with \textsuperscript{125}I-TNF. The results support the hypothesis that the prolonged presence of soluble R-II is a reflection of slow ongoing release of this soluble receptor by monocytes with relatively little additional soluble RI release \textsuperscript{144}. It has been hypothesized that the development of tolerance to repeated exposure to TNF may reflect increased shedding of TNF soluble receptors, providing a mechanism of protection \textsuperscript{146,147}. If so, the ratio of free to bound TNF may influence therapeutic efficacy.

\textbf{2.5.5.2. Structure of Soluble TNF Receptors} Human and rat TNF receptor sequences show an overall identity of 64\% \textsuperscript{148}. The sequence homology is 69\% in the extracellular domain and 60\% in the intracellular domain. Comparison of amino acid sequences obtained from the urinary TNF-binding protein with a sequence deducted from the isolated cDNA showed that TNF-BP is a soluble form of the TNF receptor \textsuperscript{148}. In competitive binding experiments, two forms of sTNF-RI have been identified with molecular weights of 28 and 32 kDa \textsuperscript{87}. Binding studies with \textsuperscript{125}I-TNF indicated that the binding was saturable and specific with a single class of binding sites with a Kd of 93 pM indicating that 2.3 molecules of sTNF-RI will bind to a TNF trimer \textsuperscript{87}. Soluble TNF-RI was also tested for its ability to inhibit the cytolytic activity of TNF-\(\alpha\) using mouse L-M fibroblasts and it was found that a relatively high concentration of soluble TNF-RI was needed to inhibit the cytotoxic effect of TNF-\(\alpha\). Gel filtration on superose 6 under non-denaturing conditions indicated that sTNF-R1 elutes at 55-60 kDa suggesting that it exists as a dimer in solution or as an elongated protein \textsuperscript{87}.

The extracellular portion of the TNF-RII receptor has also been studied in its interaction with TNF-\(\alpha\). Binding of labelled TNF-\(\alpha\) to soluble TNF-RII indicated that binding was also
specific and saturable. Chromatography on superose 6 under non-denaturing conditions indicated a monomer with a molecular mass of 36 kDa. TNF-α can bind 2 or 3 molecules of sTNF-RII giving complexes that elute at approximately 300 kDa depending on the ratio of s-TNF-RII to TNF-α.

2.5.5.3. Physiologic Roles of Soluble TNF Receptors

2.5.5.3.1. Soluble Receptors as TNF Receptor Binding Antagonists

Originally, interest in these soluble receptors focussed on their potential to block the systemic effects of TNF by binding to TNF-α in the circulation and thereby preventing the interaction of TNF-α with its cell surface receptors. In vitro studies indicated that soluble TNF receptors could inhibit the cytolytic effects of TNF-α. Early studies showed that the binding of 125I-TNF-α to FS 11 cells was decreased when human urine was present in the growth medium. In addition, decreased TNF cytotoxicity was observed with increasing concentration of these inhibitory urinary proteins. These findings were supported by work of Seckinger who showed that the urine of febrile subjects had an inhibitory effect which was specific for TNF. Seckinger also noted increased binding of 125I-TNF to TNF-inhibitory proteins due to the interaction of ligand with inhibitor. They also noted that these urinary proteins were more than just carriers of TNF as the 125I-TNF was able to dissociate from the inhibitor and show biological activity.

Higuchi and Aggarwal looked at the ability of these two soluble receptors to block the binding of labelled TNF to its cell surface receptors. They found that the soluble TNF-RI is more potent at blocking the binding of TNF to the cell surface than sTNF-RII, although both forms could inhibit the biological response of TNF. The soluble TNF-RI was found to be 50 to 100 times more effective as an inhibitor of TNF than the soluble TNF-RII. These findings were supported in work by Hale who found sTNF-RI to be 30-fold more potent at blocking TNF binding to human U937 cells than sTNF-RII. Inhibition of the biological response of TNF was found to correspond to the blocking of its binding to cell surface receptors. In another study, serum from endotoxin-treated volunteers was subjected to differing concentrations of soluble TNF receptors and the subsequent effect on TNF
bioactivity was observed. The study showed that 5ng/ml of sTNF-R1 reduced endogenous TNF-α bioactivity by 66% whereas 500 ng of soluble TNF-R11 was required to neutralize more than 50% of the endogenous TNF bioactivity. These findings suggest that the addition of a physiologic concentration of soluble TNF-R1 to plasma will significantly neutralize the cytotoxicity of endogenous levels of TNF and that the increased levels of this receptor seen in disease may reflect partial attenuation of the biological response 142. In addition to its ability to block TNF cytotoxicity, sTNF-R1 was also shown to blunt the TNF-stimulated release of IL-6. At high molar ratios (20:1, 10:1), IL-6 production was completely abolished. Soluble TNF-R-II was also capable of blunting TNF stimulated IL-6 release but was not capable of completely eliminating it 152.

2.5.5.3.2. Inhibition of TNF Activity Trials Critically ill septic patients have elevated levels of soluble TNF receptors which have been correlated with levels of immunoreactive TNF (r=0.63 ,r=0.69 RII) 153. Since there is substantial evidence that the inflammatory reaction is mediated by a cascade of cytokines and mediators, there is significant interest in the ability of soluble TNF receptors to block or attenuate this cascade in order to decrease the morbidity and mortality associated with the sepsis syndrome. Soluble TNF receptors could potentially modulate TNF activity by binding to circulating TNF thereby preventing TNF from binding to its cell surface receptors. Unfortunately we must remember that blocking the effects of specific cytokines is not a simple solution to the modulation of the septic response as we would also be inhibiting the beneficial aspects of cytokines such as their effect on host defense and wound healing.

Early reports indicated that recombinant purified soluble TNF receptors could protect against experimental sepsis 126. Soluble receptors should be superior to anti-cytokine antibodies in that they have at least 100-1000-fold higher affinities for TNF as well as being smaller, thereby allowing better distribution in the circulation 133. Animal studies by Bertini and by Van Zee both demonstrated improved survival and improved hemodynamic parameters in mice and baboons treated with soluble TNF receptors 142,154. In addition, in baboons, treatment with the soluble receptors also blocked the cascade of IL-1β and IL-6 151. In studies by Hale, sTNF-RI and sTNFR-II at high concentrations resulted in a decrease in TNF-
stimulated IL-6 production, however, at low concentrations, IL-6 production was actually enhanced. Soluble TNF receptors were found to extend the survival of mice treated with a lethal dose of IL-1β and TNF-α. Treatment with multiple doses of sTNFR-II for 12 hours post lethal injection resulted in temporary protection and a delay in mortality. However, at 24 hours post-injection, a significant clinical deterioration was observed followed by death. This group hypothesized that the prolonged survival and subsequent death might be the result of prolonged circulation of TNF:sTNFR-II complexes and the eventual release of active TNF. Mice given multiple doses of either TNF soluble receptor were completely protected against death suggesting that repeated high doses of soluble receptors may maintain sufficiently high soluble TNF receptor levels to form inhibitory complexes until a point when other synergistic factors have decayed. This study highlights the importance of the balance between circulating TNF levels and concentrations of soluble TNF receptors as well as indirectly supporting a carrier role for soluble TNF receptors. Soluble receptors are proposed to be moderators rather than inhibitors of TNF activity as large amounts of receptor (10 to 100 fold TNF alpha molar concentration) are required to inhibit TNF activity completely. Therefore, the use of sTNF-R as a therapeutic tool in human sepsis appears limited.

In continuing efforts to improve the efficacy of soluble receptors as effective blockers of TNF activity, designer molecules have been produced which have a higher affinity and improved longevity in the circulation. Bivalent fusion proteins have been constructed by joining soluble TNF receptor with the hinge region of human immunoglobulin. Preliminary animal work by Mohler indicated that these fusion proteins are substantially more efficacious than monomeric soluble TNF receptors. Mohler conducted studies on mice using monomeric soluble TNF receptors and a soluble dimeric form of the soluble TNF-RII fused to the Fc portion of human Ig G1. Using radiolabeled TNF, they demonstrated that the fusion protein had a fifty-fold higher affinity for TNF than the soluble receptor monomer. They hypothesized that this increased affinity would result in the fusion protein being a better antagonist of TNF biological activity in vivo than sTNF receptors. Mohler studied the effect of these fusion proteins on the cytotoxicity of TNF in vitro using L929 bioassay. The results indicated that while the soluble TNF receptor and the fusion protein
both inhibited TNF bioactivity in a dose-dependent fashion, the fusion protein was approximately 1000-fold more efficient than the soluble TNF receptor.

They went on to look at the protective effect in vivo in mice given a lethal dose of E. coli lipopolysacharide and demonstrated that mice treated with the fusion protein had a significantly increased survival whereas the soluble TNF-RII failed to provide any survival advantage even when given in relatively high doses (260ug). In addition, fusion proteins were able to inhibit TNF cytotoxicity even when administered up to three hours after injection of LPS. Interestingly, these researchers also found that mice given fusion proteins and subjected to LPS retained increased levels of TNF in the serum that persisted for longer periods of time suggesting that these molecules could also function as a carrier of TNF and therefore may alter the disappearance rate of TNF. This supports work by Aderka et al. who suggested that soluble TNF receptors can act as a reservoir of bioactive TNF.

Human trials are currently underway. however, preliminary results are somewhat confusing. For example, in one study the treatment group given TNF:Fc unexpectedly demonstrated increased mortality and subsequently the trial was terminated. In the final report of this trial, Fisher et al. reported that there was a significant dose response relation between TNF:Fc and mortality. The differences in mortality could not be explained by differences in baseline parameters including levels of IL-6. One hypothesis for these surprising findings was that these fusion proteins may have functioned as an intravascular carrier of active TNF-α, thereby prolonging the inflammatory response. This hypothesis is a logical extension of the work of Aderka et al. and may also explain some of the differences observed between our feeding groups in response to TNF.

Since the dose of fusion protein given in this trial was quite high, another study of the efficacy of a low dose and high dose of TNF:Fc on the response of 18 human volunteers to endotoxin challenge was conducted. As expected, TNF bioactivity was significantly inhibited by the TNF:Fc at both doses compared with placebo treatment. However, the fusion protein failed to prevent the clinical signs of shock such as decreased mean arterial pressure and increased temperature. The unexpected finding was that the low dose TNF:Fc was more effective at inhibiting the secondary cytokine and hormonal response than the high dose.
The high dose was similar to that given in the previous study where an increase in mortality was observed suggesting that a high dose of fusion protein is not as immunosuppressive as a low dose and that high doses of TNF:Fc may cause imbalances in the inflammatory response to endotoxin thereby contributing to the less positive response. This group also hypothesized that the neutralization of TNF by the fusion protein could stimulate increased production of TNF resulting in the less positive outcome observed in those receiving the higher dose. In addition, the authors observed that the fusion protein significantly extended the time that TNF was measurable in the serum (6 hours to 24 hours), supporting the hypothesis that soluble TNF receptors can serve as a carrier for potentially bioactive TNF. Timing and dosing of anti-cytokine therapies, therefore, appear to be critical when trying to modulate the response to TNF. Currently, it is impossible to determine which patients may benefit from anti-cytokine therapy. Most animal work involves treatment with antibody prior to the development of sepsis and therefore may be useful for patients with recurrent sepsis.

2.5.5.3.3. Stabilization of TNF Activity

Recent work on soluble receptors indicates that, as well as competing for cell surface receptors, soluble receptors also have the ability to stabilize TNF action. TNF rapidly degrades from trimeric to dimeric and monomeric forms. Aderka et al. found that TNF-stimulated B-CLL cell growth was affected in a bimodal fashion by the presence of soluble TNF receptors. At certain concentrations, the presence of these receptors enhanced cell growth whereas at higher concentrations they were found to inhibit growth. Aderka’s group demonstrated that the presence of soluble TNF receptors (either RI or RII) slowed the spontaneous decay of TNF in the growth medium thereby preserving its stimulatory effect. In a second set of studies, they found that the presence of soluble TNF receptors inhibited the cytocidal activity of TNF while maintaining the stability of TNF in contrast to the rapid decrease of cytocidal activity in the absence of any soluble receptor. Aderka concluded that the prevention of TNF decay allowed the effects of TNF to be augmented.

These studies indicate that the effect of soluble TNF receptors may differ depending on: i) their concentration at the site of TNF action; ii) the relation of their concentration at the site of TNF action; iii) the relation of their concentration to the local concentration of TNF; and iv)
the rates at which the soluble TNF receptors and TNF are cleared from the site of TNF action in relation to the rate of TNF decay. Therefore, in different situations, soluble receptors may inhibit the effects of TNF, serve as a carrier for TNF or act to augment the effects of TNF by prolonging its activity. In addition, these soluble receptors may mitigate the impact of overproduction of TNF by binding to it and serving as a slow release reservoir of bioactive TNF. It is important to remember that the binding of TNF to soluble TNF receptors is reversible, resulting in the release of active TNF. Ideally, recombinant soluble receptors could provide the means for specific and potent immunosuppression of the effects of TNF without affecting the actions of other cytokines.

2.5.5.3.4. Soluble TNF Receptor Production in Disease If we are to consider soluble TNF receptors as a potential contributor to the changes in TNF clearance observed in our TNF infused animals, we must first ensure that these receptors are produced in response to disease. Elevated levels of soluble TNF receptors have been noted in several disease states. Aderka et al. found that colon cancer patients had significantly increased levels of soluble TNF receptors which correlated with the staging of the disease as well as with the extent of weight loss. Aderka found that in healthy subjects, levels of sTNF-R1 and sTNF-RII were 0.79 ±0.19 and 3.2 ± 0.6 ng/ml respectively while the values were significantly higher in cancer patients (1.96ng/ml and 6.43ng/ml respectively). Aderka suggested that the excessive levels of soluble TNF receptors may be, at least in part, produced by the tumor cells. However, they may also be produced by other cells in response to tumor presence.

Elevated levels of TNF and both sTNF-RI and sTNF-RII have been observed in patients during chronic heart failure. Ferrari et al. looked at 37 patients with heart failure as well as 26 age-matched control subjects. They found correlations between the severity of heart failure and TNFα, sTNF-RI and sTNF-RII levels which were significant for the two soluble receptors. These results were found in the absence of cardiac cachexia and suggest that the levels of sTNF-RII were independently correlated with poor short term prognosis and support earlier evidence of the predictive value of sTNF-RII levels.

Elevated levels of circulating soluble TNF receptors have been measured in patients who are HIV positive, although these levels may only reflect the activation of the TNF system.
In HIV infection, a clear relation to disease progression was not observed in relation to TNF\(^{161}\). Interestingly, researchers have noted persistently high levels of soluble TNF receptors in HIV positive patients who have no outward signs of infection. Levels of soluble TNF receptors have also been studied in meningococcaemia. These researchers noted that when levels of TNF were low, there was a relationship between levels of TNF-\(\alpha\) and both soluble TNF receptors. However, when levels of TNF exceeded 500 pg/ml the levels of soluble TNF receptors did not increase proportionately as was seen previously. This led this group to hypothesize that the ratio of TNF to soluble TNF receptors may be a useful clinical tool for the prediction of fatal outcome \(^{162}\). Finally, elevated levels of soluble TNF receptors have been shown in patients with systemic lupus erythematosus, progressive systemic sclerosis and mixed connective tissue disease \(^{163}\).

### 2.6. DEVELOPMENT OF A MODEL TO STUDY TNF CLEARANCE

Previous studies in this laboratory examined the effects of a continuous infusion of TNF in animals receiving enteral and parenteral nutrition support \(^{43,44}\). TNF infusion in orally fed animals resulted in the development of the cachectic syndrome with anorexia, weight loss and protein redistribution. However, there was no mortality in these anorexic animals \(^{43}\). TPN was provided during TNF infusion in order to prevent the anorexia, weight loss and malnutrition associated with chronic low circulating levels of TNF \(^{44}\). The forced provision of nutrients by TPN resulted in a potentiation of the metabolic abnormalities characteristic of sepsis with a significantly increased mortality rate. The continuous infusion of TNF was preferred over multiple bolus injections of TNF as it more accurately reflected the chronic secretion of TNF and did not result in an inflammatory reaction at the site of injection. This model mimics the metabolic derangement of human chronic illness and has raised questions regarding the interaction of nutrition support with TNF. Since circulating levels of exogenously infused human TNF were elevated in TPN fed animals, it was hypothesized that the provision of nutrients sufficient to promote weight-gain in these TPN fed animals would result in impaired TNF clearance. Impairment of TNF clearance would result in prolonged, higher circulating TNF levels with a potentiation of the metabolic responses to TNF.
TNF is considered a pivotal mediator of both the acute stress response and the chronic wasting and anorexia observed frequently in the clinical setting. Therefore, it is reasonable to assume that many of these patients will have or have had elevated levels of circulating TNF. Since many of these patients are also receiving nutritional support, an understanding of the interaction of nutrition support with TNF action is critical. The studies presented in this thesis represent two mechanistic studies designed to determine whether the provision of nutrition support affects TNF distribution and clearance. The results of these studies will impact on the provision of nutrition support in the clinical setting.

The studies presented in this thesis also represent an extension of our earlier rat model in order to determine the effects of forced nutrient infusion on TNF metabolism, degradation and clearance. Rats were provided with sufficient nutrients via TPN to allow weight-gain thereby providing a model simulating current feeding practices. A second group of rats was provided with insufficient nutrients to allow growth, resulting in weight-loss mimicking the impaired food intake and anorexia associated with TNF. This group was included in order to determine whether hypo-caloric TPN had similar effects on TNF metabolism to optimal TPN. An orally fed group was included in order to determine if there were any additional effects on TNF metabolism due simply to the provision of nutrients via a central venous catheter. Since we wished to examine only the effects of nutrition support on TNF clearance we did not infuse TNF as in our previous model. We are assuming that if nutrition support affects the clearance of an exogenous dose of TNF, it will also affect the clearance of endogenously produced TNF resulting in the potentiation of the effects of the endogenous TNF.

The rat model was chosen as it represented an extension of our previous studies. TPN solutions consisted of a mixture of dextrose, amino acids and lipids that are also commonly used in the clinical setting. The only other model that could have been considered was a murine model, however, murine TNF receptor RII is specific for murine TNF and therefore the clearance kinetics would be different from the human in which both receptors are available to clear TNF. Rat TNF and TNF receptors show significant homology to their human counterparts suggesting that the rat represents a reasonable model. Finally, earlier work in the rat reveals that TNF clearance occurs primarily at the kidney which is similar to studies
showing impaired TNF clearance in humans with renal failure.

The clearance of a bolus of radiolabelled TNF was employed to track the movement of TNF within the circulation. As with any labelled particle, there were concerns that the labelling procedure would alter the size or the binding characteristics of the TNF molecule. These issues are addressed in the following section.

2.7. THE IODINATION OF TNF

It has been suggested that radiolabelling of TNF alters the size and activity of the molecule. Smith et al. used SDS gel electrophoresis to demonstrate that, under denaturing conditions, native TNF eluted as a polypeptide of 17 kD or 17.5 kD \(^{12}\). In non-denaturing conditions, TNF consisted of dimers or higher oligomers with Mr values of 45,000 and 70,000 respectively \(^{4,17,18}\). The Smith group ran both iodinated and native TNF preparations on Sephadex G-75 columns to establish whether iodination altered the size of the TNF molecule. They found that the elution profiles of native human and murine TNF were similar to those of human \(^{125}\)I-TNF and murine \(^{125}\)I-TNF. The elution profile of natural TNF was indistinguishable from that of recombinant human TNF and murine TNF showing a major and a minor peak as well as a peak of free iodide. Molecular weight measurement at the two peaks indicated compounds of 17,000 and 55,000, corresponding to TNF monomer and trimer \(^{12}\).

In order to demonstrate that iodination did not affect biological activity, pooled fractions of human TNF trimer and monomer were subsequently compared in competitive binding and cytotoxicity assays on an equal cpm basis. The monomer showed low binding activity and cytotoxicity compared to the trimer fraction. Binding of monomer was about 5.5-fold lower than that of the trimer as determined by competitive binding assays \(^{12}\). Scatchard plots showed the trimer to bind with Kd=90pm whereas only a small component of the monomer fraction was bound with such a high affinity \(^{12}\). In parallel cytotoxicity assays, a monomer concentration of 6-7 times greater than that of trimer was needed to elicit the same biological response when tested at low concentration. Aggarwal et al. found that labelling of TNF by the Chloramine T, Lactoperoxidase and Bolten Hunter methods provided TNF with low
specific activity and resulted, in some cases, in a >90% decline in biological activity \textsuperscript{107}. However, when TNF was labelled with the iodogen method, as ours was, a fully active compound was obtained for more than four weeks. Apparently, the labelling of TNF affected neither the size nor the biologic activity of the TNF molecule, allowing it to be used in our studies of TNF kinetics and to track the movement of TNF using compartmental analysis.

2.8. COMPARTMENTAL MODELLING

The use of models similar to compartmental models dates back to the first half of the 19th century when Fourier derived the differential equation based on the continuity of the flow of a substance \textsuperscript{164}. After the introduction of radiolabelled tracers, Fick used compartmental systems to make estimates of cardiac output. Later, Teorell used two compartment modelling to represent the pharmacokinetics involved in biological systems \textsuperscript{164}. Tracer experiments encouraged the use of compartmental models. In medicine, tests such as the radiocardiogram or the radiorenogram which involve the administration of a radioactive substance or dye depend on compartmental analysis \textsuperscript{164}. Pharmacokinetic studies are almost exclusively based on compartmental analysis. Compartments can also be non-physiological and represent groups such as species, class of age or type of industry. Therefore, compartmental analysis is widely used, not only in the field of biology, but also in ecology, economy and education.

In its most basic form, compartmental analysis is used to measure the input and output of a tracer introduced into a system. The tracer is assumed to follow the movement of the material of interest. In our case, compartmental analysis would examine the spaces into which TNF is distributed and would label them as compartments. The movement of TNF molecules between compartments is called flux. The use of compartmental modelling allows us to conceptualize a complex system as well as predict the behaviour of that system. What is designated to be a compartment depends upon the system being analyzed. In our first study, the compartments represented distribution pools of TNF, whereas in our second study the distribution of TNF into each organ or tissue studied represents a compartment. The idea of a compartment is conceptual and does not have a real anatomical counterpart and therefore the volume of distribution should not be confused with the blood volume. Using compartmental
theory relies on the assumption that the radiolabelled TNF will be evenly distributed throughout the compartment into which it is initially introduced and that the law of conservation of mass is true.

Our first set of experiments used a simple two-compartment model to examine the movement of TNF between compartments in different feeding groups. Due to the bi-exponential nature of the disappearance of TNF from the plasma, it was most appropriate to use an open two-compartment model to describe the plasma clearance (Figure 2.2). The TNF clearance curve represents two phases: the disappearance of TNF from the plasma and secondly, the appearance of TNF in the organs. The continued downward slope of the second exponential suggested that TNF was being lost to the outside (the curve tends towards 0) and therefore, we chose an open compartmental model which would allow for movement of TNF to the outside (Figure 2.2).

Using a compartmental model allowed us to examine the flow of TNF between spaces through the generation of rate constants for the movement of TNF in and out of compartments. The flow of TNF out of a compartment depends upon the concentration of TNF in that compartment and not on the concentration in the other compartments. The rate constants have the dimensions of inverse time. The standard designation for rate constants is a $K$ followed by the compartment into which the material is flowing followed by the compartment it is coming from. Therefore, the rate constant $K_{AB}$ describes the rate constant for the flow of material from compartment B to compartment A (Figure 2.2). The transfer of TNF between compartments can be described by a set of differential equations.

Our second study used a more complex modelling system. Whereas, in our first study, organ uptake data were available for only one point in time, our second study allowed us to determine not only plasma disappearance of TNF but organ uptake of TNF over time. This allowed the collection of complete flow data and calculation of the true organ uptake of TNF. We used a mammillary system to determine the movement of TNF between the organs and the plasma (Figure 2.3). In a mammillary system, there is one central compartment (in our case the plasma compartment) which exchanges material ($^{125}$I-TNF) with each additional compartment. However, there is no exchange of TNF between these other compartments. The
Figure 2.2  AN OPEN TWO COMPARTMENT MODEL - In our open two compartment model, compartment A represents the plasma-associated compartment while compartment B represents everything that is not A. The rate constant $K_{BA}$ reflects the fraction of the concentration of TNF in compartment A destined for compartment B. $K_{BA}$ reflects the fraction of the concentration of TNF in B leaving to return to A. In this model, TNF is lost from compartment A to the outside ($K_{OA}$).
Figure 2.3 THE MAMMILLARY SYSTEM - In a mammillary model there is one central compartment, in our case the plasma, which exchanges the injected material (0.3 uCi 125I-TNF + 0.5 ug unlabelled TNF) with 10 other separate compartments representing the organs and tissues collected. In this study we have used an open model as labelled TNF is lost from the system into the bile and the urine.
calculations involved in the interpretation of data from these compartmental models will be presented in the following chapters.

2.9. HYPOTHESIS DEVELOPMENT

This laboratory has developed a rat model of cachexia which mimics the wasting syndrome observed in critical and chronic human illness. In this model, the provision of a continuous infusion of TNF results in anorexia, weight loss and the redistribution of body proteins. When malnutrition due to anorexia was prevented by giving total parenteral nutrition (TPN), the same dose of TNF resulted in marked hyperglycemia, azotemia and signs of liver injury and multi-organ failure which were not observed in orally-fed wasted animals. Moreover, plasma TNF levels were highest in the parenterally-fed animals with the most severe metabolic abnormalities. Therefore, the provision of nutritional support resulted in a potentiation of the metabolic effects of TNF. These findings suggested that nutritional support sufficient to prevent cachexia in TNF-infused animals might influence TNF clearance as circulating levels of TNF were elevated in these animals.

We have hypothesized that the development of anorexia during acute and chronic illness might represent an adaptive mechanism and therefore, the provision of nutrients by TPN would upset this protective mechanism and result in a potentiation of the metabolic effects of TNF. Nutrient infusion aimed at the prevention of weight-loss and muscle wasting resulted in a potentiation of the metabolic effects of TNF. This type of nutrient infusion would mimic the quantity of nutrients typically provided in the clinical setting. Studies to date have raised several questions regarding the provision of this level of nutrition support during illness when there is likely to be circulating TNF. The studies described in this thesis will examine the influence of nutrition support on TNF clearance and distribution kinetics in an effort to explain the observed potentiation of TNF during TPN. These studies will also examine the effect of hypo-caloric nutrition support in order to determine whether an infusion of nutrients which more accurately reflects the observed anorexia during chronic or acute illness would result in improved TNF clearance kinetics in comparison with the more aggressive type of nutrition support, which more accurately reflects current feeding practice.
These studies will assist in the development of appropriate feeding protocols to be used in the clinical setting where elevated levels of TNF may be suspected.

2.9.1. General Hypothesis:

Nutritional support, given as total parenteral nutrition will affect the plasma clearance and organ uptake of radiolabeled tumor necrosis factor.

2.9.2. Specific Hypotheses:

1) Animals receiving nutrients via TPN will have TNF clearance kinetics that are different from those of orally-fed animals.

2) Animals receiving TPN sufficient to allow weight-gain will demonstrate impaired clearance of TNF in comparison with weight-gaining orally-fed animals.

3) The impaired TNF clearance in weight-gaining TPN fed animals will result in elevated serum TNF levels as well as in the potentiation of the metabolic abnormalities observed in these TPN-fed animals in previous work.

4) Animals parenterally fed nutrients insufficient to allow weight-gain (weight-loss) will demonstrate TNF clearance kinetics similar to those of animals receiving nutrients orally.

2.9.3. Objectives:

1) To determine the clearance rates of labelled TNF in enterally and parenterally fed rats.

2) To determine whether the quantity of nutrients provided by TPN affects TNF clearance kinetics.

3) To examine the organ uptake of labelled TNF in order to determine whether enteral or parenteral feeding will affect the uptake of TNF by organs, especially those presumed to be involved in TNF clearance.
CHAPTER 3.0 METHODS AND PRELIMINARY STUDIES

3.1. INTRODUCTION

This chapter will provide a detailed outline of all surgical procedures used in both clearance studies. In addition, background work which was done to complement the main studies will be presented.

3.2. SURGICAL PROCEDURES

3.2.1. Animal Preparation and Central Venous Catheter Insertion

Male Wistar rats (Charles River Canada, Inc., Quebec, Canada) were individually housed in cages and maintained at a temperature of 22°C and maintained on a 12 hour light-dark cycle. On entry to the animal facility, the rats weighed between 200-220g. All animals were maintained on a diet of laboratory chow ad libitum until four days prior to surgery. At this point the animals were provided with a liquid diet to allow acclimatization to this diet. At the time of surgery the rats had achieved weights of 240-260g.

Under general anesthesia (sodium pentobarbital, 50mg/kg intraperitoneal (i.p.)) a silastic catheter (0.037 inch OD, Dow Corning, Midland MI) was advanced into the superior vena cava through the right jugular vein using the technique of Popp and Brennan as described below \(^{165}\). The ventral aspect of the neck was clipped, prepared with 1% providone iodine solution (Betadine solution, Purdue Frederick Co. Norwalk, CT) and the site over the right internal jugular vein was surrounded by protective sterile drapes. Full sterile technique with operating masks, sterile gloves and sterile instruments was used to place the catheter through the internal jugular vein into the superior vena cava. A 1.5 cm midline incision was then extended cephalad from the suprasternal notch up the neck using a no-touch technique. The right internal jugular vein was dissected free and controlled with loops of 5-0 suture. A 1.19 mm OD silicon rubber catheter (Silastic Dow Corning, Midland MI) was threaded 2.5 cm down the internal jugular vein into the superior vena cava and tied in place. A 1.0 ml syringe with heparinized saline (10U/ml) was used to periodically withdraw blood and irrigate the
cadieter to assure unobstructed catheter position. The animal was then rolled into a prone position and an area over the back was clipped free of hair. The area was subsequently prepared with providone iodine and surrounded with sterile drapes. The catheter was tunnelled subcutaneously to the interscapular region of the back, which had been prepared, and led externally through a protective wire spring and secured to the rat by a stainless steel button (Instech Labs. Horsham PA). The button was secured with 4-0 silk suture to the suprascapular fascia as described by Goodgame et al. The entire procedure was performed in a laminar flow hood and lasted 40-50 minutes. The catheter was passed through a hole in the metabolic cage top and connected to a sterile 23 G needle which had previously been attached to the wire spring. Post-operatively, the rats were housed in plastic metabolic cages (Nalgene, Sevenoaks, Kent England) and maintained on a specially formulated liquid diet (Table 3.1) given orally ad libitum during the recovery phase. Animals also had free access to water during the entire experimental period. The patency of the catheter was maintained by a daily injection of 0.5 ml of heparinized saline (sodium heparin, 10 U/ml) into the catheter.

3.2.2 Bile Duct Cannulation

Under general anesthesia (sodium pentobarbital, 50mg/kg i.p.), an incision of approximately 5 inches was made down the midline of the rat. The skin and fur were dissected away and an incision through the underlying tissue was made from just above the penis to just under the rib cage exposing the abdominal cavity. The liver lobes were pushed away to reveal the common bile duct. The duct was isolated, and secured proximally (duodenum end) with a 4-0 silk suture. Fine cutting scissors were used to nick the bile duct allowing insertion of a PE 10 silastic cannula (Dow Corning, Midland MI). The cannula was advanced until there was a free flow of bile. The cannula was then secured with a second suture at the distal end. Bile was collected in 1.5ml Eppendorf tubes.

3.2.3. Bladder Cannulation

Following the insertion of the bile duct cannula, the bladder was isolated. An angiocath needle was inserted through the surrounding tissue and into the bladder. The
catheter needle was secured with a 4-0 silk suture. After securing the catheter, the needle was removed and the urine was allowed to flow into a 1.5ml Eppendorf tube.

3.3. DIET FORMULATION

All animals were provided with a specially formulated liquid diet either orally or parenterally. The composition of the diet is shown in Tables 3.1-3.3. The nutrients infused met the macronutrient requirements for rats and previous trials had demonstrated that this diet allowed control rats to grow at the same rate as chow-fed animals. TPN weight-gaining animals received on average 79.0 kcal/day whereas weight-losing animals received one half of that intake (39 kcal/day). Animals fed orally ad libitum received on average 79 kcal/day.

3.4. MEASUREMENT OF RADIOACTIVITY IN SAMPLES

3.4.1. Plasma Radioactivity

A sample of 50 μl of plasma was added to 0.5 ml 2% bovine serum albumin in 12 x 75 disposable glass tubes. The protein was precipitated with 1.5 ml of 15% trichloroacetic acid (TCA), centrifuged and the supernatant removed and discarded. The pellet was subsequently washed and spun a second time with 1.5 ml of 15% TCA in order to remove any non-protein-bound radioactivity. Preliminary studies showed that 90% of the plasma activity after injection of the bolus of labelled and unlabelled TNF was precipitated, indicating that most of the radioactivity was protein-bound in the plasma. The precipitate was dissolved in 1.5 ml of 1 N NaOH and counted for radioactivity. A duplicate aliquot of the standard was diluted to 1.5 ml with saline and counted in a well-type sodium-iodide crystal connected to a pulse height analyzer and scalar. In separate studies, we noted that a volume of 1.5 ml gave maximum efficiency and all samples were made up to 1.5 ml to ensure uniform geometry. Three blank tubes were counted to measure background radioactivity.

The curve of the plasma disappearance of precipitated 125I-TNF or albumin could be best fitted statistically by a bi-exponential curve using a non-linear least squares method (Figure 3.1) to the equation \[ y = c_1e^{-\beta_1t} + c_2e^{-\beta_2t}; \] where \( y \) = specific activity in counts per minute.\(^{167}\)
Table 3.1 Composition of TPN Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Percentage Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (mL)</td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td>Dextrose (g)</td>
<td>16.74</td>
<td>(72 % energy)</td>
</tr>
<tr>
<td>Amino Acids (g)</td>
<td>2.38</td>
<td>(13% energy)</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>1.25</td>
<td>(15% energy)</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>79.4</td>
<td></td>
</tr>
<tr>
<td>Total Nitrogen (g)</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Nonprotein calories/nitrogen</td>
<td>183.8</td>
<td></td>
</tr>
</tbody>
</table>

Minerals (mmol)

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>3.64</td>
</tr>
<tr>
<td>K</td>
<td>4.87</td>
</tr>
<tr>
<td>Cl</td>
<td>8.71</td>
</tr>
<tr>
<td>Ca</td>
<td>0.376</td>
</tr>
<tr>
<td>Mg</td>
<td>0.317</td>
</tr>
<tr>
<td>Zn</td>
<td>0.000454</td>
</tr>
<tr>
<td>Mn</td>
<td>0.000629</td>
</tr>
<tr>
<td>Cu</td>
<td>0.00023</td>
</tr>
<tr>
<td>Fe</td>
<td>0.0008</td>
</tr>
<tr>
<td>Se</td>
<td>0.000015</td>
</tr>
<tr>
<td>Cr</td>
<td>0.0000038</td>
</tr>
<tr>
<td>Acetate</td>
<td>3.715</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.420</td>
</tr>
<tr>
<td>Iodide</td>
<td>0.000047</td>
</tr>
</tbody>
</table>

Vitamins

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine (mg)</td>
<td>3.75</td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>0.83</td>
</tr>
<tr>
<td>Niacinamide (mg)</td>
<td>8.33</td>
</tr>
<tr>
<td>Pyridoxine (mg)</td>
<td>1.00</td>
</tr>
<tr>
<td>Pantothenate (mg)</td>
<td>2.17</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>83.30</td>
</tr>
<tr>
<td>Vitamin A (IU)</td>
<td>833.33</td>
</tr>
<tr>
<td>Vitamin D (IU)</td>
<td>83.30</td>
</tr>
<tr>
<td>Vitamin E (IU)</td>
<td>0.83</td>
</tr>
</tbody>
</table>
Table 3.2. Composition of Amino Acid Solution

2.38g of amino acids per day were provided by 10% Travasol Amino Acid Solution which included:

**Essential Amino Acids**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Leucine</td>
<td>147.58 mg</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>147.58 mg</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>138.04 mg</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>138.04 mg</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>114.24 mg</td>
</tr>
<tr>
<td>L-Valine</td>
<td>109.48 mg</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>104.72 mg</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>99.96 mg</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>42.84 mg</td>
</tr>
</tbody>
</table>

**Non-Essential Amino Acids**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>492.66 mg</td>
</tr>
<tr>
<td>Aminoacetic Acid</td>
<td>492.66 mg</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>247.52 mg</td>
</tr>
<tr>
<td>L-Proline</td>
<td>99.96 mg</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>9.52 mg</td>
</tr>
</tbody>
</table>

Table 3.3. Composition of Lipid Emulsion

The lipid was provided by 20% Intralipid which contained per 100 ml:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified Soybean Oil</td>
<td>20g</td>
</tr>
<tr>
<td>Purified Egg Phospholipid</td>
<td>1.2g</td>
</tr>
<tr>
<td>Glycerine Anhydrous in Sterile Water</td>
<td>2.2g</td>
</tr>
</tbody>
</table>
Figure 3.1 THE CLEARANCE OF LABELLED ALBUMIN - A bolus of 5ug of 125I - albumin was injected into control rats via a central venous cannula. Blood samples were withdrawn over a 20 minute period.
3.4.2. Radioactivity in Whole Blood, Bile and Urine

A sample of 100 µl of whole blood was placed in 12x75 glass tubes for gamma counting. The pipet tip was rinsed with 0.9% saline to ensure that all of the blood was removed. The protein was precipitated with 1.5 ml of 15% TCA, centrifuged at 3000 RPM for 5 minutes and the supernatant removed and discarded. This process was repeated to ensure the removal of all non-protein bound-radioactivity. The remaining pellet was dissolved in 1.5 ml of 3N NaOH and counted for radioactivity in a dual channel gamma counter (Beckman Gamma 5500).

Samples of 100 µl of bile and urine were placed in 12 x 75 glass tubes and were analyzed for radioactivity prior to precipitation. The volume of bile and urine collected was also measured and recorded. Following initial radioactivity analysis, bile and urine samples were precipitated with 15% TCA and spun for 5 minutes at 3000 RPM. The supernatant was removed and the pellet was dissolved in 3 N NaOH and counted for radioactivity.

3.4.3. Uptake of $^{125}$I-TNF by Organs

Organ samples were homogenized with normal saline using a mortar and pestle followed by precipitation with 1.5 ml of 15% TCA. Precipitated samples were centrifuged for 5 minutes at 3000 RPM and the supernatant was subsequently removed and discarded. This procedure was repeated once to ensure the removal of any non-protein-bound radioactivity. The pellet was dissolved in 1.5 ml 10 N NaOH for 24 hours and counted for radioactivity as described above.

3.5 ANALYSIS OF CLEARANCE DATA

Analysis of the data from the bi-exponential fits of the $^{125}$I-TNF clearance data was done in two separate ways, namely compartmental analysis and linear systems analysis. Linear systems analysis does not make any assumptions based on compartmental models.

3.5.1. Compartmental Modelling

The clearance of tumor necrosis factor is best described as a biexponential function which
is similar to the clearance of other plasma proteins\textsuperscript{168}. Accordingly, we suggest a two-compartment model (Figure 2.2), which gives rise to clearance data that could be fitted by the sum of two exponential functions\textsuperscript{168}. In this model, compartment A is the compartment containing the plasma while compartment B is extravascular. Irreversible loss occurs by organ uptake and excretion of radioactivity.

The $K$'s are rate constants, bearing the dimensions of $[t^{-1}]$. $K_{BA}$ is the coefficient governing the flow of cachectin from compartment A to compartment B. $K_{AB}$ governs the flow from B to A. $K_{OA}$ governs the rate of irreversible loss of TNF from compartment A. By curve-fitting a biexponential function of the form

$$C_A(t) = C_1 e^{\beta_1 t} + C_2 e^{\beta_2 t}$$

(3.1)

to the clearance data, where $C_A$ is the concentration of $^{125}$I-TNF in compartment A in counts per minute per ml, we obtain values for the parameters $C_1$, $C_2$, $\beta_1$ and $\beta_2$. Using these parameter values, we can calculate the rate constants $K_{AB}$, $K_{BA}$ and $K_{OA}$. Details concerning the derivation of these rate constants can be found in Appendix 1. Values for the rate constants averaged for weight-gaining (WGR) and weight-losing (WLR) parenterally fed animals as well as for orally fed animals (OFR) are given in Table 4.1. If we consider the system to be near equilibrium during the second exponential phase, then the ratio of these rate constants ($K_{BA}/K_{AB}$, BARATIO) reflects the ratio of concentration of TNF in compartment B relative to that in compartment A (Appendix 2).

The total volume of distribution ($V$) may be estimated in a compartment-dependent model from the ordinate intercept of the second exponential equation:

$$V = \frac{D}{C_2}$$

(3.2)

where $D$ [cpm] represents the injected dose of $^{125}$I-TNF. This estimation of volume is acceptable when spatial gradients in TNF at steady state are small\textsuperscript{169}. The volume of compartment A can be estimated by dividing the injected dose $D$ by the combined ordinate
intercepts (cpm) for both exponential equations. The volume of compartment B can then be calculated by subtraction of the volume of compartment A from the total volume of distribution ($V_A, V_B$, Table 4.1). In addition, the ratio of $V_A/V_B$ may be used with the BARATIO to estimate the relative mass of TNF-α in compartments A and B (Appendix 2).

The fractional clearance rate of TNF ($FCR'$) can be determined using this compartmental model. In the steady state, the $FCR'$ is an estimate of the rate of irreversible loss of TNF from compartment A ($K_{OA}$, Appendix 1) and can be estimated using the equation:

$$FCR' = \frac{\beta_1 \beta_2}{A_1 \beta_2 + A_2 \beta_1} \quad \text{[time}^{-1}] \quad (3.3)$$

where $A_1=\frac{C_1}{C_1+C_2}$ and $A_2=\frac{C_2}{C_1+C_2}$.

Finally, a metabolic clearance rate ($MCR'$) of TNF can be determined using this compartmental model. The metabolic TNF clearance rate can be interpreted as the flow out of compartment A (Appendix 1) and can be estimated using the equation:

$$MCR' = K_{OA} \times V_A \quad \text{[vol*time}^{-1}] \quad (3.4)$$

3.5.2. Linear Systems Analysis

The clearance of $^{125}$I-TNF can be calculated solely from a linear systems analysis of the time-invariant, labelled-TNF system, without explicit reference to any compartmental model. The metabolic clearance rate, $MCR$, of TNF is defined by:

$$MCR = \frac{D}{\int_0^\infty C_2 \, dt} \quad \text{[vol*time}^{-1}] \quad (3.5)$$

where $D$ [cpm] represents the injected dose of $^{125}$I-TNF. The integral in the denominator represents the area under the clearance curve. It may be measured by any technique, but is conveniently estimated from the biexponential function (3.1). Thus
\[ MCR = \frac{D}{\frac{C_1}{\beta_1} - \frac{C_2}{\beta_2}} \quad (3.6) \]

\( MCR \) and \( MCR' \), while not identical, reflect the volume of the distribution of TNF cleared per unit time. The total volume of distribution (TVD) can be calculated with no assumptions regarding compartments using the equation:

\[ TVD = \frac{D \int_0^\infty C_A^* \, dt}{\left(\int_0^\infty C_A^* \, dt\right)^2} \quad [\text{vol}] \quad (3.7) \]

The mean residence time, \( t \), may be estimated from the expression:

\[ t = \frac{\int_0^\infty C_A^* \, dt}{\int_0^\infty C_A^* \, dt} \quad (3.8) \]

By dividing MCR by TVD, we obtain a measure of fractional clearance per unit time, \( FCR \). The results of these calculations are shown in Table 4.2.

\[ FCR = \frac{MCR}{TVD} = \frac{1}{t} \quad [\text{time}^{-1}] \quad (3.9) \]

\( FCR \) is not expected to be equal to \( FCR' \) as calculated by compartmental analysis. It can be shown that:

\[ FCR' = FCR \left[1 + \frac{C_2}{C_1}\right] \quad (3.6) \]
3.6. ALBUMIN STUDY

Information on the clearance of other plasma proteins was obtained for comparison by measuring the clearance of a bolus of radiolabelled albumin. In addition, the volume of distribution of labelled albumin will provide an approximation of the blood volume of our experimental animals. Three separate chow-fed rats were anaesthetized and a central venous catheter inserted as described previously. Rats were injected with a 5 µg (3.83 x 10^5 cpm) bolus of ^125^I-albumin (NCI Biochemicals, Mississauga, Ontario) and blood samples removed over a twenty minute period in a manner identical to that described previously (Figure 3.1). Blood samples were centrifuged and the plasma separated and stored at -70°C until analyzed.

The bolus of labelled albumin was rapidly distributed in the plasma over 0.15 min (Table 3.4). The volume of distribution corresponds to the plasma volume of a 250-300g rat (Table 3.4) \(^{170}\). After this initial phase, the concentration of labelled albumin remained constant for the duration of the study. The plasma volume determined by albumin corresponds to the \(V_A\) of orally fed rats confirming that compartment A corresponds to the plasma (Tables 4.1 and 3.4). Since the final exponent was not determined by a 20 minute observation, the metabolic clearance rate could not be calculated.

3.7. THE STABILITY OF TUMOR NECROSIS FACTOR-α

3.7.1. Denaturation of TNF

The nature of the TNF molecule predisposes it for non-covalent linkages which result in the formation of oligomers and polymers. TNF oligomers are either trimeric or dimeric in structure, although the trimeric form is favoured by most researchers \(^{12}\). Therefore, in a physiologic state, TNF is widely accepted to exist primarily as a stable trimer with a small fraction being present as monomers \(^{11}\). Denaturation of TNF reveals that it undergoes a single reversible transition between folded (native) trimer and unfolded (denatured) monomer in 3.8M urea at pH 9 and 8 °C \(^{171}\).

In order to study the stability of TNF, Petersen et al. applied freshly prepared labelled TNF to a Sephadex G-200 column \(^{19}\). The elution profile revealed that 85% of the radioactivity eluted at \(K_v = 0.52\) indicating a molecular weight (Mr) of 33 kDa, correlating to TNF dimer
Table 3.4. ALBUMIN CLEARANCE KINETICS

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>ALBUMIN GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLOPE $\beta_1$</td>
<td>4.406 ± 0.307</td>
</tr>
<tr>
<td>SLOPE $\beta_2$</td>
<td>0.016 ± 0.002</td>
</tr>
<tr>
<td>T1/2 Exp. 1 (min)</td>
<td>0.159 ± 0.01</td>
</tr>
<tr>
<td>T1/2 Exp. 2 (min)</td>
<td>44.85 ± 6.41</td>
</tr>
<tr>
<td>Vol. Dist.(ml)</td>
<td>7.509 ± 0.916</td>
</tr>
<tr>
<td>Blood Volume(ml)</td>
<td>13.880 ± 1.692</td>
</tr>
</tbody>
</table>

Vol. Dist. represents the volume of distribution. Blood volume was calculated from the volume of distribution and the hematocrit. Values expressed as mean ± SEM.
or a compact trimer. There were also peaks representing multimers at $Kav = 0$, and $Kav = 1.0$, comprising 0.8% and 7.8% respectively. Apart from a modest increase in the peaks at $Kav=0$ and $Kav=1$, similar results were obtained with stock solutions of $^{125}$I-TNF which had been stored at $-20^\circ C$ for up to 28 days. In addition, repeated freeze thawing did not have a great effect on the elution profile of TNF.

The stability of $^{125}$I-TNF during binding studies was also determined by Petersen et al. TNF was incubated at 4°C with BSA buffer prior to chromatography. This treatment resulted in significant changes to the elution profile of TNF. After 24 hours of incubation, a peak could be detected at $Kav=0.71$ (Mr 16kDa), along with a simultaneous decrease in the oligomer peak. After five days of incubation, more than 55% of the total radioactivity which initially eluted at $Kav = 0.52$ had been relocated to $Kav = 0.71$ and less than 25% eluted as oligomers. Changing the pH from 6.0 to 8.0 resulted in similar changes that were even more drastic.

### 3.7.2. Gel Filtration Chromatography of TNF

To investigate the possible denaturation of labelled TNF in vivo during our experimental period, gel filtration chromatography using Sephadex G-200 (Pharmacia, Uppsala, Sweden) was performed on serum samples collected at 1, 30, and 60 minutes post-injection of labelled TNF into an experimental animal. A 60 x 1.5 cm column was calibrated using a low molecular weight marker kit (Pharmacia) with the elution of blue dextran indicating the void volume (Appendix 3). Serum samples were applied to the column and eluted with a buffer of 50 mM sodium phosphate ($NaH_2PO_4$)/L and 1 mol sodium chloride/L (pH 7.1) at a flow rate of 4.0 mL/hour. Fractions of 1.0 ml were collected and analyzed for radioactivity.

### 3.7.3. Column Preparation

The G-200 sephadex was added to three times the volume of the bed of buffer. The gel was allowed to swell over 72 hours according to the manufacturers instructions. The gel was resuspended and allowed to settle for twenty minutes. The excess buffer was removed by suction and replaced with an equal volume of fresh buffer. The gel slurry was allowed to
reach room temperature before column packing began. The gel matrix was not degassed prior to packing the column.

The void volume of the column was determined by using the elution volume of Blue Dextran 2000. A fresh solution of Blue dextran (1 mg/ml) was dissolved in eluent buffer. The blue dextran was applied to the column followed by 2 ml of 10% sucrose buffer to aid in obtaining a well defined peak. One ml fractions were collected and the absorbance at 280 nm was read. The void volume was determined to be 14.8 ml (Appendix 3). Subsequently, the column was calibrated using the low molecular weight markers which included ovalbumin (7 mg/ml), ribonuclease A (10 mg/ml), chymostrypsinogen A (3 mg/ml) and albumin (7 mg/ml). The volume of sample applied to the column corresponded to 1% of the total bed volume (380 µl).

3.7.4. Developing a Calibration Curve

The molecular weight calibration curve defines the relationship between the elution volume of a set of standard proteins and the logarithm of their respective molecular weights. This kit uses $K_v$ as the elution parameter as it is less sensitive to errors which are introduced as a result of variations in column preparation and column dimensions and does not require the calculation of the column internal volume. The $K_v$ is calculated for each protein according to the equation:

$$K_v = \frac{Ve - Vo}{Vt - Vo}$$

Where $Ve$ = elution volume of the protein

$Vo$ = column void volume (from blue dextran)

$Vt$ = total bed volume

The $K_v$ for each protein is plotted on the linear scale against the corresponding molecular weight on the logarithmic scale using semilogarithmic paper. A straight line of best fit is drawn between the points.
3.7.5. Molecular Weight Determination of Unknown Samples

The elution volume and the $K_v$ of the unknown protein are calculated. Using the standard calibration curve, the point on the curve which corresponds to the $K_v$ value of the protein is located. The volume on the logarithmic scale which corresponds to this point is the estimated molecular weight of the protein (Figure 3.2a).

3.7.6. Results of Plasma Chromatography

The elution patterns of labelled TNF prior to injection, one. 30 and 60 minutes post-injection can be seen in Figure 3.2b. Chromatographic separation revealed 2 peaks with molecular weights of approximately 48,000 kDa and 17,000 kDa. These molecular weights are consistent with those of TNF trimer and monomer (Figure 3.2b). The elution profiles are similar, even after 60 minutes in circulation, indicating that the label remains on the TNF molecule and is not transferred to any other serum protein. In addition, these results indicate that during this experiment, TNF trimer also remains stable.

3.8. GEL ELECTROPHORESIS OF BILE AND URINE

An sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) system for small proteins was used for the separation of bile and urine in order that samples of these could be applied directly to the gel without prior concentration. The method employed was designed for the separation of low-molecular weight proteins (1-100 kDa) and was based on the method of Laemmli.

3.8.1. Gel Preparation

Stock solutions were prepared for gel electrophoresis with a 30% concentration of both monomers (acrylamide and bisacrylamide). The composition of these acrylamide mixtures and of all gels is defined by the letters T and C according to Hjerten. T denotes the total percentage of acrylamide and bisacrylamide whereas C denotes the percentage concentration of crosslinker relative to the total concentration T. There was a 2.6% concentration of crosslinker relative to the total concentration. All solutions were kept at room temperature.
Figure 3.2b CHROMATOGRAPHY OF TNF - The elution profiles of 125I-TNF in plasma collected 1, 30 and 60 minutes post-injection. Injected 125I-TNF read off right axis (Y2) all others from left axis (Y1).

Figure 3.2a STANDARD CURVE - The standard curve of the low molecular weight standards: a = Ribonuclease A, b = Chymotrypsinogen, c = Ovalbumin, d = Albumin.
except for the acrylamide:bisacrylamide mixtures, which were stored at 4°C. The 16.5% T-2.5% C gel was used as a uniform 5.5cm-long separating gel and was overlaid with a 4% T-2.6% C 1.5 cm stacking gel. Gels were polymerized by the addition of 150μl of a 10% ammonium persulfate solution and 15ul TEMED/30 ml. It is essential that the separating gel polymerizes first.

3.8.2 Sample Preparation

The bile and urine samples were incubated for 30 minutes at 40°C in a mixture of 4% SDS, 12% glycerol (w/v), 50 mM Tris. 2% mercaptoethanol (v/v) and 0.01% bromphenol blue adjusted to pH 8.9. Bromphenol blue was used as the tracking dye. The sample, 0.5-2 ug per protein band, was laid under the cathode buffer (0.1M Tris. 0.1 M Tricine (N-tris (hydroxymethyl) methylglycine). 0.1 % SDS at pH 8.25) using a microliter syringe. A solution of 0.2M Tris at pH 8.9 was used as the anode buffer. The gel was run in a vertical position electrophoresis at room temperature. All runs were started at 30 volts and when the samples had entered the stacking gel, the voltage was increased to a constant of 100V. The gels were run for approximately two hours. Low molecular weight markers for silver staining (Bio-rad. Hercules CA) were used to allow calculation of molecular weight. Labelled and unlabelled TNF were also run on the gels in order to compare the location of the label with the location of the radioactivity present in the bile or urine. The low molecular weight standards included phosphorylase B - 97.4 kDa, bovine serum albumin - 66.2 kDa, ovalbumin - 45.0 kDa, carbonic anhydrase - 31.0kDa, soybean trypsin inhibitor - 21.5 kDa and lysozyme - 14.4 kDa (Figure 3.3).

3.8.3 Fixing, Staining and Destaining

The gels were stained using a commercial silver stain kit (Bio-rad Inc., Hercules, CA). The protein bands were fixed in a solution containing 50% methanol, 10% acetic acid, 30% deionized water and 10% fixative enhancer concentrate. Gels were placed in this solution and agitated gently for 20 minutes. Following the fixation, the gels were rinsed twice with deionized water. A staining solution was prepared by mixing deionized water with silver
Figure 3.3 SDS PAGE OF BILE SAMPLES - The separation of bile samples from the three feeding groups at three hours post-TNF injection under reducing and non-reducing conditions. Lane 1 - bile from oral fed animals, lane 2 - bile from TPN fed wt.-gaining animals, lanes 3&4 bile from TPN fed wt.-losing animals. Hot TNF is found in lane 5. Low molecular weight markers (Pharmacia) are seen in lane 6. Lanes 7-10 are identical to lanes 1-4 with the addition of mercaptoethanol.

Figure 3.4 AUTORADIOGRAM OF GEL IN FIGURE 3.3 - After seven weeks of exposure, bands representing the presence of intact labelled TNF were seen in the bile of all feeding groups. Lanes match those of gel above.
complex solution, reduction moderator solution and image development reagent. Just prior to staining, a development accelerator solution was added and the entire solution was poured over the gels. The gels were allowed to stain until the bands were visible at a desired intensity (approximately twenty minutes). At this time, the staining solution was removed and replaced with a 5% acetic acid solution to stop the reaction. The gels were subsequently dried using the BioGelWrap gel drying system (Biodesign Inc, New York) and then exposed to x-ray film (Kodak X AR 5, 8" x 10") at -70°C. The film was developed after 2 months of exposure.

3.8.4. Results of Gel Electrophoresis

Exposure of the x-ray film to the gel resulted in a large band representing the $^{125}$I-TNF standard. Although somewhat weak, there were definite bands of radioactivity present in the bile samples that corresponded to the labelled TNF (Figure 3.4). This labelled TNF was present in bile samples collected at one hour and at three hours post-injection of TNF. No such bands were observed with the urine, suggesting that TNF is degraded in the kidney with the subsequent release of radioactive iodine. Due to the weakness of the radioactive TNF band, it was impossible to distinguish any differences in TNF concentration in the bile between experimental groups.

3.9. CHROMIUM LABELLING OF RED BLOOD CELLS

The circulating red cell volume of man and animals has been investigated by the injection of red cells labelled with radioisotopes of iron (Fe $^{55}$ and Fe $^{59}$) and phosphorous (P$^{31}$) $^{175-177}$. However, the labelling of red blood cells with radioactive chromium can also be used to determine the circulating red cell volume. When this isotope is added to erythrocytes in vitro as Na$_2$Cr$^{51}$O$_4$, it is taken up avidly by the red cells which were found to retain their radioactivity without significant loss to the plasma for periods of one day or more after injection into experimental animals $^{178}$. This method has been tested in humans and found to be simple and accurate. Measurements of red cell volume ten minutes after injection into 12 human volunteers agreed (±3-5%) with red cell volume determined 24 hours later $^{179}$. The
accuracy of the Cr\textsuperscript{51} method, as verified by hemorrhage and transfusion experiments with measured volumes of blood, was within 3-5%, which compares favourably with other isotope dilution methods\textsuperscript{179,175,176}. In addition, Hall et al. have demonstrated that Cr\textsuperscript{51} labelling of red blood cells in rats provides a useful method to approximate blood volume and erythrocyte survival\textsuperscript{180}.

### 3.9.1. Labelling Protocol

One day prior to experimentation, 3 male wistar rats were sacrificed and their blood collected thorough a central venous line for chromium labelling. Blood samples were spun and the plasma separated from the red blood cells. Radioactive chromium (Amersham, Ontario, specific activity 250-500mCi/mg Cr), Cr\textsuperscript{51} as Na\textsubscript{2}Cr\textsuperscript{51}O\textsubscript{4} in sterile saline was added and samples were incubated for one hour at 37°C. Within the red cell, the hexavalent Cr\textsuperscript{51}O\textsubscript{4}\textsuperscript{2-} is reduced to the trivalent \textsuperscript{51}Cr\textsuperscript{3+} which then binds to the hemoglobin molecule\textsuperscript{178}. After the incubation period, cells are washed twice with cold normal saline followed by 10nM ascorbic acid (in saline), plasma and finally twice with saline in order to remove any non-bound Cr\textsuperscript{51}. Ascorbic acid reduces extracellular Cr\textsuperscript{6+} to Cr\textsuperscript{3+} which can bind to plasma proteins. Therefore, the sequential ascorbic acid/plasma washes facilitate the removal of unbound Cr and prevent further labelling. A stock solution of labelled chromium and 9% sterile sodium chloride (500 µl) was prepared and a sample (25µl) was counted to determine the concentration of radioactive Cr\textsuperscript{51}. A concentration of approximately 1.0x10\textsuperscript{5} cpm was desired. Subsequently, the stock solution was added to the required volume of red blood cells (depending on the number of experimental animals) in order to attain a concentration of approximately 1.2 x 10\textsuperscript{3} cpm in 200 µl of red blood cells. A standard of red blood cells from each batch of animals was used as a standard.

Cr\textsuperscript{51} has a half life of 26.55 days and emits a \(\gamma\)-ray of 4.92 Kev which is much higher of that of \textsuperscript{125}Iodine. Since the primary gamma energy ray of Cr\textsuperscript{51} attains a lower energy state by deflecting off neighbouring molecules, the final energy profile consists not only of the primary energy band but also of several energy states below, which overlap with that of \textsuperscript{125}I-TNF. This phenomenon is referred to as the "Compton Scatter"\textsuperscript{181}. To correct for this
radioactivity that was counted in the iodine channel was used to correct the counts for the plasma and organ samples. The window was set on the dual gamma counter in order to maximize the recovery of iodine and chromium and resulted in approximately 10% backscatter from chromium to iodine channels.

3.9.2. Calculation of Blood Volume

On the day of experimentation, rats were injected with 200-300μl of labelled red blood cells (amount adjusted in order to attain desired activity) through the central venous catheter. The blood was allowed to distribute for ten minutes, which has been shown by Sterling and Gray to be an adequate amount of time to achieve equilibration. Ten minutes post-injection, 300μl of blood was drawn into a heparinized syringe for measurement of radioactivity and to calculate the hematocrit. A 100μl sample of whole blood was precipitated and counted according to the methods described previously for plasma. A standard of labelled blood was counted separately to provide the injected dose. The dilution of the chromium dose allowed the calculation of the blood volume. In order to calculate blood volume, based on only one point, we had to verify that the hematocrit remained stable throughout the experimental period. This is described in the following section.

3.10. HEMATOCRIT STABILITY

In order to determine whether the hematocrit and therefore the packed red cell volume remained stable over the time course of the experiment, hematocrits were collected from control rats over a period of three hours. It is essential that the volume of red cells remains relatively stable over the time course of the experiment. If the hematocrit is relatively stable then one blood collection for the determination of the hematocrit would be acceptable otherwise blood would have to be taken at several times.

3.10.1. Hematocrit Collection Protocol

Control rats (4) underwent surgical implantation of central venous catheters as described previously. Blood samples were collected over one hour (2 rats) and up until three hours (2
rats) for hematocrit determination. The blood was drawn into heparinized syringes and placed into labelled eppendorf tubes. Hematocrits were determined using heparinized capillary tubes sealed with plasticine. The hematocrit tubes were spun at 2000 rpm for seven minutes and the percent of the total blood volume as red blood cells and plasma was determined (red blood cells compromised 45-60% of the total blood volume depending on the animal). The times of blood collection were recorded by a computerized stop watch program. All packed cell volumes were normalized to the initial value and regressed against time in order to determine if there were any significant changes over time. The results are shown in Figure 3.5. The regressions of the normalized packed cell volumes against time were not statistically significant. Accordingly, we are able to use one hematocrit value at 10 minutes post injection to adequately reflect the hematocrit and therefore blood volume of the rat.

3.11. TNF ELISA

In order to clarify whether circulating levels of TNF-α were different among the three groups of experimental animals prior to the experiment, plasma samples were analyzed for rat TNF. Since we had injected human recombinant TNF, this ELISA would provide some indication of the quantity of endogenous rat TNF produced in response to the experimental protocol.

3.11.1 TNF ELISA Protocol

Rat TNF-α was measured using a commercially available kit. (Cytoscreen, Biosource International). This ELISA will recognize both natural rat TNF-α and recombinant rat TNF-α. This assay is a solid-phase sandwich enzyme-linked immuno-sorbent assay (ELISA). An antibody specific for TNF-α has been coated onto the wells of a microtiter plate. The TNF-α present in the sample or standard binds during the first incubation to the immobilized (capture) antibody on the one site and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a
Figure 3.5  HEMATOCRIT TEST - Hematocrits were measured in four control rats over a three hour period in order to ensure that the hematocrit of experimental animals remained relatively stable throughout the experimental period.
second incubation and washing to remove all of the unbound enzyme, a substrate solution is added which reacts with the bound enzyme to produce colour. The intensity of this color product is directly proportional to the concentration of TNF-α present in the original specimen. The standards are prepared from 15.6 pg/ml to 1000 pg/ml. All samples were run in duplicate. The sensitivity of this assay was determined by adding two standard deviations to the mean optical density obtained when the zero standard was assayed 30 times. The minimum detectable dose of TNF-α is greater than 4 pg/ml. Cross reactivity was observed with mouse TNF-α (100%) and human TNF-α (0.15%), however no other cross reactivities were noted for any other rat, human or mouse cytokine. The precision of this assay was also determined and the coefficient of variation between samples was 2.6%.

3.11.2. TNF ELISA Results

Samples from 28 TPN-fed weight-losing animals, 23 weight-gaining TPN-fed animals and 27 control animals were analyzed for rat TNF. The mean levels of circulating rat TNF were found to be 324 ± 43 pg/ml in weight-losing TPN fed animals, 327 ± 56 in weight-gaining TPN-fed animals and 197 ± 49 pg/ml in control animals. Four animals (1 weight-losing, 2 weight-gaining, 1 control) had values that lay outside the standard curve calculations and were excluded from the final analysis. The distribution of plasma TNF levels is shown in Figure 3.6. Analysis of variance indicated no statistically significant differences between groups { F (2.70) = 2.36, NS}.

Circulating TNF levels were not significantly different between groups prior to the experiment and therefore would not explain differences in clearance and organ uptake of TNF between groups.

3.12. STATISTICAL ANALYSIS

Groups of rats were compared using analysis of variance (ANOVA). Comparisons between specific groups were done using the Tukey-Kramer test for unplanned comparisons. All results are expressed as the mean plus or minus the standard error of the mean. The level
Figure 3.6  THE LEVEL OF CIRCULATING RAT TNF - The level of circulating rat TNF was measured in the experimental animals using a commercially available ELISA kit for rat TNF. The mean TNF concentration was 323.9 pg/ml in TPN fed wt. losing rats, 327.4 pg/ml in TPN fed wt. gaining rats and 196.5 pg/ml in orally fed animals.
of significance was set at $p<0.05$ for the comparison of groups. Data for the fractional clearance of TNF were analyzed using the Wilcoxon Test for non-parametric data.
CHAPTER 4.0 INITIAL STUDY OF TUMOR NECROSIS FACTOR CLEARANCE IN PARENTERALLY AND ENTERALLY FED RATS

4.1 INTRODUCTION

Cachectin/tumor necrosis factor (TNF) is a 17kDa polypeptide which, when infused continuously into rats, will result in a metabolic response similar to that observed with human sepsis. In addition, cachectic patients with heart failure and septic patients have raised circulating TNF levels. Previous studies in rats have shown that a continuous infusion of TNF results in anorexia and weight loss, however, these malnourished animals have minimal increases in plasma glucose and blood urea nitrogen. When malnutrition due to anorexia was prevented by giving total parenteral nutrition (TPN) the same dose of TNF resulted in marked hyperglycemia, azotemia and signs of liver injury. Moreover, plasma TNF levels were highest in the parenterally fed animals with the most severe metabolic abnormalities. These findings suggest that nutrition support sufficient to prevent cachexia in TNF infused animals may influence TNF clearance.

We therefore hypothesize that both the route by which nutrition support is provided as well as the nutrient density of that support will affect the clearance kinetics of a bolus of radiolabeled TNF. Specifically, we hypothesize that animals receiving nutritional support sufficient to allow weight-gain will clear TNF more slowly than those animals receiving 50% of the optimal nutrients needed for growth. We believe that the anorexia observed previously in septic animals may be a protective mechanism and that the results of these studies will assist in the development of appropriate feeding protocols to be used in the clinical setting where elevated levels of TNF may be suspected.

4.2 MATERIALS AND METHODS

4.2.1 Animal Preparation

Male Wistar rats (Charles River Canada, Inc., Quebec, Canada) were individually housed in cages at a temperature of 22°C maintained on a 12 hour light-dark cycle. Rats on entry to the animal facility weighed between 200-220g. All animals were maintained on a diet of
laboratory chow ad libitum until they achieved a weight of 240-260g. Under general anesthesia (sodium pentobarbital, 50mg/kg intraperitoneal (i.p.)) a silastic catheter (0.037 inch OD, Dow Corning, Midland MI) was placed into the right jugular vein and advanced to the superior vena cava using the sterile technique of Popp and Brennan. The external portion of the catheter was tunneled subcutaneously to the interscapular region of the back and led externally through a protective wire spring and secured to the rat by a stainless steel button (Instech Labs, Horsham PA). The entire procedure was performed in a laminar flow hood and lasted 40-50 minutes. Post-operatively, the rats were housed in plastic metabolic cages (Nalgen, Sevenoaks, Kent England) and maintained on a specially formulated liquid diet (Tables 3.1-3.3) given orally ad libitum during this recovery phase. Animals also had free access to water during the entire experimental period. Patency of the catheter was maintained by a daily injection of 0.5 ml of heparinized saline (sodium heparin, 10 U/ml) into the catheter.

4.2.2. Cachectin/TNF

Recombinant human cachectin/TNF was the generous gift of the Chiron Corporation, Emeryville, CA. The endotoxin content was <25ng lipopolysaccharide/mg cachectin (according to the product specifications). The TNF was diluted according to the manufacturer's instructions and stored in 2 ml aliquots each containing 25 μg of TNF at -70°C until use.

4.2.3. Diet Formulation

All animals were provided with a specially formulated liquid diet either orally or parenterally. The composition of the diet is shown in Tables 3.1-3.3. The nutrients infused met the nutritional requirements for rats and previous trials had demonstrated that this diet allowed control rats to grow at the same rate as chow fed animals. TPN weight-gaining animals received on average 79.0 kcal/day whereas weight-losing animals received approximately one half of that intake (37 kcal/day). Animals orally fed ad libitum received on average 79 kcal/day.
4.3. EXPERIMENTAL PROTOCOL

The surgical protocol for central venous catheter insertion is described in section 3.2. On day 0 of experimentation, the catheter and spring were connected to a swivel device (Instech Labs, Horsham PA) which allowed movement of the animal about the cage. The swivel device was suspended above the metabolic cage by a metal rod. Rats were randomly assigned to one of two experimental groups: i) maintained on oral feeding ii) fed the identical liquid diet via the central venous catheter (TPN). The TPN solution was aseptically prepared every day, placed in 60cc syringes (Beckton-Dickinson. Rutherford, N.J.) and continuously infused by an infusion pump at a rate of 2.5 cc/hour (Pump 22: Harvard Apparatus, Wellesley, MA). Orally-fed control animals received 0.9% saline at the same rate. Details of the composition of the liquid diet are found in Tables 3.1-3.3.

The group receiving total parenteral nutrition was subdivided into two sub-groups: those receiving optimal nutrient intake to promote growth (weight-gaining rats, WGR) and those receiving 50% of the optimal nutrient intake (weight-losing rats, WLR).

On day 4 of experimentation, rats were anaesthetized with an injection of 25mg/kg sodium pentobarbital i.p. A bolus of 0.5μg TNF (4.2.2) + 0.3μCi of $^{125}$I-cachectin ($[^{125}\text{I} \text{iodotyrosyl tumor necrosis factor-\alpha. Amersham Canada Inc.,Oakville,Ontario, specific activity 800 Ci/mmol}$) adjusted to a specific radioactivity of 15.0μg/μCi was injected via the central venous catheter. Catheters were pre-injected with 0.5 ml of 2% bovine serum albumin to coat the lumen and prevent adherence of the radiolabeled material. In separate experiments, it was shown that catheters treated in this way did not have any significant residual radioactivity following injection of the labelled TNF as described above. Blood samples were withdrawn from the central venous catheter into heparinized syringes over a twenty minute period at exact timed intervals noted in a computer stopwatch program. Over the first few minutes, the plasma radioactivity fell sharply, and after about two minutes, fell at a monoexponential rate. Twenty minutes of observation therefore allowed sufficient time for the pattern of clearance to be determined. For each experimental group, a duplicate aliquot of the injected dose was kept as a standard. At each time point, 0.3 ml of blood was taken and the catheter flushed with 0.2 ml of saline. The blood was immediately centrifuged and the plasma separated and
stored at -70°C until analyzed. Preliminary results had indicated that negligible amounts of radioactivity were present in the red blood cells which was expected as red blood cells lack TNF receptors. At the end of the experiment (20 minutes post-injection) the animals were sacrificed and the heart, liver, kidneys and spleen were removed and weighed. Organ samples were taken for analysis of radioactivity as well as for determination of dry weight (described in section 3.4.3).

Organ counts per minute were corrected for background radioactivity as well as for the time difference between plasma measurements and organ measurements. This was done using information on the rate of decay of the radioisotope. Finally, organ data were corrected for mean plasma activity and expressed as counts per gram of dry weight per minute. Mean plasma activity was calculated by integrating the area under the curve from t(0) to t(20) minutes and dividing by 20.

The experimental protocol and procedures were approved by the Animal Care Committee of the University of Toronto.

4.4. MEASUREMENT OF RADIOACTIVITY IN SAMPLES

4.4.1. Plasma

A sample of 50 μl of plasma was added to 0.5 ml 2% bovine serum albumin in 12 x 75 disposable glass culture tubes. The protein was precipitated and washed with 1.5 ml of 15% trichloroacetic acid (TCA) to remove any radioactivity due to free iodide. Preliminary studies indicated that 90% of the plasma activity after injection was precipitated indicating that most of the plasma radioactivity was not free iodide. The precipitate was dissolved in 1.5 ml of 1 N NaOH and counted for radioactivity. A duplicate aliquot of the standard was diluted to 1.5 ml with saline and counted in a well-type sodium-iodide crystal connected to a pulse height analyzer and scalar. In separate studies we noted that a volume of 1.5 ml gave maximum efficiency and all samples were made up to 1.5 ml to ensure uniform geometry. Three blank tubes were counted to allow the calculation of and the correction for background radioactivity.

The curve of the plasma disappearance of precipitated 125I-TNF or albumin could be best fitted statistically by a two exponential curve using a non-linear least squares method.
(Figures 4.1-4.3) with the equation $y = c_1e^{\beta_1t} + c_2e^{\beta_2t}$. Where $y =$ specific activity in counts per minute. Single and three exponential fits gave statistically worse fits.

4.4.2. Organs

Organ samples were homogenized with normal saline, precipitated by centrifugation and washed twice with 1.0 ml 15% TCA to remove excess blood and free iodide. Samples were dissolved in 1.5 ml 2N NaOH for 24 hours and counted for radioactivity as described above.

Organ counts per minute were corrected for background radioactivity as well as for the time difference between plasma measurements and organ measurements. This was done using information on the rate of decay of the radioisotope. Finally, organ data were corrected for the mean plasma activity and expressed as counts per gram dry weight per minute. Mean plasma activity was calculated by integrating the area under the curve from $t(0)$ to $t(20)$ minutes and dividing by 20.

4.5. GEL FILTRATION OF PLASMA AND $^{125}$I-TNF

To investigate the possible dissociation of labelled TNF, gel filtration chromatography using Sephadex G-200(Pharmacia, Uppsala Sweden) was performed on serum samples collected at 1.30 and 60 minutes post-injection of labelled TNF into an experimental rat. The column was calibrated using a low molecular weight marker kit (Pharmacia) with the elution of blue dextran indicating the void volume. Serum samples were applied to the column and eluted with a buffer of 50mM sodium phosphate (NaH$_2$PO$_4$) and 1mM sodium chloride (pH 7.1) at a flow rate of 4.0 ml/hour. Fractions of 1 ml were collected and analyzed for radioactivity.

4.6. ANALYSIS OF CLEARANCE DATA

Analysis of the data from the biexponential fits of the TNF clearance data was done in two unique ways, namely compartmental analysis and linear systems analysis. Linear systems analysis does not make any assumptions based on compartmental models. These different methods both gave congruent results helping to support the overall conclusion.
Figure 4.1 TNF CLEARANCE IN TPN FED WT. - GAIN ANIMALS -
The plasma clearance of a bolus of 0.3uCi TNF + 0.5ug unlabelled TNF in TPN fed weight-gaining rats.
Figure 4.2 TNF CLEARANCE IN TPN FED WT. - LOSE ANIMALS - The plasma clearance of a bolus of 0.3 μCi 125I-TNF + 0.5 μg unlabelled TNF in TPN fed weight-losing animals.
Figure 4.3  TNF CLEARANCE IN ORALLY FED ANIMALS -
The plasma clearance of a bolus of 0.3uCi 125I-TNF +
0.5ug unlabelled TNF in orally fed animals.
4.6.1. **Compartmental Modelling**

The clearance of tumor necrosis factor is best described as a biexponential function, which is similar to the clearance of other plasma proteins. Therefore, we suggest a two compartmental model (Figure 4.4), which would give rise to clearance data that could be fitted by the sum of two exponential functions. In this model, compartment A is the compartment containing the plasma while compartment B is extravascular. Irreversible loss occurs by organ uptake and excretion of radioactivity.

The $K$'s are rate constants, bearing the dimensions of $[t^{-1}]$. $K_{BA}$ is the coefficient governing the flow of cachectin from compartment A to compartment B. $K_{AB}$ governs the flow from B to A. $K_{OA}$ governs the rate of irreversible loss of TNF from compartment A. By curve-fitting a biexponential function of the form

$$C_A(t) = C_1 e^{\beta_1 t} + C_2 e^{\beta_2 t}$$

(4.1)

to the clearance data, where $C_A$ is the concentration of $^{125}$I-TNF in compartment A, we obtain values for the parameters $C_1$, $C_2$, $\beta_1$, and $\beta_2$. Using these parameter values, we can calculate the rate constants $K_{AB}$, $K_{BA}$, and $K_{OA}$. Details concerning the derivation of the these rate constants can be found in Appendix 1. Averaged values for the rate constants for weight-gaining (WGR) and weight-losing parenterally fed animals (WLR) as well as the orally fed animals (OFR) are given in Table 4.1. If we consider the system to be near equilibrium during the second exponential phase, then the ratio of these rate constants ($K_{BA}/K_{AB}$, BARATIO) reflects the ratio of concentration of TNF in compartment B relative to A (Appendix 2).

The total volume of distribution may be estimated in a compartment dependent model from the ordinate intercept of the second exponential equation:

$$V = \frac{D}{C_2}$$

(4.2)

where $D$ [cpm] represents the injected dose of $^{125}$I-TNF. This estimation of volume is acceptable when spatial gradients in TNF at steady state are small. The volume of
Figure 4.4 COMPARTMENTAL MODELS - These models represent the flux of tracer, in each experimental group, between the plasma associated compartment (A) and the non-plasma compartment (B) with the irreversible loss of tracer through compartment A. The rate constant KBA represents the fractional change in the concentration of TNF in A destined for B while KBA represents the fraction of the concentration of TNF in B destined for A.
Table 4.1. Compartmental Analysis of the TNF Clearance Kinetics

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>TPN WT. GAIN</th>
<th>TPN WT. LOSE</th>
<th>ORAL CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{AB}$</td>
<td>0.50 ± 0.097</td>
<td>0.59 ± 0.060</td>
<td>0.47 ± 0.054</td>
</tr>
<tr>
<td>$K_{BA}$</td>
<td>0.30 ± 0.059</td>
<td>0.99 ± 0.23 †</td>
<td>0.68 ± 0.28</td>
</tr>
<tr>
<td>$K_{OA}$</td>
<td>0.040 ± 0.007§‖</td>
<td>0.083 ± 0.018</td>
<td>0.091 ± 0.036</td>
</tr>
<tr>
<td>BARATIO</td>
<td>0.72 ± 0.14</td>
<td>2.09 ± 0.061</td>
<td>1.89 ± 1.06</td>
</tr>
<tr>
<td>VOLUME TVD(ml)</td>
<td>20.77 ± 1.43 §</td>
<td>16.29 ± 0.90</td>
<td>15.13 ± 0.53</td>
</tr>
<tr>
<td>$V_A$ (ml)</td>
<td>12.57 ± 0.98§‖</td>
<td>6.97 ± 0.92</td>
<td>8.18 ± 1.52</td>
</tr>
<tr>
<td>$V_B$ (ml)</td>
<td>9.26 ± 1.46</td>
<td>10.44 ± 1.43</td>
<td>8.12 ± 1.45</td>
</tr>
<tr>
<td>$MCR'$ (ml/min)</td>
<td>0.50 ± 0.056</td>
<td>0.44 ± 0.036</td>
<td>0.46 ± 0.020</td>
</tr>
<tr>
<td>$FCR'$ (min⁻¹)</td>
<td>0.040 ± 0.007§‖</td>
<td>0.080 ± 0.018</td>
<td>0.091 ± 0.036</td>
</tr>
</tbody>
</table>

Values expressed as Mean ± SEM.

† Significantly different from WEIGHT GAINERS P<0.05.
§ Significantly different than WEIGHT-LOSERS P<0.01
‖ Significantly different than ORAL CONTROLS P<0.05
BARATIO is the ratio of the rate constants $K_{BA}/K_{AB}$.
compartment A can be estimated by dividing the injected dose $D$ by the combined ordinate intercepts for both exponential equations. The volume of compartment B can then be calculated by subtraction of the volume of compartment A from the total volume of distribution ($V_A, V_B$, Table 4.1). In addition, the ratio of $V_A/V_B$ may be used with the BARATIO to estimate the relative mass of TNF in compartments A and B (Appendix 2).

The fractional clearance rate of TNF ($FCR'$) can be determined using this compartmental model. In the steady state, the $FCR'$ is an estimate of the rate of irreversible loss of TNF from compartment A ($K_{OA}$, Appendix 1) and can be estimated using the equation:

$$FCR' = \frac{\beta_1 \beta_2}{A_1 \beta_2 A_2 \beta_1}$$  \hspace{1cm} \text{[time}^{-1}] \hspace{1cm} (4.3)$$

where $A_1 = C_1/C_1 + C_2$ and $A_2 = C_2/C_1 + C_2$.

Finally, a metabolic clearance rate ($MCR'$) of TNF can be determined using this compartmental model. The metabolic TNF clearance rate can be interpreted as the flow out of compartment A (Appendix 1) and can be estimated using the equation:

$$MCR' = K_{OA} \times V_A$$  \hspace{1cm} \text{[vol*time}^{-1}] \hspace{1cm} (4.4)$$

4.6.2. Linear Systems Analysis

The clearance of $^{125}$I-TNF can be calculated solely from a linear systems analysis of the time-invariant, labelled-TNF system, without explicit reference to any compartmental model. The metabolic clearance rate, $MCR'$. of TNF is defined by:

$$MCR' = \frac{D}{\int_0^\infty C_\alpha' dt}$$  \hspace{1cm} \text{[vol*time}^{-1}] \hspace{1cm} (4.5)$$
Where \( D \) [cpm] represents the injected dose of \(^{125}\text{I}-\text{TNF}\). The integral in the denominator represents the area under the clearance curve. It may be measured by any technique, but is conveniently estimated from the biexponential function (4.1). Thus

\[
MCR = \frac{D}{\frac{1}{\beta_1} + \frac{1}{\beta_2}} \tag{4.6}
\]

\( MCR \) and \( MCR' \), while not identical, reflect the volume of the distribution of TNF cleared per unit time. The total volume of distribution (TVD) can be calculated with no assumptions regarding compartments using the equation:

\[
TVD = \frac{D \int_0^\infty C_A \, dt}{(\int_0^\infty C_A \, dt)^2} \text{ [vol]} \tag{4.7}
\]

The mean residence time, \( t \), may be estimated from the expression:

\[
t = \frac{\int_0^\infty C_A \, dt}{\int_0^\infty C_A \, dt} \tag{4.8}
\]

By dividing \( MCR \) by \( TVD \), we obtain a measure of fractional clearance per unit time, \( FCR \). The results of these calculations are found in Table 4.2.

\[
FCR = \frac{MCR}{TVD} = \frac{1}{t} \text{ [time}^{-1}] \tag{4.9}
\]

\( FCR \) is not expected to be equal to \( FCR' \) as calculated by compartmental analysis. It can be shown that:
Table 4.2 Clearance Kinetics of Orally and Parenterally Fed Animals Using Linear Systems Analysis (LSA)

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>TPN WT. GAIN</th>
<th>TPN WT. LOSE</th>
<th>ORAL CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLOPE $\beta_1$</td>
<td>0.81 ± 0.13 ‡</td>
<td>1.63 ± 0.24</td>
<td>1.21 ± 0.30</td>
</tr>
<tr>
<td>SLOPE $\beta_2$</td>
<td>0.023 ± 0.003</td>
<td>0.026 ± 0.002</td>
<td>0.029 ± 0.001</td>
</tr>
<tr>
<td>INT A</td>
<td>867.36 ± 114.4</td>
<td>838.04 ± 76.13</td>
<td>943.00 ± 132.82</td>
</tr>
<tr>
<td>INT B</td>
<td>616.02 ± 106.49</td>
<td>2145.53 ± 877.02</td>
<td>1605.32 ± 665.30</td>
</tr>
<tr>
<td>RAT WEIGHT (g)</td>
<td>282.3 ± 3.9 §</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{1/2}$, $\beta_1$ (min)</td>
<td>1.42 ± 0.42</td>
<td>0.50 ± 0.07</td>
<td>0.77 ± 0.14</td>
</tr>
<tr>
<td>$T_{1/2}$, $\beta_2$ (min)</td>
<td>30.41 ± 3.10</td>
<td>28.19 ± 2.05</td>
<td>23.89 ± 0.82</td>
</tr>
<tr>
<td>MCR (ml/min)</td>
<td>0.50 ± 0.056</td>
<td>0.44 ± 0.036</td>
<td>0.46 ± 0.020</td>
</tr>
<tr>
<td>FCR (min$^{-1}$)</td>
<td>0.023 ± 0.003</td>
<td>0.027 ± 0.0019</td>
<td>0.030 ± 0.001</td>
</tr>
<tr>
<td>RES. TIME (min)</td>
<td>42.86 ± 4.35</td>
<td>39.46 ± 2.84</td>
<td>33.29 ± 1.33</td>
</tr>
<tr>
<td>TVD (ml/min)</td>
<td>20.77 ± 1.43 ‡</td>
<td></td>
<td>16.29 ± 0.90</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>12</td>
<td>7</td>
</tr>
</tbody>
</table>

**FCR** represents fractional clearance rate. **RES. TIME** refers to the mean residence time of one molecule of TNF in the system. **MCR** represents the metabolic clearance rate, **TVD** represents the total volume of distribution and **N** represents the number of animals per experimental group. $T_{1/2}$ is calculated by dividing 0.692 by the slope of the exponent. Values expressed as mean ± SEM.

‡ Significantly different from WEIGHT-LOSERS $P<0.05$ § $P<0.01$

|| Significantly different from ORAL CONTROLS $P<0.05$
\[ FCR' = FCR \left[ 1 - \frac{C_2}{C_1} \right] \]

4.7. STATISTICAL ANALYSIS

Groups of rats were compared using analysis of variance (ANOVA). Comparisons between specific groups were done using the Tukey-Kramer test for unplanned comparisons. Results are expressed as the mean plus or minus the standard error of the mean. The level of significance was set at \( p < 0.05 \) for the comparison of groups. Data for the fractional clearance of TNF were analyzed using the Wilcoxon Test for non-parametric data.

4.8. RESULTS

4.8.1 Weight Change

The relative weight changes of the orally and TPN-fed rats during the experimental phase are shown in Figure 4.5. There were no significant differences in initial weights between any of the three groups \( (F(2.28)=1.25, \text{ N.S.}) \).

There were significant group differences in final body weights at the end of the experimental period \( (F(2.28)=20.06, p<0.01) \). The changes were significant between weight-gaining and weight losing TPN fed animals \( (p<0.01) \) as well as between weight-gaining TPN-fed and control animals \( (p<0.01) \).

4.8.2 Plasma Clearance of \( ^{125}\text{I}-\text{Tumor Necrosis Factor} \)

The mean slope of the rate of disappearance of labelled TNF for the first exponential phase was significantly different between study groups \( (F(2.28)=4.19, p<0.05, \text{ Figures 4.1-4.3}) \). The weight-losing parenterally fed rats had significantly steeper slopes for this exponent (Table 4.1) in comparison with their weight-gaining counterparts \( (p<0.05) \).

4.8.2.1 Compartmental Analysis

Analysis of the calculated rate constants indicated that the forward rate constant \( K_{BA} \) was significantly different between study groups.
Figure 4.5  Rat Body Weights - Mean body weights of animals over the experimental period. Day 0 indicates the mean weights of experimental animals at the initiation of enteral or parenteral feeding. Day four indicates the mean weights of experimental animals at the end of the study period. Mean body weights ± SEM.
(F(2.28)=3.68,p<0.05) with the weight-losing parenterally fed animals (WLR) having higher rates (Table 4.1) in comparison with the weight-gaining rats (WGR)(p<0.05). $K_{OA}$ and the $FCR'$ (which are numerically equal) were significantly different between study groups (Table 4.1. p<0.05). In contrast, there was no difference between groups for the reverse rate constant $K_{AB}$ (Table 4.1. F(2.28)=0.667,N.S.) and the $MCR'$ (F(2.28)=0.626, N.S.). Weight-gaining animals(WGR) had a smaller BARATIO, indicating a higher concentration of labelled TNF in compartment A relative to B (Figure 4.4)

Since there was negligible circulating TNF in our animals (normal rats), and the injected labelled TNF was of a low specific radioactivity introduced into the plasma, it is reasonable to assume that the labelled material was of a uniform specific activity in all compartments in which it was distributed. On the basis of the above considerations, the calculated ratio of the mass of TNF in compartments A and B indicates that WGR had a significantly larger mass of TNF in compartment A versus compartment B than either WLR or OFR (Appendix 2).

The total volume of distribution (TVD, Table 4.1) as well as estimates for the volumes of compartments A ($V_A$) and B ($V_B$) were made (Table 4.1) and these calculations indicate that WGR had a significantly larger TVD (Table 4.1) as well as a significantly larger volume of compartment A ($V_A$, Table 4.1) than either WLR or OFR.

**4.8.2.2. Linear Systems Analysis** Analysis of the data without reference to compartmental models supports the findings by compartmental analysis and indicates that WGR had a significantly larger total volume of distribution (TVD, Table 4.2) than WLR or OFR (F(2.28)=6.549,p<0.05). Fractional TNF clearance rates ($FCR$) and the metabolic clearance rates were similar for all groups (Table 4.2).

**4.8.2.3. Organ Uptake** Organ uptake of labelled TNF, expressed as a percent of total injected dose per gram of dry weight (Figure 4.6) indicates that the kidney had the highest concentration of labelled compound followed by the liver, heart and spleen. Overall, optimally fed weight-gaining TPN animals had lower organ uptakes of labelled TNF in comparison with sub-optimally fed TPN animals or control animals. Significant differences
Figure 4.6. The organ uptake of TNF: The uptake of 125I-TNF by 4 organs collected 20 minutes post-TNF injection. 125I-TNF uptake is expressed as a percent of the injected dose on a per gram dry weight basis.

+ p < 0.01 from wt losers

* p < 0.05 from orally fed rats

ORALLY FED
WT LOSERS
WT GAINERS

% OF INJECTED DOSE PER GRAM DRY WEIGHT

LIVER
HEART
KIDNEY
Spleen
between groups were noted for the kidney and heart \((F(2,28)=3.77,3.71, p<0.05)\). Weight-gaining TPN animals demonstrated significantly less uptake of labelled TNF by the heart than weight-losing TPN animals \((p<0.01)\). In addition, kidney uptake of labelled TNF was also significantly lower in weight-gaining TPN animals in comparison with the orally-fed group \((p<0.05)\).

4.8.2.4. Gel Filtration of Plasma and \(^{125}\text{I}-\text{labelled TNF}\) The elution patterns of labelled TNF before injection and 1, 30 and 60 minutes after injection can be seen in Figure 3.2. Chromatographic separation reveals two peaks with molecular weights of approximately 48,000 and 17,000 kDa. These molecular weights are consistent with those of TNF trimer and monomer respectively. The elution profiles are similar even after 60 minutes in circulation, indicating that the label remains on the TNF molecule and is not transferred to any other protein.

4.9. DISCUSSION

In this study, we have three different states of nutrition: rats growing optimally on TPN (WGR), rats losing weight on hypocaloric TPN (WLR) and orally fed rats (OFR). The WGR and WLR are similar except for protein-calorie intake and the orally fed rats are gaining weight at the same rate as WGR but are fed by a different route. In addition, the distribution of TNF was examined by two different methods; linear systems analysis and compartmental modelling. These methods produced compatible results supporting a compartmental model for TNF clearance whereby labelled TNF leaves the system via compartment A. The premise that compartment A represents the plasma-associated compartment is supported by the fact that its volume of distribution \((V_A)\) in orally-fed animals is similar to that calculated through studies with radiolabeled albumin (Table 3.4 and 4.1).

Data from the TNF clearance curves analyzed by either linear systems analysis or by compartmental modelling indicated that the total volume of distribution of TNF in WGR was significantly elevated and that this was due to an 80% increase in the plasma compartment \((V_A)\) for WGR as compared with WLR or OFR in which little change was noted. This
expansion of the volume of distribution in WGR can not be explained as being due simply to an expansion in the plasma volume as a result of the difference in body weight between WGR and WLR as this difference was only 18%. Therefore, the marked difference in $V_A$ represents an expansion of the space of distribution of TNF closely related to the plasma. The effect of anaesthetic on TNF clearance kinetics is unknown. However, both control and TPN animals received a standardized dose of anaesthetic based on body weight. In addition, the use of anaesthetic prevented differences in activity levels between groups during the experiment, which may also affect clearance kinetics, and it facilitated rapid organ collection at the end of the experiment.

Compartmental analysis further supports an expansion of the plasma associated space. In WGR there is a low BARATIO indicating a higher concentration of labelled TNF within compartment A in relation to compartment B. From this ratio, we have shown (Appendix 2) that in WGR there is an increased proportion of the mass of TNF in compartment A relative to compartment B. This is not seen in WLR or OFR which have high BARATIOS indicating low proportions of the mass of TNF in compartment A relative to B (Figure 4.4 and Appendix 2).

The expanded plasma-associated TNF compartment in WGR is associated with a significantly slower fractional clearance rate of TNF ($FCR' = K_{OA}$) but similar metabolic clearance ($MCR$ and $MCR'$) as compared with WLR and OFR. This observation is easily understood since the rate of irreversible loss of TNF is equal to: $K_{OA} * M_A = FCR' * M_A$ Thus this product, which is equal to $MCR$, can be constant when $FCR$ decreases while $M_A$ increases.

The above findings can be explained on the basis of two alternative possibilities. First, in view of the known rapid clearance of TNF, the observed increase in mass of TNF in compartment A in WGR suggests that TNF was being held in this space (Table 4.1) with unchanged $MCR'$. In this situation, the slower $FCR'$ is a reflection of unchanged absolute clearance ($MCR'$) from a larger pool. An increase in the number of soluble plasma receptors in WGR as compared with WLR or OFR provides one explanation why TNF may be bound and retained in the plasma-associated pool to a greater extent in WGR.
Circulating soluble TNF receptors have been identified in the serum and urine of healthy humans\textsuperscript{131,135}. In addition, elevated levels of these soluble receptors have been associated with a variety of clinical states including cancer, sepsis, renal failure and in systemic lupus\textsuperscript{138,162,182,183}. These soluble receptors are thought to be shed forms of the cell surface TNF receptors (TNF-R1 and TNF R2)\textsuperscript{184}. However, the mechanisms that result in the shedding of TNF receptors are as yet unknown. Injection of TNF, phorbol esters and heparin have been shown to result in increased release of TNF receptors in humans\textsuperscript{134,140,185}.

The presence of both soluble TNF receptors has been demonstrated in mice, however, in rats, only the soluble TNF-R1 (p60) receptor has been identified\textsuperscript{85,148}. In mice, the binding kinetics of human and murine TNF are similar - saturable and specific, however, the TNF-R2 is highly specific for murine TNF\textsuperscript{88,10}. In mice, the soluble TNF-R1 has a $K_d$ of 200 pM and the soluble TNF-R2 has a $K_d$ of 50pM\textsuperscript{108}. In mice, injection of lipopolysaccharide (LPS) causes a rapid shedding of soluble TNF receptors with peak levels of sTNF-R1 occurring within 30 minutes whereas sTNF-R2 peaks somewhat slower at approximately 8 hours post-injection\textsuperscript{137}. This group also demonstrated that injection of a bolus of human recombinant TNF was able to elicit the same shedding of soluble receptors as murine TNF. The possibility that the infusion of nutrients or some individual component of nutrition support might result in increased receptor shedding is of great therapeutic interest as these receptors are thought to be important modulators of TNF bioactivity. The provision of nutrients via total parenteral nutrition has previously been associated with structural and physiologic changes in the host, including intestinal mucosal atrophy, bacterial translocation and impaired host response to infection\textsuperscript{186}. Fong et al. found that the provision of TPN was associated with a magnified counter-regulatory hormone response to bacterial infection as well as enhanced systemic and splanchnic production of cytokines\textsuperscript{187}. This enhanced metabolic response to LPS seen in TPN-fed animals provides further evidence that the provision of TPN may result in physiological changes resulting in the "priming" of the animal to TNF. This "priming" effect may lead to alterations in receptor binding and/ or shedding not seen in orally-fed animals\textsuperscript{187}.

Soluble tumor necrosis factor receptors have been suggested to function as physiological inhibitors of TNF activity. Therefore, great interest has arisen regarding their potential
therapeutic use as TNF inhibitors. However, Aderka et al. have recently shown that soluble TNF receptors, both type I and type II, stabilize TNF. Within a certain range, increasing the soluble receptor concentration reduces spontaneous denaturation of the TNF, thereby prolonging its biological actions. Aderka et al. have postulated that these receptors provide a "slow release reservoir" of bioactive stable tumor necrosis factor. Our kinetic data are consistent with an increase in circulating receptor binding in weight-gaining TPN-fed animals resulting in a failure of the concentration of TNF in the circulation to decrease as would be expected if TNF was being effectively cleared from this compartment. In this situation, the enhanced biological effects we noted previously in weight-gaining TPN fed animals continuously infused with TNF would be consistent with the findings of Aderka et al. The lower tissue uptake observed in this study in WGR can be explained by postulating that the increased circulating mass of soluble TNF receptors would compete with tissue receptors for TNF binding.

The second possibility is that rather than increased binding of TNF to plasma receptors, the $FCR'$ of TNF is reduced in WGR. In this situation, since the inflow of TNF (our injection) is the same in all groups, at equilibrium, the mass of TNF in the plasma-associated compartment would be set at a level at which $MCR'$ matched the inflow. Therefore, WGR with the lowest $FCR'$ would have the highest $M_A$. One possible mechanism which could reduce $FCR'$ is binding of TNF to activated $\alpha_2$-macroglobulin. Studies have demonstrated that plasmin-activated $\alpha_2$-macroglobulin can bind and clear TNF through hepatocyte receptors. The recent isolation of a low-density-lipoprotein receptor related protein (LRP) on hepatocytes provides a possible link between nutrient infusion and TNF clearance. These studies have proposed that LRP is identical to the $\alpha_2$-macroglobulin receptor on hepatocytes although a specific role for LRP in lipoprotein metabolism has yet to be confirmed. One can speculate that activated $\alpha_2$-macroglobulin/TNF complexes may be cleared through these hepatocyte receptors thereby competing for receptor binding sites with low density lipoproteins. Provision of optimal nutrition support (increased lipid density) may enhance competition for binding, resulting in slower plasma clearance by this route and in higher circulating concentrations of TNF. These elevated levels of serum tumor necrosis factor may
be linked to the potentiation of the metabolic abnormalities seen in TNF-infused animals receiving TPN.

Recently, it has become evident that the lipid composition of the nutrition support as well as the route of delivery of nutrients may affect metabolic processes. Humans given TPN with lipid emulsions containing exclusively long-chain triglycerides had a significantly higher production of TNF from mononuclear cells in response to LPS stimulation in comparison with those who received a blend of medium and long-chain triglycerides. In addition, the degree of saturation as well as the unsaturated fatty acid composition has been shown to affect cytokine induction from peritoneal macrophages in response to LPS. These changes in cytokine production are thought to be the result of increased production of proinflammatory eicosanoids through the arachidonic acid pathway. Therefore, it is reasonable to hypothesize that modulation of TNF kinetics may be related to the lipid composition and concentration of the nutrition support. The induction and modulation of cytokine activity is complex and there may be other factors which influence the metabolism of TNF which we have not addressed in this paper.

The organ uptake data indicate that weight-gaining animals have lower organ uptakes of labelled TNF in the kidney, heart and perhaps the liver than either sub-optimally fed TPN animals or orally fed animals. These findings also support a concentrating or "pooling" of TNF in spaces other than the tissues or binding to α2-macroglobulin in WGR, resulting in lower organ concentrations of labelled material than WLR or OFR. The enhanced tissue uptake seen in the weight-losing animals appears to be mainly in the kidney. The kidney has previously been shown to be an important site of TNF catabolism. These findings are supported by evidence that the kidney participates in the clearance of many low molecular weight proteins such as insulin and growth hormone. In addition, a study of acutely nephrectomized animals demonstrated an impaired ability of these animals to clear cachectin in comparison to their sham operated controls. Bemelmans et al. have also utilized the murine model of nephrectomy in order to demonstrate the importance of the kidney in the clearance of TNF. They have utilized the specificity of the murine TNF-R2 for murine TNF to demonstrate the importance of the kidney in the clearance of soluble receptor (TNF-
R2)/TNF complexes. If the kidney uptake is indeed a prime mediator of TNF metabolism and breakdown, then the observed differences in labelled uptake of TNF support a beneficial effect of oral or sub-optimal TPN feeding over the provision of optimal nutrition support.

In conclusion, analysis of the clearance data using linear systems or compartmental analysis gave similar results and indicate that animals given total parenteral nutrition to allow weight-gain have an increased vascular compartment associated pool of TNF. The change of distribution is coupled with lower $FCR'$ and organ uptakes of TNF but unaltered $MCR$ and $MCR'$ in weight-gaining TPN fed animals. The data are consistent with the possibility that there is increased binding of TNF to circulating soluble receptors in these optimally TPN-fed animals or that there is reduced $FCR$. In turn, this state would result in improved TNF availability and prolonged biological activity. This reservoir of TNF would explain the enhanced biological activity we have observed in previous work. Additional work will be needed to determine the exact nature and implications of organ uptake as well as the role of soluble plasma receptors and the role of renal and hepatic failure in preventing TNF clearance. This is the first study to demonstrate an interaction of nutrition support with TNF clearance kinetics. It may have important implications on the type of nutrition support provided to patients with sepsis or heart failure because our data suggests that clinical states with increased circulating TNF levels may be adversely affected by currently available nutritional practices.
CHAPTER 5.0 TISSUE DISTRIBUTION AND CLEARANCE OF $^{125}$I-TUMOR NECROSIS FACTOR IN ENTERALLY AND PARENTERALLY FED RATS

5.1 INTRODUCTION

In previous studies, TNF infused animals provided with TPN in amounts sufficient to allow weight-gain showed potentiation of the metabolic abnormalities characteristic of sepsis with increased mortality and morbidity in comparison with TNF infused anorexic orally fed animals. Since circulating levels of TNF were highest in the TPN fed animals with the most severe metabolic abnormalities, it was hypothesized that the provision of nutrients via TPN might affect the clearance of TNF. Impaired clearance of TNF would result in higher circulating levels of TNF and would possibly explain the potentiation of the metabolic effects of TNF previously observed when nutrients were provided by TPN. Our initial work, described in chapter four, focussed on the plasma clearance kinetics of labelled TNF in animals provided with enteral or parenteral nutrition support. In these studies, we have also included the effect of nutritional status by providing animals with either sufficient nutrients to allow growth (wt.-gain) or with only 50% of the nutrients required for growth (wt.-lose) resulting in weight loss.

The results of this work suggested that animals provided with TPN sufficient to allow weight-gain had a slower clearance of TNF than either weight-losing TPN fed animals or orally fed animals. We observed a retention of the TNF in the plasma associated space in TPN fed weight-gaining animals as indicated by a significantly larger volume of distribution in comparison with either TPN fed weight-losing animals or orally fed animals. Compartmental analysis of the clearance data indicated that an increased proportion of the mass of TNF was found in the plasma associated compartment in weight-gaining animals supporting a pooling of TNF in the plasma compartment. Organ uptake of labelled TNF at twenty minutes post-injection also supported a retention of TNF in the plasma in weight-gaining animals as organ uptake was generally lower in comparison with weight losing or orally fed rats. Impaired TNF clearance by organs is further supported by the finding of a lower uptake of TNF by the kidney, in TPN weight-gaining rats. The kidney is accepted to be...
one of the major organs involved in the clearance of TNF$^{108,109}$.

Enhanced binding of TNF to circulating soluble TNF receptors in weight-gain animals provides one possible explanation for the observed retention of TNF in the plasma associated compartment. TNF binding to soluble TNF receptors would prevent circulating TNF from binding to cell surface receptors thereby resulting in less organ uptake and degradation. In addition, retention of TNF in the plasma compartment could prolong the presence of potentially bioactive TNF, which upon release, could be responsible for the potentiation of the metabolic abnormalities previously observed in TPN fed animals.

Our initial study focussed on the plasma clearance of TNF with organ uptake being examined at only one time point, 20 minutes post-TNF injection. One of the limitations of this study was our inability to accurately assess the blood volume of the rats. We had to use an indirect measure of blood volume which was determined from our albumen experiments. Knowledge of the blood volume is important when discussing the volume of distribution because these two measures are often mistaken to be the same. However, in these studies, the volume of distribution is greater than the blood volume as it represents all binding sites for TNF. A second limitation was that the organ data represented only a snapshot of the entire organ throughput of TNF and information regarding the true uptake of labelled TNF was not attainable from this study. Therefore, an in-depth organ study was designed to complement the plasma clearance data as well as to determine the true uptake of each organ over time. The dilution of chromium labelled red blood cells allowed not only the calculation of blood volume but also the correction of tissues for residual blood content, thereby making this a technically more advanced and accurate study. This study represents an extension of our first study and was designed to investigate the potential differences in tissue uptake and degradation of TNF in enterally and parenterally fed animals in an attempt to explain the previously observed differences in TNF clearance kinetics.

5.2 OVERALL HYPOTHESIS:

Nutritional support, given as total parenteral nutrition, can affect the distribution, uptake, and degradation of TNF over time.
5.2.1. Specific Hypotheses:
1) The tissue distribution and uptake of labelled TNF will follow different patterns over time in weight-gaining and weight-losing TPN fed animals, with weight losing and orally fed animals showing improved uptake by tissues primarily involved in TNF clearance.

2) The rate of clearance of a bolus of tumor necrosis factor will be affected positively by a decrease in the energy density of the nutrition support provided, as observed previously.

5.3 OBJECTIVES
1. To determine the rate of uptake of $^{125}$I-TNF-α in specific organs of enterally and parenterally fed rats.
2. To determine the effects of adequate vs inadequate (in regard to weight gain) nutrition support on the distribution and rate of $^{125}$I-TNF-α uptake.
3. To use compartmental modelling to compare the complete distribution of TNF-α over time in weight gaining and weight losing TPN fed animals in comparison with orally fed animals.

5.4. EXPERIMENTAL METHODS

5.4.1. Study Protocol

The animal preparation procedures as well as the surgical protocol for central venous catheter insertion are found in section 3.0. In addition, all animals received the same specially formulated liquid diet as found in Tables 3.1-3.3 which was provided orally during the recovery phase.

On day 0 of experimentation the catheter and spring were connected to a swivel device (Instech Labs. Horsham PA) which allowed movement of the animal about the cage. The swivel device was suspended above the metabolic cage by a metal rod. Rats were randomly assigned to one of three experimental groups: i) maintained on oral feeding ii) fed via central venous catheter sufficient nutrients to allow growth (weight -gain) or iii) fed via central venous catheter 50% of the nutrients of the weight gain animals (weight-lose). The TPN
solution was aseptically prepared every day, placed in 60cc syringes (Becton-Dickinson, Rutherford, N.J.) and continuously infused by an infusion pump at a rate of 2.5 cc/hour (pump 22: Harvard Apparatus, Wellesley, MA).

After five days of nutrient infusion, rats were anaesthetized with an injection of 25 mg/kg sodium pentobarbital i.p. A bolus of 100 mg of sodium iodide was given to block uptake of radioactive iodine by the thyroid. Following this, 200μl of labelled red cells were injected and allowed to distribute for ten minutes. Chromium labelled blood was prepared in advance using red cells from separate rats as described in section 3.10. A sample of 200μl of red cells was counted as a standard injected dose of chromium. A sample of 0.3 ml of blood was collected into a heparinized syringe for hematocrit determination as well as analysis of radioactive chromium in order to determine blood volume. These procedures are outlined in section 3.9.

The abdomen was subsequently opened and the bile duct and the bladder were cannulated according to the protocols in section 3.2 for the collection of bile and urine. Catheters were pre-injected with 0.5 ml of 2% bovine serum albumin to coat the lumen of the tube thereby preventing adherence of radioactive material to the lumen of the cannula. Subsequently, a bolus of 0.3 μCi of¹²⁵I-tumor necrosis factor (¹²⁵I iodotyrosyl tumor necrosis factor-α, Amersham Canada Inc., Oakville, Ontario) and 0.5μg of unlabelled tumor necrosis factor-alpha (Upstate Biochemical Co) was injected via the central venous catheter. Blood samples were withdrawn from the central venous catheter into heparinized syringes at exact timed intervals noted in a computer stop watch program. For each experimental group, a duplicate aliquot of the injected dose was kept as a standard. At each time point 0.3ml of blood was taken and the catheter flushed with 0.2ml of 0.9% saline. The experiment was terminated at five different time points post-injection of labelled TNF and eleven tissues and organs were collected for analysis of radioactivity as outlined in section 3.4.3. Rats were sacrificed at 4, 10 30, 60 and 180 minutes post-TNF injection by an overdose of sodium pentobarbital given through the central venous catheter. Death was instantaneous, however, the chest cavity was immediately opened and the aorta severed to prevent any further blood flow to the tissues. Bile and urine that were collected over the experimental period were analyzed for
radioactivity as outlined in section 3.4.2. The results represent the average uptake of approximately 5-6 animals per feeding group at each of the five end points post-TNF injection, resulting in data being available for approximately 25 animals per feeding group. Organ uptake data were corrected for chromium backscatter into the iodine channel as described in section 3.0 and the mean of each group of animals for each time point was calculated. The results for each organ in each of the experimental groups were plotted and the total area under the uptake curve was calculated by calculating the area under the curve between each time point and subsequently adding all the calculated areas together. The variation in the areas was calculated by considering the standard deviation of all of the individual time points and calculating the area when those errors were added to or subtracted from the mean uptake.

5.4.2. Linear Systems Analysis

The equations used for the linear systems analysis of the clearance kinetics were discussed in chapter 3. However, in our previous work we had no method to evaluate the blood volume of the animals except from the volume of distribution. In these experiments, chromium labelling of red blood cells allows us to determine the approximate blood volume. Therefore, if we assume that at time 0, when the TNF is just injected, there is an almost instantaneous mixing of the labelled TNF within the circulation, then dividing the injected dose by the blood volume would provide an estimate of the concentration of label in the blood at time 0. This concentration reflects a more accurate 0 time intercept than that generated from the slope of the first exponential. Therefore, this calculated 0 intercept has been used in the calculation of the slope of the line as well as in subsequent calculations using linear systems analysis.

5.4.3. Radioactivity in the Bile and Urine

In this experiment, different animals were sacrificed at different time points post-TNF injection and the organs and muscles were collected and analyzed for radioactivity. This
process was also carried out for the bile and urine collected during each experimental period. Therefore, the data represent an amalgamation of approximately 5 rats per feeding group per time period or 25 rats total per feeding group. However, the data collected were quite variable due to the number of different rats used and the difficulty in collecting urine (Figures 5.1, 5.2). There was also a great deal of variability in the amount of urine collected which influenced these results. Therefore, a second experiment was designed to enhance the previous one. In this experiment, 8 rats (3 TPN wt. gain, 3 TPN wt. lose and 2 orally fed) were followed from time 0 to 180 minutes post-TNF injection and the bile and urine collected for discrete time intervals during the experimental period. Bile samples were collected in eppendorf tubes which were changed at the end of the time intervals used previously - 4, 10, 30, 60, 180 minutes post TNF injection. It was anticipated that collecting the bile and urine output in the same animals throughout the entire three hour experimental period would result in less variation and allow a more useful comparison between groups. The samples were analyzed for radioactivity as described in section 3.4.2. Furthermore, two additional tissues, adipose tissue and skin, were collected at the end of this three hour time point in order to determine the location of residual radioactivity.

5.4.4. Compartamental Modelling of Organ Uptake Data - A Mammillary System

The mammillary system is one type of compartmental system which is used to construct a model for the interaction of TNF with the various organs and tissues. In this type of model, there is one central compartment (the plasma) which exchanges material (TNF) with each additional compartment (Figure 2.3). The organ uptake data were used as an initial base for the calculation of the rate constants for the movement of TNF between the organ and the plasma compartment. The uptake of labelled TNF at each of the five time points, for each group of animals and for each organ was amalgamated into a clearance curve (Appendix 4). In addition, this method required the equation of the plasma disappearance. These equations were determined by curve fitting the plasma disappearance data from each of the three feeding groups using a specially written computer program. A three exponential equation was used instead of the previously used two exponential equation as it gave a better fit in the lowest
Figure 5.1  TNF IN THE BILE  - The mean loss of radioactivity into the bile in the three feeding groups over the three hour experimental period.

Figure 5.2  TNF IN THE URINE  - The mean release of radioactivity into the urine in the three feeding groups over the experimental period.
portion of the clearance curve, with a lower sum of squares of the residuals. Therefore, the general equation that governs the plasma disappearance curve is of the order:

$$M_p^* = M_1 e^{\beta_1 t} + M_2 e^{\beta_2 t} + M_3 e^{\beta_3 t}$$

(5.1a)

The value of $M_p^*$ was determined from the plasma disappearance data from time $= 0$ to 180 minutes while each subsequent $M$ represents one intercept, in this case as a percentage of the initial mass of TNF injected. In addition, $\beta_1$, $\beta_2$ and $\beta_3$ are all >0. In this method, it was assumed that when the tracer was given at $t=0$, 100% of the injected dose was present in the plasma. This results in a “ski slope” type of elimination curve with a rapid drop in TNF from 0 to 4 minutes. Therefore, in this model, the three intercepts, $M_1$, $M_2$ and $M_3$ were constrained to sum to 100. The curve fitting was done using a specially designed computer program which allowed a visual display of the various curve fits. This allowed a visual check of the generated curve to the actual data points. The best fit was determined by the least squares criterion. The next step was the calculation of the rate constants for the flow of TNF between the organs and the plasma.

5.4.4.1. Calculation of the Rate Constants For the Flow of TNF between Compartments

Calculation of the rate constants for the flow of TNF between the organs and the plasma was done by considering the interaction of each organ with the plasma within a ten compartment mammillary system (Figures 5.3-5.4). The change in the mass of TNF in the plasma compartment at any point in time reflects the sum of the masses of TNF leaving and returning to this compartment and is of the form:

$$\frac{dM_p^*}{dt} = (K_{PS} x M_S^*) + (K_{PL} x M_L^*) + (K_{PD} x M_D^*) - M_p^*(K_{SP} - K_{LP} - K_{DP})$$

(5.1b)

Therefore, the mass of TNF going to the organs or back to the plasma is determined by the product of the rate constant and the mass of TNF in the plasma or in each organ respectively. Effectively, we dealt with 10 interdependent, two-compartment models. Using the equations
Figure 5.3 A SIMPLE COMPARTMENTAL MODEL - Labelled TNF enters the plasma compartment and exchanges with various organs and tissues, such as the heart. The two rate constants represent the fraction of the mass of TNF in the plasma going to the heart (KHP) as well as being returned to the plasma from the heart (KHP).

Figure 5.4 A COMPLEX COMPARTMENTAL MODEL - Labelled TNF enters the plasma compartment and exchanges with the various organs such as the liver and kidney. In this model, labelled TNF is released into the bile or the urine. The rate constant KNL represents the fraction of the mass of TNF in the liver that is released into the bile.
of a simple two-compartment model as outlined in Appendix 1. we can see that for any organ, the heart for example, the differential equations would be of the form:

\[
\frac{dM_p^*}{dt} = \lambda_1 M_p^* + \lambda_2 M_H^*
\]  \hspace{1cm} (5.2)

\[
\frac{dM_H^*}{dt} = \lambda_3 M_p^* + \lambda_4 M_H^*
\]  \hspace{1cm} (5.3)

where \(M_p^*\) and \(M_H^*\) are the masses of TNF in the plasma and heart. The solutions of these differential equations are always given by exponential functions (Appendix 2, equation A2). The particular form of these equations that refer to the movement of TNF between the plasma and the heart are:

\[
\frac{dM_p^*}{dt} = K_{pH} M_H^* - K_{HP} M_p^*
\]  \hspace{1cm} (5.4)

\[
\frac{dM_H^*}{dt} = K_{HP} M_p^* - K_{pH} M_H^*
\]  \hspace{1cm} (5.5)

Equation 5.1b provides the mass of TNF in the plasma (\(M_p^*\)) in its explicit form, which we can use to solve for each of the ten organ masses. Using our previous example of the heart, we can re-arrange equation 5.5 to the form:

\[
\frac{dM_H^*}{dt} + K_{PH} M_H^* = K_{HP} M_p^*
\]  \hspace{1cm} (5.6)

Since \(M_p^*\) is known explicitly as a function of \(t\) (equation 5.1), we can solve equation (5.6) as a linear, ordinary differential equation. Multiplying both sides of equation (5.6) by the integrating factor \(e^{K_{PH} t}\), followed by integration and the introduction of the initial condition, \(M_H^*(0)=0\) (assumes that there is no tracer in the heart at time = 0), results in:
where $T$ is a dummy variable. By substituting in the known function $M_p^*$ from equation (5.1) into equation (5.7) we may evaluate the integral.

The right-hand side of equation (5.7) is a known function of time containing the two rate constants $K_{H^P}$ and $K_{P^H}$. The left-hand side of equation (5.7) is the quantity $M_h^*$, which has been measured experimentally. We can now curve-fit the measured $M_h^*$-data to the known function of time given by the right-hand side of equation (5.7), and by the least squares criteria determine the best values for the rate constants $K_{H^P}$ and $K_{P^H}$. This process was repeated for each of the ten compartments. The calculated rate constants allow the construction of a model of TNF flux and reflect our best estimate of the true model. Since the true model is unknown, we cannot determine the error associated with these calculated rate constants.

5.4.4.2. Determination of the Rate Constants for the Loss of TNF in the Bile and Urine

The movement of TNF in and out of the liver and the kidney is represented by three rate constants instead of the usual two as there are additional losses to the bile and the urine from these organs. Therefore, they form a slightly different compartmental model (Figure 5.4). The rate constants for the probability of movement of TNF into to the liver ($K_{l^P}$) as well as the rate constant for the probability of movement of TNF out of the liver ($K_{P^L} + K_{NL}$) were calculated as described for the other organs. A similar set of rate constants were calculated for the kidney. However, the losses of TNF from the liver and kidney were subsequently separated to allow the determination of both the probability of movement of TNF back to the plasma ($K_{P^L}$) and the loss of TNF to the bile ($K_{NL}$).

In order to calculate this additional rate constant for the loss of TNF to the bile or the urine, a two exponential fit of the kidney or liver uptake curve was done. This curve comes directly from the appearance and disappearance of labelled TNF in the organ over time. We did not use the three exponential fit used previously, as a simple two exponential fit more accurately reflected the TNF uptake by the kidney and liver without restraining it to the
plasma curve. Therefore, these calculations are independent of the plasma curve. We will represent the mass of TNF in the bile as N. The differential equation for the one way loss of TNF to the bile is defined by:

\[
\frac{dN}{dt} = K_{NL} M_L^*
\]

where \( M_L^* \) is the mass of TNF in the liver. \( N(t) \) can be defined as the rate constant multiplied by the integral of the mass of TNF in the liver from 4 to 180 minutes. Values are assigned to the rate constant and the calculated data are compared with the measured data from the bile uptake curve until the best fit is achieved. Calculation of the rate constants was done both in log space and in linear space as fits only in the linear space resulted in a weighting of the points in favour of the higher uptake points with disregard for the lower points. This occurred as the computer was trying to minimize the sum of squares. Therefore, analysis in log space resulted in a more even fit of all points with a lower sum of squares of residuals. This rate constant is subsequently subtracted from the sum of the rate constants for exiting material determined earlier to provide two separate rate constants for the loss of TNF from the liver and kidney.

5.4.4.3. Interpretation of Rate Constants We will take the heart to represent the interaction of a typical compartment with the plasma. The equation for the change in mass of TNF in the heart is governed by the equation:

\[
\frac{dM_H^*}{dt} = K_{HP} M_p^* - K_{PH} M_H^*
\]  

(5.8)

Equation 5.8 can subsequently be divided into two parts:

\[
\frac{dM_H^*}{dt} \quad \text{gain of TNF from plasma to heart} \quad \frac{dM_H^*}{dt} \quad \text{loss of TNF to plasma from heart}
\]  

(5.9)
Alternatively, this equation can be expressed using the plasma compartment:

\[
\frac{dM_H^*}{dt} = \frac{dM_p^*}{dt} - \frac{dM_h^*}{dt} = \begin{cases} 
\text{loss of TNF from plasma to heart} & \text{loss of TNF to} \\
\text{M}_p^* & \text{to plasma from heart} 
\end{cases} \quad (5.10)
\]

Now we can identify the terms in equation 5.8 with the corresponding terms in equation 5.10.

\[
\frac{dM_p^*}{dt} = \begin{cases} 
\text{loss of TNF from plasma to heart} & \text{loss of TNF from} \\
K_{HP} & \text{M}_p^* 
\end{cases} \quad (5.11)
\]

\[
\frac{dM_h^*}{dt} = \begin{cases} 
\text{loss of TNF to plasma from heart} & \text{loss of TNF to} \\
K_{PH} & \text{M}_h^* 
\end{cases} \quad (5.12)
\]

By rearranging equations 5.11 and 5.12 we can see that:

\[
\frac{\Delta M_p^*/M_p^*}{\Delta t} = \begin{cases} 
\text{fractional loss of TNF from plasma to heart} & \text{fractional loss of TNF} \\
K_{HP} & \text{to heart} 
\end{cases} \quad (5.11a)
\]

\[
\frac{\Delta M_h^*/M_h^*}{\Delta t} = \begin{cases} 
\text{fractional loss of TNF to plasma from heart} & \text{fractional loss of TNF} \\
K_{PH} & \text{to plasma} 
\end{cases} \quad (5.12a)
\]

\(K_{PH}\) is the rate constant representing the fraction of the mass of TNF leaving the heart per unit time. Since TNF does not exchange between individual organ compartments (Figure 2.3), the rate constant \(K_{PH}\) represents the only loss of TNF from the heart. Therefore, this rate constant does not require normalization. The same reasoning can be applied to all other organ compartments allowing the direct comparison of their respective rate constants, \(K_{PH}\). The liver and the kidney compartments have additional rate constants representing the fraction of the mass of TNF being lost into the urine or into the bile per unit time, however, these rate constants can be added to the rate constants for the movement of TNF back to the plasma thereby allowing a direct comparison with the other organs. The rate constant \(K_{XP}\), however, is only a part of the fraction of the mass of TNF leaving the plasma compartment to the periphery per unit time and therefore we need to normalize this rate constant in order to allow comparison between individual \(K_{XP}\) values. This normalization facilitates the comparison of
this rate constant between organs of differing sizes. The sum of all $K_{xp}$ for a given interval of
time represents the total fraction of the mass of TNF leaving from the plasma and going to the
periphery.

5.5.1 Weight Change of Animals

The relative changes in body weight during the course of the experiment can be seen in
Figure 5.5. Surgical insertion of the cannula occurred on day 0 and subsequently the rats were
allowed to recover for 5 days. During this time, all animals were provided with the liquid diet
described in chapter 3 which resulted in an average gain of approximately 3.6 g of body
weight per day. On day 5, animals were randomly allocated to receive the liquid diet by TPN
or to remain on oral feeding, at this time there were no statistically significant differences in
body weight between animals ($F(2.75)=2.38, NS$). Those allocated to receive TPN were
subsequently divided into two groups: those receiving the full nutrition support in order to
promote weight-gain or those receiving 50% of that given to weight-gain rats resulting in
weight-loss. Animals continued on enteral or parenteral feeding for an additional five days
during which time the weight-gaining rats gained approximately 3.4 g/day. Weight-losing
animals lost on average 1.4 g/day of body weight. The weight-gain rats were not started at full
strength TPN immediately, therefore some weight-loss occurred initially which has resulted in
a lower overall average weight-gain for the experimental period. Control animals gained an
average 3.0 g/day. The body weights were significantly different between groups on day 8
and day 10 of experimentation with orally fed and TPN fed weight-gaining rats having
significantly higher body weights in comparison with TPN fed weight-losing animals ($F(2.75)
=68.4, p<0.01$).

5.5.2 Plasma Clearance Kinetics of $^{125}$I-Tumor Necrosis Factor

The disappearance of $^{125}$I-TNF from the blood can be seen in Figure 5.6. The analysis of
the clearance data from the bi-exponential fits of the $^{125}$I-TNF disappearance curves was done
using linear systems analysis and supports the results of our previous study (Table 5.1). The
rate of the disappearance of labelled TNF for the first exponential was significantly different
Figure 5.5  RAT BODY WEIGHTS - The mean body weights of animals in each experimental group over the experimental period. On day 0 animals underwent surgical placement of a central venous cannula. On day 5 animals were randomized to receive either TPN or to remain on oral feeding. On day 10 the study was terminated and animals were sacrificed.
Figure 5.6 PLASMA CLEARANCE OF TNF - The plasma clearance of a bolus of 0.3 uCi 125I-TNF + 0.5 ug of unlabelled TNF in the three feeding groups over the three hour experimental period.
### Table 5.1  CLEARANCE DATA

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>TPN WT. LOSE</th>
<th>TPN WT. GAIN</th>
<th>ORAL FED</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLOPE β1 (min⁻¹)</td>
<td>1.70 ± 0.23</td>
<td>1.08 ± 0.14*</td>
<td>2.0 ± 0.22</td>
</tr>
<tr>
<td>SLOPE β2 (min⁻¹)</td>
<td>0.023 ± 0.004</td>
<td>0.023 ± 0.008</td>
<td>0.01 ± 0.004</td>
</tr>
<tr>
<td>BLOOD VOLUME (ml)</td>
<td>12.67 ± 0.47</td>
<td>13.89 ± 0.54</td>
<td>13.21 ± 0.61</td>
</tr>
<tr>
<td>VOLUME OF DISTRIBUTION (ml)</td>
<td>32.82 ± 1.27</td>
<td>38.55 ± 1.40*</td>
<td>32.39 ± 1.20</td>
</tr>
<tr>
<td>VOLUME OF COMPARTMENT A (ml)</td>
<td>8.79 ± 0.27</td>
<td>10.14 ± 0.32</td>
<td>9.49 ± 0.33</td>
</tr>
<tr>
<td>VOLUME OF COMPARTMENT B (ml)</td>
<td>24.04 ± 1.20</td>
<td>28.42 ± 1.27</td>
<td>22.89 ± 1.16+</td>
</tr>
<tr>
<td>METABOLIC CLEARANCE (ml/min)</td>
<td>0.68 ± 0.08</td>
<td>0.65 ± 0.08</td>
<td>0.56 ± 0.07</td>
</tr>
<tr>
<td>FRACTIONAL CLEARANCE</td>
<td>0.11 ± 0.06</td>
<td>0.06 ± 0.12</td>
<td>0.047 ± 0.01</td>
</tr>
<tr>
<td>MEAN RESIDENCE TIME (min)</td>
<td>6.02 ± 0.61</td>
<td>6.22 ± 0.50</td>
<td>4.85 ± 0.37</td>
</tr>
<tr>
<td>N</td>
<td>28</td>
<td>23</td>
<td>27</td>
</tr>
</tbody>
</table>

* P< 0.01 FROM ORAL FED  + P< 0.01 FROM TPN WT. GAIN  MEAN ± SEM
between groups \( F(2.75) = 5.55, p<0.01 \) with TPN fed weight-losing rats having a significantly steeper slope than weight-gaining rats. Weight-gaining orally fed rats also had a significantly larger slope of the disappearance of labelled TNF in comparison with TPN fed weight-gaining rats even though both groups gained approximately the same amount of weight. There were no significant differences between the rate of disappearance of \(^{125}\text{I}\)-TNF for the second exponential \( F(2.75)=0.89, \text{NS} \). Table 5.1.

While there were no significant differences in blood volume determined by the dilution of \( \text{Cr}^{51} \), the volume of distribution was significantly different between experimental groups \( F(2.74)=6.71, p<0.01 \). The volume of distribution (calculated from equations in section 3) was significantly larger in TPN fed weight-gaining rats than in either TPN fed weight-losing or orally fed animals. This expansion of the volume of distribution in weight-gaining animals was also seen in our first study. Differences in the volume of distribution cannot solely be attributed to the difference in body weight as orally fed animals that gained weight had a similar volume of distribution to the weight-losing animals. This difference in the volume of distribution was reflected in a significantly larger volume in the plasma associated space (volume A. Table 5.1) in weight-gaining TPN fed animals in comparison with weight-losing TPN or orally fed animals \( F(2.75)=4.57, p<0.01 \). These results support our previous findings of an expanded volume of distribution associated with the plasma compartment in weight-gaining animals and suggesting that there is a retention of TNF in this plasma associated space.

Clearance kinetics revealed no statistically significant differences in metabolic clearance rate (MCR) between groups \( F(2.75)=0.59, \text{NS} \). Although the fractional clearance rate in TPN fed weight-losing rats is numerically double that of the weight-gaining rats, the differences were not statistically significant \( F(2.75)=0.844, \text{NS} \). Finally, calculation of the mean residence time of one molecule of TNF indicated no significant differences between groups \( F(2.75)=2.05, \text{NS} \).

### 5.5.3. Organ Uptake and Distribution of \(^{125}\text{I}\)-Tumor Necrosis Factor

Organ uptake data are presented as both uptake per total organ and per gram organ
The expression of the organ uptake on a per gram dry weight basis allows a comparison to be made between organs of differing sizes and could reflect the density of TNF cell surface receptors. The overall total organ uptake of labelled TNF for all organs as well as on a per gram basis can be found in Appendix 4. In addition, the areas under the uptake curves were calculated and appear in Tables 5.2 and 5.3.

Examination of the data for the organ uptake of labelled TNF reveals two major patterns. First, there are the organs that accumulate TNF rapidly in the first 30 minutes followed by a steady loss or degradation of labelled TNF. Secondly, there are the organs that show a slow but continuous accumulation of TNF over the entire three hour period. Peripheral muscle, the carcass, the intestine and the stomach were all found to accumulate labelled TNF over time whereas the visceral organs, the diaphragm and lung had a rapid initial accumulation of TNF followed by continuous loss of labelled TNF over the rest of the experimental period. The liver and kidney were found to take up the largest percentage of the injected dose followed by the lung, spleen and intestine. Overall organ uptake accounted for 20 to 25% of the injected dose.

There were several interesting differences in TNF uptake between the experimental groups. The TPN weight-losing animals showed consistently higher uptakes (area under uptake curve) of labelled TNF in peripheral muscle as demonstrated by the uptake of TNF by the extensor digitorum longus (EDL), soleus and diaphragm than either oral fed or TPN weight-gaining rats. This difference was statistically significant for the diaphragm (Appendix 4, Figures 7-8). The spleen and the heart showed a different pattern of TNF uptake with TPN fed animals having similar uptakes of labelled TNF which were larger than the uptake of labelled TNF in orally fed animals (Appendix 4, Figures 9-12).

The stomach and the intestine continue to accumulate TNF slowly over the study period. The orally fed and TPN weight-gaining animals showed a rapid initial accumulation of TNF followed by a relative plateau phase where there was almost no change in the mass of TNF over the rest of the experimental period. This pattern is different from that of the TPN weight-losing animals which demonstrated a slow continuous accumulation of TNF by the intestine. The results are significant per gram dry weight of intestine (Appendix 4, Figures
<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>TPN WT. GAIN</th>
<th>TPN WT. LOSE</th>
<th>ORAL FED</th>
</tr>
</thead>
<tbody>
<tr>
<td>URINE</td>
<td>201 ± 22 *</td>
<td>472 ± 146</td>
<td>628 ± 116</td>
</tr>
<tr>
<td>BILE</td>
<td>583 ± 47 *</td>
<td>527 ± 11 *</td>
<td>1250 ± 171</td>
</tr>
<tr>
<td>TOTAL ORGAN</td>
<td>1162 ± 414</td>
<td>1886 ± 245</td>
<td>1631 ± 177</td>
</tr>
<tr>
<td>PER GRAM</td>
<td>1049 ± 314</td>
<td>2415 ± 298</td>
<td>2307 ± 260</td>
</tr>
<tr>
<td>ORGAN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIDNEY</td>
<td>258 ± 20</td>
<td>239 ± 29</td>
<td>317 ± 18</td>
</tr>
<tr>
<td>DIAPHRAGM</td>
<td>23 ± 2</td>
<td>36 ± 5</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>HEART</td>
<td>60 ± 11</td>
<td>58 ± 7.4</td>
<td>41 ± 3.9</td>
</tr>
<tr>
<td>INTESTINE</td>
<td>175 ± 30</td>
<td>268 ± 33</td>
<td>258 ± 53</td>
</tr>
<tr>
<td>STOMACH</td>
<td>74 ± 8.1 +</td>
<td>41 ± 5.7</td>
<td>87 ± 12 ++</td>
</tr>
<tr>
<td>LIVER</td>
<td>1064 ± 120</td>
<td>976 ± 65 *</td>
<td>718 ± 54</td>
</tr>
<tr>
<td>SLPEEN</td>
<td>87 ± 14</td>
<td>81 ± 12</td>
<td>44 ± 6</td>
</tr>
<tr>
<td>EDL</td>
<td>4.8 ± 1.3</td>
<td>9 ± 2.3</td>
<td>5.9 ± 2.2</td>
</tr>
<tr>
<td>SOLEUS</td>
<td>6 ± 1.7</td>
<td>8.3 ± 1.4</td>
<td>4.9 ± 0.7</td>
</tr>
<tr>
<td>LUNG</td>
<td>334 ± 59</td>
<td>400 ± 89</td>
<td>256 ± 31</td>
</tr>
</tbody>
</table>

* P<0.01 from ORAL FED
\( \dagger \) P<0.05 from TPN WT LOSE
++ P<0.01 from TPN WT LOSE
### Table 5.3 AREAS UNDER THE ORGAN UPTAKE AND PLASMA DISAPPEARANCE CURVES

**PER GRAM OF ORGAN (counts per 180 minutes)**

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>TPN WT. GAIN</th>
<th>TPN WT. LOSE</th>
<th>ORAL FED</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIDNEY</td>
<td>500 ± 37</td>
<td>761 ± 65 *</td>
<td>695 ± 70 *</td>
</tr>
<tr>
<td>DIAPHRAGM</td>
<td>114 ± 13 +</td>
<td>159 ± 5</td>
<td>92 ± 11 ++</td>
</tr>
<tr>
<td>HEART</td>
<td>176 ± 31</td>
<td>213 ± 17</td>
<td>146 ± 15</td>
</tr>
<tr>
<td>INTESTINE</td>
<td>91 ± 11 +</td>
<td>165 ± 20</td>
<td>105 ± 15 +</td>
</tr>
<tr>
<td>STOMACH</td>
<td>170 ± 18</td>
<td>102 ± 9.5</td>
<td>142 ± 25</td>
</tr>
<tr>
<td>LIVER</td>
<td>334 ± 32</td>
<td>358 ± 28</td>
<td>293 ± 21</td>
</tr>
<tr>
<td>SPLEEN</td>
<td>168 ± 22</td>
<td>191 ± 22</td>
<td>188 ± 24</td>
</tr>
<tr>
<td>EDL</td>
<td>152 ± 41</td>
<td>184 ± 27</td>
<td>163 ± 2</td>
</tr>
<tr>
<td>SOLEUS</td>
<td>175 ± 48</td>
<td>215 ± 33</td>
<td>130 ± 21</td>
</tr>
<tr>
<td>LUNG</td>
<td>400 ± 55</td>
<td>497 ± 70</td>
<td>625 ± 3</td>
</tr>
<tr>
<td>CARCASS</td>
<td>191 ± 2.3</td>
<td>205 ± 62</td>
<td>361 ± 89</td>
</tr>
</tbody>
</table>

* *p* < 0.01 from TPN WT GAIN  
** *p* < 0.01 from TPN WT LOSE  
++ *p* < 0.05 from TPN WT LOSE
13.14). These results are in marked contrast to the uptake of TNF by the stomach in which TPN weight-losing animals failed to accumulate TNF whereas the orally fed and the TPN weight-gaining animals showed a continuous accumulation of labelled TNF. These results reached statistical significance on a per gram dry weight basis (Appendix 4. Figures 15.16).

The liver and the kidney are widely accepted to be the main sites of TNF clearance. The differences in liver uptake of labelled TNF support increased uptake by TPN fed animals over orally fed animals (Figures 5.7-5.8). It is therefore surprising to find that the orally fed animals had significantly more radioactivity in the bile than TPN fed animals (Figures 5.9, 5.10). Autoradiography of SDS gel separated bile proteins support the presence of intact labelled TNF in the bile of all experimental groups (Figures 3.3-3.4).

The kidney uptake of labelled TNF is highest in the TPN weight losing and orally fed animals in comparison with TPN fed weight-gaining rats. The differences achieve statistical significance when examined on a per gram basis (Figures 5.11.5.12). These results are supported by the release of radioactivity in the urine (Figures 5.13). The release of radioactivity in the urine was significantly greater in the orally fed animals than in the weight-gaining TPN fed animals. The difference between the TPN fed weight-gaining and weight-losing animals did not achieve statistical significance. Autoradiography of the SDS gel electrophoresis of the urine failed to reveal the presence of any intact labelled TNF.

5.5.4 Compartmental Analysis of Rate of Uptake of Labelled TNF by Organs

A mammillary system of compartmental analysis was used to describe the interchange of TNF with the ten organs and tissues (Figure 2.3). Using this model, we calculated the rate constants for the movement of TNF from the plasma to the organ as well as from the organ back to the plasma (Table 5.4).

The rate constant $K_{xp}$ (where $x$ is any organ) reflects the fraction of the mass of TNF in the plasma going to organ $x$ per unit time whereas the rate constant $K_{px}$ reflects the fraction of the mass of TNF in organ $x$ leaving to go back to the plasma per unit time. Therefore, the $K_{xp}$ can be thought of as the pull of the organ for TNF. the larger the $K_{xp}$ the stronger the "pull" of the organ for TNF. This rate constant may primarily reflect blood flow but may also reflect
Figure 5.7 TNF UPTAKE BY THE LIVER - The mean uptake of 125I-TNF by the liver in the three feeding groups over the three hour experimental period.

Figure 5.8 TNF UPTAKE PER GRAM LIVER - The mean uptake of 125I-TNF per gram dry weight of liver in the three feeding groups over the three hour experimental period.
Figure 5.9 TNF IN THE BILE - The mean release of radioactivity into the bile in the three experimental groups over the experimental period.

Figure 5.10 TNF IN PRECIPITATED BILE - The mean release of 125I-TNF into precipitated bile in the three feeding groups over the experimental period.
Figure 5.11  TNF UPTAKE BY THE KIDNEY - The mean uptake of 125I-TNF by the kidney in the three feeding groups over the three hour experimental period.

Figure 5.12  TNF UPTAKE PER GRAM KIDNEY - The mean uptake of 125I-TNF per gram dry weight of kidney in the three feeding groups over the three hour experimental period.
Figure 5.13 TNF IN THE URINE - The mean release of radioactivity into the urine of the three feeding groups over the experimental period.
Table 5.4. RATE CONSTANTS FOR THE MOVEMENT OF TNF BETWEEN COMPARTMENTS

<table>
<thead>
<tr>
<th>Organ</th>
<th>TPN WT GAIN time⁻¹</th>
<th>TPN WT LOSE time⁻¹</th>
<th>ORAL CONTROL time⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine KIP*</td>
<td>0.033</td>
<td>0.023</td>
<td>0.044</td>
</tr>
<tr>
<td>KPI</td>
<td>0.013</td>
<td>0.0049</td>
<td>0.0098</td>
</tr>
<tr>
<td>Lung KPB</td>
<td>0.97</td>
<td>2.94</td>
<td>1.94</td>
</tr>
<tr>
<td>KPB</td>
<td>0.081</td>
<td>0.38</td>
<td>0.13</td>
</tr>
<tr>
<td>LIVER KLP</td>
<td>2.64</td>
<td>20.20</td>
<td>18.21</td>
</tr>
<tr>
<td>KPL</td>
<td>0.26</td>
<td>2.14</td>
<td>1.83</td>
</tr>
<tr>
<td>KNL</td>
<td>0.0030</td>
<td>0.0028</td>
<td>0.0018</td>
</tr>
<tr>
<td>Spleen KSP</td>
<td>0.50</td>
<td>0.58</td>
<td>0.32</td>
</tr>
<tr>
<td>KPS</td>
<td>0.11</td>
<td>0.15</td>
<td>0.089</td>
</tr>
<tr>
<td>Diaphragm KPD</td>
<td>1.02</td>
<td>13.85</td>
<td>1.03</td>
</tr>
<tr>
<td>KPD</td>
<td>0.47</td>
<td>0.00069</td>
<td>0.50</td>
</tr>
<tr>
<td>EDL KEP</td>
<td>0.0000014</td>
<td>0.046</td>
<td>0.047</td>
</tr>
<tr>
<td>KPE</td>
<td>0.010</td>
<td>0.0049</td>
<td>0.0032</td>
</tr>
<tr>
<td>Heart KHP</td>
<td>0.60</td>
<td>1.20</td>
<td>1.57</td>
</tr>
<tr>
<td>KPH</td>
<td>0.12</td>
<td>0.26</td>
<td>0.43</td>
</tr>
<tr>
<td>Soleus KMP</td>
<td>0.0054</td>
<td>0.046</td>
<td>0.0083</td>
</tr>
<tr>
<td>KPM</td>
<td>0.0051</td>
<td>0.0049</td>
<td>0.0043</td>
</tr>
<tr>
<td>KIDNEY KRP</td>
<td>2.55</td>
<td>3.69</td>
<td>2.37</td>
</tr>
<tr>
<td>KPR</td>
<td>0.18</td>
<td>0.27</td>
<td>0.12</td>
</tr>
<tr>
<td>KUR</td>
<td>0.0047</td>
<td>0.0067</td>
<td>0.016</td>
</tr>
<tr>
<td>Stomach KFP</td>
<td>0.021</td>
<td>0.026</td>
<td>0.030</td>
</tr>
<tr>
<td>KPF</td>
<td>0.00091</td>
<td>0.011</td>
<td>0.021</td>
</tr>
</tbody>
</table>

* all Kₓ, where x is any organ. values represent normalized values according to section 5.4.4.3.
the balance between soluble plasma TNF receptors and cell-surface TNF receptors. On the other hand, the $K_{px}$ reflects the ability of the organ to hold on to TNF preventing its return to the plasma. A small $K_{px}$ suggests that the organ has a high affinity for TNF and therefore does not want to release the TNF back to the plasma. This rate constant may also reflect the density or the affinity of the cell-surface receptors for TNF. The balance of the two rate constants can result in either a net accumulation of TNF in the organ ($K_{xp} > K_{px}$) or a net loss of TNF ($K_{xp} < K_{px}$). The overall accumulation and or loss (appearance and disappearance) of TNF over the three hour period is referred to as the uptake of the organ.

Interesting differences between weight-gaining TPN fed animals, weight-losing TPN fed animals and orally fed animals were observed. In the intestine, the weight-gaining TPN fed animals and the orally fed animals showed a rapid accumulation of TNF from the plasma. All groups showed a low $K_{pf}$ suggesting a high affinity for TNF by the intestine resulting in TNF retention. Retention of TNF was greatest in the orally fed and weight-losing TPN fed groups resulting in a larger net accumulation of TNF in the intestine in these animals. $K_{pf}$ was greatest in the TPN weight-gain animals suggesting that a larger fraction of the mass of TNF in the intestine was leaving the organ for the plasma resulting in a lower net accumulation of TNF in this organ. All groups had similar pulls for TNF towards the stomach. however, the stomach in TPN fed weight-gaining animals also had a high affinity for TNF (very low $K_{pf}$) resulting in a larger net accumulation of TNF in the stomach in comparison with TPN weight-losing and orally fed animals. This finding is interesting as a recent study by our group found the development of inflammatory changes in the stomach during forced enteral feeding by gastrostomy during TNF infusion.

In the lung, both TPN weight-lose rats and orally fed rats showed strong pulls for TNF suggesting increased loss of TNF from the plasma to the lung in these animals. In addition, these animals showed a relatively large $K_{pp}$ suggesting that the lung had little affinity for TNF resulting in rapid loss of TNF back to the plasma with no net accumulation. However, the TPN fed weight-gaining rats demonstrated a lower pull for TNF towards the lung as well as a
stronger affinity for TNF to remain in the lung resulting in a net accumulation of TNF in this tissue.

The kidney and the liver are the two organs associated with TNF clearance. The rate constant $K_{lp}$ is very large in TPN fed weight-losing and orally fed animals suggesting that there is a very strong pull for TNF by the liver. However, both of these groups also show a large fractional change in the mass of TNF within the organ ($K_{pl}$) suggesting that TNF is not retained in the liver (low affinity for TNF) in these animals but is released back to the plasma and out into the bile. The TPN fed-weight-gaining animals demonstrated a weaker pull for TNF from the plasma which was coupled with a relatively higher affinity for TNF by the liver ($K_{pl}$). These results suggest that TNF is retained to a greater extent in the liver of TPN weight-gaining animals and is not as rapidly released back to the plasma or to the bile. The mass of TNF released into the bile reflects the product of the rate constant for the release of TNF into the bile and the mass of TNF in the liver. Even though each of the rate constants are similar for the loss of TNF to the bile, the mass flux of TNF present in the liver in weight-lose TPN fed and orally fed animals is huge compared to that of the weight-gaining animals. Therefore, as a fractional change, much more TNF is lost into the bile in these animals than in the weight-gain animals, who have a lower pull for TNF to begin with (Figures 5.9-5.10).

The rate constant for the pull of TNF towards the kidney suggests that the kidneys of the TPN-fed weight-losing animals have the strongest pull for TNF with the kidneys of the TPN weight-gain animals and orally fed animals having a weaker but similar pull. However, the TPN weight-losing and control animals also have a relatively high $K_{pr}$ indicating that the kidney has little affinity for TNF resulting in a net loss of TNF back to the plasma or out into the urine. The loss of TNF into the urine is also higher for these animals in comparison with the weight-gain animals (Figure 5.13).

It is interesting to note that organ and plasma interactions can be divided into three patterns which are reflected in the rate constants for the movement of TNF between compartments. There are the organs such as the spleen, lung and the heart which show some pull for TNF from the plasma but little affinity for TNF to remain within them resulting in a net accumulation of TNF followed by a net loss which will at some point reach equilibrium with
the plasma. In addition, there are organs such as the liver and the kidney which show a strong pull for TNF from the plasma resulting in an initial net accumulation of TNF within the kidney which is followed by a rapid release of TNF back to the plasma and into the urine resulting in a continuous loss of TNF with little affinity for TNF to remain within the organ. These organs tend to be the visceral organs and show a large turnover of TNF. The total mass flux of TNF appears to be greater in the weight-lose TPN fed and control animals in comparison with the TPN fed-weight gain animals. The final pattern is found predominantly in the skeletal muscle, stomach, intestine and in the diaphragm. These organs show a very high affinity for TNF to remain in the tissue with a very low $K_{ex}$. This high affinity results in the net accumulation of TNF within these organs over time. This finding is interesting in light of the commonly observed action of TNF on muscle wasting.

5.6. DISCUSSION AND CONCLUSION

This study represents the first in-depth study of organ and tissue uptake, distribution and degradation of TNF over time in vivo and complements our first plasma clearance study. In this study, the organ uptake of labelled TNF in enterally fed animals was compared to that of parenterally fed animals in order to determine whether nutritional status affects TNF distribution in tissues. TNF is widely accepted to mediate the cascade of events leading to the development of anorexia, wasting and malnutrition in both the critically and chronically ill patient. Therefore, if nutrition support can further influence the metabolism or action of TNF then this interaction must be considered in the clinical setting where elevated levels of TNF are likely. Typically, nutrition support in the clinical setting is aimed at the prevention of muscle wasting through the provision of sufficient energy to meet or exceed metabolic requirements. However, we have speculated that the observed decrease in food intake observed during acute and chronic illness is an adaptive response and therefore, the provision of an insufficient nutrient intake via TPN might more appropriately reflect the physiological response to infection, inflammation and bacterial invasion.

Our results of the analysis of the disappearance of labelled TNF from the blood supports our previous study in which TPN fed weight-gaining rats were found to have impaired
clearance of TNF in comparison with orally fed weight-gaining animals or TPN fed weight-losing animals. In addition, we also see a significantly expanded volume of distribution in the parenterally fed weight-gaining animals which is reflected in a significant expansion of the plasma associated space. These findings are also similar to those found in our first study and suggest that TNF is retained in the plasma associated space thereby preventing it from binding to cell-associated receptors and being internalized and degraded. Impaired clearance of TNF could result in higher, more prolonged circulating levels of TNF and subsequently in the potentiation of the metabolic effects of TNF previously observed in animals receiving TPN. Increased levels of circulating soluble TNF receptors in TPN weight-gain animals provides one possible explanation for the observed pooling of TNF in the plasma associated space. Since soluble TNF receptors represent shed cell-surface receptors, we hypothesize that an increase in soluble TNF receptors would be reflected in changes in TNF cell-surface receptor numbers. Therefore, we examined the pattern of TNF uptake in tissues over a three hour period.

The uptake of TNF by organs and tissues revealed two distinct patterns. There are organs which show a net accumulation of TNF during the first thirty minutes post-TNF injection followed by a rapid release of TNF or its degradation products back to the plasma over the remainder of the three hour study period. These organs show a strong pull for TNF from the plasma as well as a large release of TNF back towards the plasma or into the bile or the urine resulting in little retention of TNF within them. The organs that follow this pattern tend to be the visceral organs. Secondly, there are organs and tissues that have a high affinity for TNF (very low $K_{pv}$) which results in TNF being retained within these organs and therefore in a net accumulation of TNF throughout the entire three hour study period. This pattern of uptake is found predominantly in the muscle tissue as well as in the stomach, intestine and in the carcass (Appendix 4. Figures 13-16, 19.3-6).

During the first 20 minutes post-TNF injection, approximately 60% of the injected dose is distributed between the blood and the tissues that were examined. However, by the end of the three hour period, less than 20% of the TNF could be accounted for in the tissues and blood. In an attempt to locate the remaining TNF, adipose tissue and skin were examined at
the three hour time point in the rats used for the additional bile and urine experiment described above. These animals were found to have, on average, 50 grams of skin and fur which took up anywhere from 32% to 55% of the injected dose of TNF. Adipose tissue contained less than 0.7% of the injected dose of TNF at three hours suggesting that it is not a major site of TNF accumulation. Therefore, when the carcass and the skin are considered as a whole, these two sites would most likely account for the remaining part of the injected dose.

Evidence from TNF binding and internalization studies suggests that TNF, after binding to cell surface receptors, is quickly internalized and degraded. Beutler et al. in their classic study of TNF distribution in the mouse, found that gel electrophoresis of tissues eight minutes after TNF injection contained no intact TNF band, suggesting that TNF had been rapidly degraded and released as small molecular weight peptides\textsuperscript{35}. Additional support for the rapid degradation of TNF comes from studies by Yoshie et al. who examined the pattern of TNF uptake in cells in vitro\textsuperscript{35}. In this study, Hela cells were incubated with labelled TNF in order to achieve maximal binding. Following binding, the cells were placed in fresh culture medium and the uptake of TNF into the cell. the TNF released spontaneously into the medium as well as the cell surface bound TNF (removed by an acid wash) were observed over an additional 120 minutes. These authors demonstrated that Hela cells in culture would continue to internalize labelled TNF for approximately 60 minutes after replating. The internalization of TNF was seen as an increase in cell associated radioactivity which could not be removed by acid treatment. Cell membrane bound TNF was released by acid treatment and was observed to rapidly decrease from time of replating. Spontaneously released TNF showed a bi-phasic increase over the entire experimental period. Initially there was a large spontaneous release of cell bound TNF followed by the release of internalized and/or degraded TNF which corresponded to the loss of internalized TNF. Further evidence that TNF was degraded within the cell comes from the precipitation of cell culture supernatants. Initially, 92% of the spontaneously released TNF was acid precipitable, whereas at 120 minutes, only 24% was acid precipitable suggesting that TNF was being degraded inside the cell with the release of label\textsuperscript{35}. Degradation of TNF within the organs is suggested in our study, as initially, acid precipitation accounted for approximately 90% of the total blood
radioactivity. However, by three hours post-TNF injection, the precipitable radioactivity accounted for only approximately 50% of the total radioactivity, suggesting that TNF is degraded within tissues with a simultaneous increase in released free iodide.

Organ uptakes of labelled TNF revealed some interesting differences between enterally and parenterally fed animals as well as between the weight-gaining and weight-losing TPN fed animals. The liver and kidney were found to take up the largest percentage of the injected dose followed by the lung, spleen and intestine. These findings are supported by distribution studies by Beutler and Pang who found the liver, skin, kidney and spleen to be major sites of TNF uptake.

The TNF kinetics of the gastrointestinal tract revealed some interesting differences between groups. There has been some concern that the uptake of labelled material by the stomach and intestine simply reflects the accumulation of labelled iodide by these organs, however, the tissue samples were homogenized and acid precipitated prior to analysis of the radioactivity which should eliminate the possibility that the radioactivity present in the samples was not protein bound. The uptake of TNF by the stomach was very different between the study groups with TPN weight-gaining animals and orally fed animals showing significantly increased TNF uptake by the stomach in comparison with the weight-losing TPN fed animals. In addition, the rate constants for the loss of TNF from the stomach to the plasma suggests that although all groups have a similar pull for TNF to the stomach, there is a much smaller fractional change in the mass of TNF within the stomach in TPN weight-gain animals than in either control or TPN weight-lose animals resulting in an accumulation of TNF within the stomach in these weight-gain animals. This high affinity for TNF by the stomach is interesting in light of our previous findings of an inflammatory reaction in the gastrointestinal tract of TNF infused animals provided with a continuous infusion of nutrients by gastrostomy but not in orally fed animals. This study supports our hypothesis that the forced provision of nutrients to animals which would normally develop anorexia results in a potentiation of the inflammatory response.

In contrast to the stomach, the TPN weight-losing animals show a continuous accumulation of TNF in the intestine whereas both TPN weight-gaining and orally fed
animals show a plateau suggesting a relative equilibrium with no net accumulation or loss of TNF over time. Rate constants for TNF uptake by the intestine also indicate that TPN fed weight-losing animals have a strong affinity for TNF resulting in an accumulation of TNF within this organ over time. This accumulation of TNF is also seen to a lesser extent in the orally fed animals. The intestine of the weight-gaining TPN fed animals shows a lower affinity for TNF with an increased fraction of the mass being returned back to the plasma, resulting in a lower retention of TNF in the intestines of these animals. A study by Ogle et al. found significantly increased levels of TNF mRNA and IL-6 mRNA in rat jejunal and caecal segments of intestine after 7 days of TPN in comparison with chow fed animals. Increased local cytokine production in TPN may have down-regulated TNF cell surface receptor expression leading to limited TNF uptake. However, this hypothesis does not explain why the intestines of all animals showed a similar affinity for TNF (similar $K_{ip}$) or why there was a sustained uptake in weight-losing animals. In addition, intestinal segments used in this study were duodenal segments which were not specifically investigated in the Ogle study.

The liver and the kidney are accepted to be the main sites of TNF clearance. Studies using perfused rabbit and monkey kidneys and livers revealed that the uptake of TNF from perfusing blood by the kidneys was mono-exponential, demonstrating the importance of the kidney in the clearance of TNF. In addition, studies of nephrectomized mice and rats have demonstrated impaired TNF clearance in animals subjected to bilateral nephrectomy in comparison with sham operated control animals. Our uptake data suggest that weight-losing TPN fed animals and orally fed animals have an increased mass flux of TNF by the kidney. In these animals, there is a strong pull for TNF from the plasma which is coupled with a larger fraction of the mass of TNF in the kidney being lost back to the plasma or into the urine. This rapid flux of TNF is supported by the release of radioactivity into the urine which was significantly higher in orally fed animals in comparison with the TPN fed weight-gaining animals. These results suggest increased uptake and degradation of TNF in the kidney of these animals. The radioactivity in the urine was not visible as a distinct 17kDa TNF band when subjected to gel electrophoresis suggesting that urine radioactivity is a reflection of the degradation and subsequent release of radioactive iodide. The rapid movement of TNF
back into the plasma suggests rapid filtration, uptake and degradation of TNF in TPN fed weight-losing and orally fed animals.

The uptake of TNF by the liver follows a similar pattern to that of the kidneys and suggests that, in orally fed and TPN fed weight-losing animals, there is a strong pull for TNF by the liver with little retention of TNF within the liver. The livers of the weight-gaining TPN fed animals have a weaker pull for TNF from the plasma coupled with a lower fractional change of the mass of TNF within the liver suggesting an increased retention of TNF within the liver with less TNF being lost back to the plasma or into the bile. This is supported by the release of TNF into the bile which is largest for the weight-lose and orally fed animals. Gel electrophoresis revealed that the radioactivity in the bile was associated with intact TNF. The presence of intact TNF in the bile may reflect one mechanism where TNF is recirculated through the enterohepatic circulation.

It is very interesting to note that the visceral organs, predominantly the kidney and liver, show an increased pull for TNF from the plasma especially in weight-losing TPN fed and orally fed animals but that the TNF is not retained within these organs but is released to the plasma or to the bile or urine. In contrast, skeletal muscle, intestine, diaphragm, and stomach show a net accumulation of TNF over time primarily due to the very high affinity for TNF to remain within these organs. Although there is no clear pattern within these retaining type organs, in general, the weight-losing TPN fed and orally fed animals show a greater retention of TNF in comparison with the TPN fed weight-gaining animals. This scenario does not hold true for the stomach and the heart.

The finding of these two very distinct patterns of TNF uptake may reflect the relative balance between the affinity of the plasma soluble TNF receptors for TNF and the cell-associated TNF receptors. The binding of TNF to cell-surface receptors may reflect the number or the affinity of the receptors for TNF. The strong pull for TNF by the visceral tissues in weight-losing and orally fed animals may reflect differences in the balance between cell-surface and soluble plasma TNF receptors. The weaker pull of organs for TNF in TPN fed weight-gaining animals may reflect an increased affinity or number of soluble TNF receptors in relation to cell-surface receptors. Therefore, our data is consistent with the hypothesis that
the route and the density of nutrition support can affect the balance of plasma soluble TNF receptors in relation to cell-associated receptors. In addition, the binding of TNF may have important implications in the development of the physiologic effects of TNF such as the anorexia and muscle wasting commonly observed during chronic TNF infusion. The increased retention of TNF by muscular tissues may also suggest that TNF binding to artery walls may have a profound effect on blood pressure and on the development of the vasodilation and hypotension that is common during sepsis. The physiological significance of increased tissue binding or uptake of TNF is unknown and would provide an avenue for further studies of tissue binding. In addition, studies of tissue binding and the relationship of the nutritional status to TNF cell-surface receptor binding would be an exciting area to pursue given the results of these organ uptake studies.

The study of TNF uptake and distribution is technically difficult and therefore has some limitations on its interpretation. Organ uptake studies that require the use of independent animals result in a larger variation in uptake within groups. Due to this variation, several clearance curves might generally be fitted to the data with comparable accuracy. The number of animals that would be required to tighten these curves would be unrealistic and might not give answers significantly different from those obtained. Unfortunately, it is not possible to study the same animals throughout the three hour period using this methodology. The improvement in bile and urine data seen when the same animals were studied over the three hour period supports the importance of individual variability.

Overall, these studies suggest that the quantity of nutrients provided, as well as how they are provided, enterally or parenterally, can affect not only the plasma clearance kinetics of TNF but also the organ distribution and degradation. Since TPN is routinely provided in the clinical setting where elevated levels of TNF are likely present, these studies should impact on the decision of what constitutes optimal nutrition support within that setting. Our data suggest that TPN in quantities sufficient to maintain growth impairs TNF clearance and organ uptake which might result in higher circulating TNF levels and more profound metabolic effects. The most reasonable explanation for the observed changes in TNF clearance appears to be related to the production of soluble TNF receptors. Enhanced shedding of cell surface
receptors to their soluble form would result in the binding and retention of TNF in the plasma as well as in fewer cell-associated TNF receptors, resulting in lower organ uptake of TNF and an expanded volume of distribution. Changes in TNF cell-surface receptor expression could be reflected in the lower affinities of the organs for TNF (except the intestine and spleen) in weight-gain animals. Currently, work is continuing in our laboratory with the hope of determining at a molecular level the changes in TNF receptor expression on tissues as a result of feeding practices.
CHAPTER 6.0 SUMMARY AND CONCLUSIONS

6.1 INTRODUCTION

Tumor necrosis factor-α is widely accepted to be the pivotal mediator in the cascade of cytokines and humoral factors released in response to injury or infection. Acute over-production of TNF results in hemodynamic collapse, shock and death. Chronic production of TNF is responsible for the anorexia, weight loss and accelerated muscle protein catabolism associated with chronic and acute illness. This wasting syndrome, or cachexia, can lead to dramatic losses of critical body proteins thereby threatening survival. Recognition of the profound effects of this wasting syndrome has led to the widespread implementation of nutrition support in the clinical setting. Nutrients are provided in order to preserve lean body mass and prevent weight-loss and malnutrition. However, despite the routine provision of parenteral and enteral nutrition support in the clinical setting, the possibility that this support can affect the underlying disease process has been virtually ignored.

Our laboratory has developed a rat model of chronic wasting and malnutrition which mimics the human cachectic syndrome. Preliminary evidence, using this model, has suggested that the aggressive provision of nutrition support by TPN can result in a potentiation of the metabolic abnormalities of TNF. This thesis is based on the completion of two mechanistic studies aimed at determining whether nutrition support can influence TNF distribution and clearance. In addition, it also examines whether less aggressive nutrition support might provide some advantage over aggressive nutrition support with respect to TNF metabolism. The results of these studies will contribute not only to our understanding of the kinetics and degradation of TNF but will assist in the development of protocols for the provision of nutrition support in the clinical setting.

These final sections will review the overall results of the two studies completed. In addition, it will explore possible mechanisms which could explain why we have observed changes in TNF clearance kinetics in response to both the route of nutrition support (enteral vs parenteral) as well as the quantity of nutrients provided by that support.
6.2 SUMMARY OF OVERALL CONCLUSIONS

6.2.1. Background Studies Leading to Current Hypothesis

In previous work, this laboratory has focussed on the effects of nutritional status on TNF action. Rats provided with a continuous infusion of TNF developed anemia, anorexia, weight-loss and wasting of peripheral muscle which mimics the cachexia of chronic and acute illness in humans. Therefore, using this model allows us to manipulate the nutritional status of these animals in order to define how nutrients and TNF interact. Early work indicated that animals provided with TNF and allowed to eat ad libitum developed the classic cachexic syndrome with anorexia weight-loss and wasting of muscle proteins. However, there was no mortality in these wasted animals. Subsequently, animals receiving TNF were provided with TPN in order to determine whether the provision of nutrition support could prevent the malnutrition and wasting observed in orally fed animals. Surprisingly, several of these TPN fed, TNF infused animals developed a hyperglycemic, hyperosmolar diuresis and subsequently died. Such results did not occur in orally fed, wasted animals receiving the identical dose of TNF. Therefore, we observed a potentiation of the metabolic abnormalities characteristic of sepsis when TNF and TPN were provided together in amounts sufficient to allow weight-gain and prevent malnutrition. These animals had higher circulating levels of the human recombinant TNF-α, which was infused, suggesting that the clearance of this exogenous TNF was impaired. The higher circulating level of TNF was hypothesized to be responsible for the observed potentiation of the metabolic effects of TNF in these animals.

6.2.2. Current Hypothesis

The studies outlined in this thesis were designed to determine whether the provision of nutrients in quantities sufficient to maintain growth would impair TNF clearance. Since the anorexia associated with the presence of TNF seemed to be somewhat protective, we also included a group of animals that received hypocaloric TPN in order to compare the clearance of TNF in two nutritional states. Nutrition support in the clinical setting is often aggressive in order to preserve lean body mass, however, preliminary results using this animal model of
chronic disease suggest that this may not be the optimal approach. The results of these
clearance studies should therefore assist in the provision of nutrition support in the clinical
setting where elevated levels of TNF may already be present.

6.2.3. Summary of Results of Current Study

This thesis reports on two mechanistic studies in which the clearance kinetics and tissue
distribution of TNF were examined in three nutritional states: enterally fed animals receiving
an oral liquid diet, animals receiving the identical liquid diet by central venous catheter
(TPN) in sufficient quantities to allow weight-gain and TPN fed animals receiving 50% of the
diet sufficient for growth resulting in weight-loss. The results of these two studies support
one another and suggest that the provision of nutrients via TPN in amounts sufficient to allow
weight-gain results in impaired TNF clearance. We have observed a significantly slower
initial distribution of TNF in these TPN fed weight-gaining animals as indicated by the
smaller slope of the first exponential function (Tables 4.2 & 5.1). In addition, we have
demonstrated in both studies, an expanded volume of distribution in TPN weight-gaining rats
in comparison with orally fed or weight-losing TPN fed rats which cannot be explained solely
by differences in body weight.

Separation of the volume of distribution into two compartments suggests that it is the
plasma associated compartment which is expanded in these weight-gaining animals (Tables
4.2 & 5.1). Compartmental analysis has allowed the calculation of rate constants for the
movement of TNF between plasma and non-plasma associated compartments. The ratio of the
movement of TNF in and out of the plasma associated compartment, the BARATIO, reveals
that the movement of TNF out of the plasma associated compartment is low in TPN fed
weight-gaining animals in comparison with TPN weight-losing or orally fed animals (Figure
4.5). In addition, by using the BARATIO and the calculated volumes of the two
compartments ($V_A$ and $V_B$) we have calculated the ratio of the masses of TNF present in the
two compartments (Appendix 2). This ratio is high in the TPN fed weight-gaining animals in
comparison with the low ratios found in TPN fed weight-losing or orally fed rats. These mass
ratios further support a retention of TNF in the plasma associated compartment. It was hypothesized that retention of TNF in the plasma associated compartment could affect the ability of tissues to take up and degrade TNF resulting in the prolonged presence of TNF in the circulation and subsequently a potentiation of the metabolic abnormalities observed previously in weight-gaining TPN fed animals.

Our initial study focused on the clearance kinetics of TNF including an examination of the tissue uptake of TNF at one time point, twenty minutes post-TNF injection. The uptake of TNF by four key organs suggested that TNF uptake was impaired in weight-gaining TPN fed animals with a significant reduction in uptake by the kidney. Reduced TNF uptake by the kidney is important as the kidney has been previously shown to be one of the key organs of TNF degradation.

This first study focussed on the clearance kinetics of labelled TNF. The study was limited by the fact that there was no direct measure of blood volume. In addition, organ data were available for only one time point thereby making any determination of true organ uptake impossible. For these reasons, the second study presented in this thesis represented an in-depth organ study of TNF uptake over time in enterally and parenterally fed animals. It was hypothesized that changes in TNF clearance kinetics would be reflected in differences in the distribution, uptake and degradation of TNF in tissues, especially those organs associated primarily with clearance, possibly reflecting changes in surface receptor number and soluble TNF receptor shedding.

The in-depth study of labelled TNF uptake by eleven organs and tissues over three hours revealed two major patterns of TNF uptake in these tissues. The first pattern was characterized by a strong pull for TNF by the organ resulting in a rapid accumulation of TNF within the organ during the first thirty minutes post-TNF injection. This rapid accumulation of TNF was followed by a large subsequent release, loss or degradation of TNF from the organ resulting in a steady release of tissue associated TNF. These organs not only had a strong pull for TNF as indicated by the large fractional change in the mass of TNF in the plasma but they also had an increased fractional change in TNF mass within the organ
suggesting that TNF was not retained in these organs but was rapidly released back to the plasma or into the bile and the urine. This pattern was found predominantly in the visceral organs, the liver and the kidney.

The second pattern of uptake was found primarily in the intestine, stomach, peripheral muscle and carcass which showed a continuous slow accumulation of TNF over the entire three hour time period (Appendix 4, Figures 3-6,13-16,19). These organs had a low fractional change in TNF mass within the organ suggesting a high affinity for TNF resulting in a net retention of TNF within the organ. These organs are all primarily smooth or striated muscle tissue. Therefore, it is interesting to try and link the influence of the type of uptake pattern of an organ or tissue on the physiologic effects of TNF. When all organs are considered on a per gram dry weight basis, there was a tendency for the TPN fed weight-losing animals to have a higher uptake of TNF for all organs except the stomach in comparison with TPN weight-gaining animals. Statistically significant differences in organ uptake are difficult to achieve due to the large individual variations between different animals.

TNF uptake by the organs primarily associated with clearance, the liver and the kidney, was lowest in TPN fed weight-gaining animals. In addition, the loss of radioactivity into the urine was also decreased in the TPN weight-gaining animals which suggests that uptake and degradation of TNF by the kidney is impaired in these TPN fed weight-gaining animals. Rate constants for the movement of TNF suggested that in orally fed and TPN fed weight-lose animals there was a stronger pull for TNF to leave the plasma for the liver and kidneys resulting in an increased mass flux of TNF in these organs. However, there was also increased fractional changes in TNF mass within these organs suggesting that TNF was not retained but was released back to the plasma or into the bile or urine in these animals. The rate constants for the weight-gain TPN fed animals suggest a lower pull for TNF by the liver and kidney in comparison with weight-lose animals as well as a lower turnover of TNF within the organ with increased retention of TNF. Increased retention of TNF may in part explain some of the metabolic effects associated with the provision of TNF and TPN.

Overall, these two studies support each other and suggest that TNF clearance is impaired
at the primary sites of TNF clearance, the kidney and the liver. This finding, coupled with the plasma clearance data, allows us to conclude that TNF metabolism is affected by the route and nutrient density of the nutrition support, resulting in impaired clearance and retention in the plasma compartment. Retention of TNF in the plasma would result in elevated circulating levels and provides one possible explanation for the observed potentiation of the metabolic response to TNF in these animals.

**6.3 CONCLUSIONS**

1. The provision of sufficient nutrients by TPN to allow growth affects the plasma clearance kinetics of TNF in rats. This is evident from the differences in TNF clearance between weight-gaining orally fed animals and weight-gaining TPN fed animals. Weight-gaining TPN fed animals show:
   * slower distribution of TNF in plasma disappearance curve (slope of 1st exponential)
   * an expanded volume of distribution, which is associated with the plasma compartment
   * impaired flux of TNF to the non-plasma compartment with an increased mass ratio of TNF in compartment A, suggesting retention of TNF in the plasma compartment with impaired clearance.

2. The provision of sufficient nutrients by TPN to allow weight gain also affected the tissue distribution of TNF. The organs of weight-gaining TPN fed animals had a lower affinity for TNF (except the stomach). This was evident in the kidney and liver which are the primary sites of TNF clearance. TPN fed weight-gaining animals also showed impaired clearance of TNF into the bile and the urine.

3. The nutrient density of the nutrition support also affects TNF clearance. Animals provided with 50% of the nutrients sufficient to allow weight-gain (weight-losing rats) by TPN showed plasma clearance kinetics and tissue distribution patterns that were similar to the orally fed animals and not to those of the TPN weight-gaining animals.

4. The provision of nutrition support by TPN in amounts sufficient to allow weight-gain is
associated with impaired clearance and altered tissue distribution of TNF which may be detrimental to the host.
CHAPTER 7.0 DISCUSSION OF THE INTERACTION OF NUTRITION WITH THE CLEARANCE KINETICS OF TNF

7.1 INTRODUCTION

There are now two questions that must be raised. The first question is how could differences in the route of nutrition support affect TNF distribution and clearance. The second is why does the provision of fewer nutrients result in TNF clearance kinetics that more closely resemble TNF clearance kinetics in orally fed animals. In order to answer these questions we must explore the physiologic differences between the provision of nutrients by either the enteral or parenteral route. In addition, we must determine whether the physiological changes associated with each type of nutrition support can be linked to TNF production or clearance. Finally, we must also determine whether individual nutrients can affect TNF production, distribution or clearance. The following sections will first examine the effect of altering the density of the macronutrients on TNF production and/or clearance. They will also examine some interesting work suggesting a between excess body weight and TNF mRNA expression. The final sections will examine the physiologic changes associated with the provision of nutrients by TPN as well as how these changes might affect TNF clearance. These studies will be examined in light of our current findings and will provide some potential explanations for the observed changes in TNF metabolism with TPN.

7.2 THE EFFECT OF THE QUANTITY OF NUTRIENTS ON TNF PRODUCTION

7.2.1. Intravenous Lipid Emulsions

Of the three major components of TPN, the composition of the lipid fraction has received more study in relation to cytokine production than either the dextrose or amino acid fraction. Originally, studies of the effect of intravenous lipid emulsions focussed on evidence that TPN with lipid was detrimental to the host's defence system. Lipids were hypothesized to exert negative effects on the ability of neutrophils, macrophages and the reticuloendothelial system to kill bacteria. A classic study by Freeman et al. found that infants receiving lipid
emulsions had a higher incidence of bacteremia than those not receiving lipids. These researchers hypothesized that one explanation for their observations was not an effect of the lipid emulsion on the immune system but was that the lipid might serve as a nutrient for staphylococci on the inner walls of the intravenous cannula. In a recent critical review, Palmblad concluded that there were no consistent, clear effects of lipid emulsions on the host immune system that would be significant enough to warrant discontinuation of their use.

Since these original studies, research on intravenous lipid emulsions has shifted its focus to the ability of various fatty acids to affect cytokine production. Dietary fats are precursors for the formation of EPA (eicosapentanoic acid, 20:5, n-3), which is similar to arachidonic acid (20:4, n-6), and competes as a substrate for cyclooxygenase and lipoxygenase pathways leading to the production of bioactive leukotrienes and prostaglandins. Therefore, there has been some speculation that the type of fat consumed can influence the production of these pro-inflammatory eicosanoids.

In animal studies, enterally fed rats were provided with diets of differing fatty acid composition by gastrostomy. Rats received either a diet high in the n-6 polyunsaturated fatty acid, linoleic acid, from safflower oil or a diet rich in n-3 fatty acids (EPA) from menhaden oil. Animals were provided with these diets for five days following which they were given an injection of bacterial lipopolysaccharide. The production of TNF-α did not significantly differ between diet groups following treatment with LPS nor were there any differences in survival. However, menhaden oil treated rats had a significantly lower level of PGE₂ and 6-keto-PGF₁α (a stable metabolite of prostacyclin) production than the group receiving the safflower based diet. Therefore, consumption of diets rich in linoleic acid or arachidonic acid may lead to the production of biologically active proinflammatory mediators such as PGE₂, thromboxanes and leukotriene B₄.

In a second study, the effect of n-3 versus n-6 fatty acids on TNF production by peripheral blood mononuclear cells was conducted to determine whether the chain-length of the fatty acid subsequently affected the TNF response. This was a randomized prospective study in which two lipid emulsions were compared. One lipid emulsion was a mixture of
50% LCT and 50%MCT whereas the other lipid emulsion was 100%LCT. TPN was given to 20 malnourished subjects for at least 30 days and production of TNF from peripheral blood monocytes was observed after 15 and 30 days of TPN. In the LCT fed group, there was a significant increase in cell-associated and secreted TNF when challenged with endotoxin or phytohemagglutinin (PHA) whereas 30 days of MCT/LCT emulsion had no effect on monocyte TNF production. This study investigated the effect of the chain length on TNF production and the results suggest that LCT alone, as a source of lipid, does not result in the appearance of TNF in the circulation but does affect TNF synthesis after LPS challenge. They were not able to demonstrate increased levels of TNF in the supernatant, therefore, although TNF synthesis may be increased it is not necessarily released. These changes may reflect changes in eicosanoid production by arachidonic acid or alternatively may reflect changes in membrane phospholipid composition affecting fluidity and intracellular messengers in different ways between LCT and MCT.

Alternatively, dietary fats could potentially modulate the inflammatory response by altering the sensitivity of cytokine producing cells (macrophages, monocytes and fibroblasts) to inflammatory stimuli and/or by changing the sensitivity of target cells to the actions of cytokines. A study in which healthy humans consumed a fish oil supplement for 6 or more weeks demonstrated that monocytes from these fish oil treated volunteers were able to produce less IL-1, IL-6 and TNF in response to a challenge with bacterial endotoxin in comparison with non supplemented volunteers. In order to investigate the effects of different fatty acids on TNF production, rats were fed for 4 or 8 weeks on rat chow or one of five experimental diets. The diets consisted of a 10% fat from corn oil with remaining from butter, coconut oil, corn oil, fish oil or olive oil. After feeding these rats for 4 to 8 weeks, peritoneal macrophages were challenged with LPS to observe the effect on cytokine production. They observed a rapid production of IL-1 and TNF by macrophages with a relatively slower release of IL-6. After 8 weeks, the olive oil diet showed marked suppression of TNF production in relation to chow fed animals (47%), an effect which was unique to this diet. However, both the olive oil and the corn oil diets increased IL-6
production after LPS stimulation in comparison with other diets (22%). Fish oil and coconut oil both resulted in an inhibitory effect on TNF production. IL-1 production was reduced by all fats but in particular by fish oil which resulted in a drop in production by 31%. Fish oil was seen to give the most consistent effects on IL-1 and IL-6. Therefore, it is clear that dietary fats can modulate cytokine release. However, the relationship is complex and seems to depend upon several factors including how long a particular diet is fed. In our study, the lipid used was identical between enterally and parenterally fed animals, thereby making it unlikely that the type of lipid is a factor in our results. However, one consideration is that TPN fed weight-losing animals received only half of the lipid of the weight-gain animals and therefore the quantity of lipid infused may also provide an explanation for differences in TNF responses in this group.

The quantity of lipid emulsion present might influence TNF kinetics by competing for clearance with activated alpha-2-macroglobulin at the liver. Studies have demonstrated that plasmin activated alpha-2-macroglobulin can bind TNF and clear it through receptors at the liver. The recent isolation of a low-density lipoprotein receptor related protein (LRP) on hepatocytes suggests one mechanism whereby intravenous lipid emulsions could influence TNF clearance (Figure 2.1). Studies have proposed that the LRP is the same as the alpha-2-macroglobulin receptor. Therefore, we can speculate that activated alpha-2-macroglobulin:TNF complexes might be cleared through these hepatocyte receptors as well as competing for clearance with low density lipoproteins at this site. Provision of nutrition support sufficient to allow weight-gain would provide 50% more lipid than that of the TPN fed weight-losing animals. Therefore, the weight-gaining animals might experience enhanced competition for clearance of alpha-2-macroglobulin-TNF complexes at the liver. Impaired clearance of TNF complexes would result in higher circulating concentrations of TNF and would explain our observed findings. However, the difficulty in accepting this theory lies in the ability of the orally fed animals to maintain TNF clearance in the presence of an equal intake of intravenous lipid.
7.2.2. The Effect of Protein

The effect of various levels of protein intake in enterally fed animals with
gastrostomies was examined in a study by Peck et al. In this study, guinea pigs were
provided with feeding gastrostomies followed by implantation of osmotic pumps filled with a
mixture of \textit{E.coli} and \textit{Staphylococcus aureus} which were released into the peritoneum over a
seven day period. This infusion of bacteria provided a model of bacterial peritonitis. The
guinea pigs were divided into four diet groups which were provided 5%, 10%, 15% and 20%
of energy from protein sources. There was a significant effect of the protein content of the
diet on overall mortality with the lowest protein diet having a 46% survival rate whereas only
a 15% survival rate was found in animals on the highest protein diet. Therefore, the authors
concluded that the provision of a low-protein diet (5% of calories) in their model of bacterial
peritonitis resulted in improved survival. Unfortunately, TNF levels were not measured.
They equated their protein intake to humans by considering the body surface area of the
guinea pig and concluded that their experimental diet would be equivalent to a human diet of
<0.5g protein/kg/day. The author's hypothesized that the improved survival of animals on a
low-protein diet was related to either modulation of the immune response or alternatively to
an effect on bacterial virulence. None of their diets contained sufficient protein to offset
the hypercatabolism of sepsis and all animals lost weight. Finally, the mechanism by which
protein restriction was able to positively influence survival is as of yet undetermined. It is
possible that differences in the protein content of the diet between parenterally fed weight-
gaining and weight-losing animals might affect their subsequent response to a bolus of labeled
TNF and could in part explain some of the effects associated with the nutrient density of the
nutrition support.

7.2.3. The Effect of Energy on TNF Clearance

In a similar experiment by Alexander et al., the effect of energy intake was observed in
guinea pigs provided with enteral feedings of varying levels of energy intake. Guinea pigs
underwent surgical placement of gastrostomy feeding tubes and after recovery were
subsequently provided with diets of 175 kcal/kg, 150 kcal/kg, 125 kcal/kg, or 100 kcal/kg body weight of energy. The highest level of energy intake had previously been found to be optimal for burned guinea pigs. Animals were implanted with osmotic pumps that delivered a mixture of *E. coli* and *S. aureus* into the peritoneum over 7 days. The lowest energy group lost a significant amount of weight - in excess of 15% of total body weight. However, despite this, these wasted animals had a survival rate of 57.2%. This survival rate was significantly greater than that of guinea pigs provided with 175 kcal/kg or 150 kcal/kg which had a 0% survival rate. The observed differences in survival rates suggest that overfeeding has an adverse effect whereas underfeeding results in improved survival even though it is associated with significant weight-loss.

These findings support our initial hypothesis that the anorexia associated with the presence of circulating TNF is a protective adaptation to sepsis and that forced provision of nutrients disrupts this adaptive response. These authors suggest that although increased nutrition may improve some nutritional parameters, it may also stimulate bacterial virulence or provide more substrates such as iron which are preferentially used by bacteria. In this study, as in ours, all components of the diet were decreased proportionately. Therefore, we are unable to determine whether the effect is due simply to the decrease in energy or to some specific component of the nutrition support such as the lipid or the protein fraction.

### 7.3 TNF PRODUCTION IN RELATION TO EXCESSIVE WEIGHT AND WEIGHT LOSS

In addition to the above evidence suggesting that overfed animals have an impaired ability to respond to a bacterial challenge, there is also evidence that excessive amounts of body fat can affect TNF production. The cytokine TNF is expressed in the adipose tissue of rats and mice and is expressed at a higher level in the adipose tissue from genetically obese rodents. This overexpression of TNF is thought to be, in part, responsible for insulin resistance in rodent obesity since after infusion of TNF binding protein, insulin sensitivity improves owing to improved insulin receptor kinase activity. The production of TNF by adipose tissue could be a local regulator of fat cell size, and the overproduction of TNF by
adipocytes of obese animals could represent a form of adipostat.

TNF expression was investigated in a study of 39 subjects who underwent adipose tissue biopsies. Eleven of these subjects underwent adipose tissue biopsies before and after a weight loss program was instituted. Adipose tissue biopsies were used to determine TNF mRNA using competitive RT-PCR methods. Tumor necrosis factor-α messenger RNA levels were highest in those subjects with mild to moderate obesity (BMI 27-45). Lower levels of TNF mRNA were found in those grossly obese subjects with a BMI > 45. When all subjects were compared, there was so much variation in the levels of TNF mRNA between subjects that the results failed to show statistical significance. However, when the group with BMI’s >45 were removed, there was a statistically significant relationship between TNF mRNA and BMI (r=0.37). There was also a significant correlation between percent body fat, as measured by bioelectric impedance, and TNF mRNA (r=0.46).

Interestingly, there was a consistent effect of weight reduction on TNF mRNA. Subjects lost an average of 34.7 kg (26.6% of initial body weight) and after maintaining this new weight for three months had adipose tissue biopsies repeated. These investigators observed that the reduction in weight of their subjects corresponded to a statistically significant reduction in TNF mRNA levels to 58.7% of their baseline values. The observed decrease in TNF mRNA was associated with a fall in the TNF protein level as measured by ELISA. TNF level in the adipose tissue was also found to correlate significantly with LPL activity. Subjects with the highest level of TNF also had the lowest level of LPL activity. The finding of lower TNF mRNA in the grossly obese subjects is interesting; however, even in these individuals, a reduction in body weight resulted in a drop in TNF mRNA which was consistent with the other subjects.

The effect of weight loss on adipose tissue mRNA has been examined in several subsequent studies which support the previous findings. Hotamisligil et al. also conducted a study to investigate the expression of TNF in adipose tissue. In this study, thirty-seven premenopausal females, 18 lean and 19 obese were studied. These women had an overall BMI of >30. After initial fat biopsies, 9 of the obese women went on weight loss programs during which time they lost 17% of their BMI. Northern blot techniques were used to identify TNF mRNA in adipose tissue. Analysis of adipose tissue revealed mRNA levels 2.5 fold higher in obese women in comparison with lean women. This effect was not observed for any other cytokines. In order to determine whether adipose tissue from obese women
secreted more TNF in vitro they explanted adipose tissue samples and measured the released TNF by ELISA. Adipose tissue from obese women was found to secrete significantly more TNF than that from lean women which might reflect local amounts of circulating TNF. The authors also observed a decrease in TNF mRNA expression in obese women after weight reduction. Therefore, there is a strong body of evidence demonstrating that elevated TNF mRNA expression in adipose tissue is correlated with obesity which is accompanied by significant insulin resistance. This overexpression of TNF mRNA is also seen in many animal models of obesity such as the db/db mouse as well as the ob/ob, tub/tub and Zucker fa/fa rats.

It is interesting to speculate that the provision of TPN in amounts that allow weight-gain might somehow stimulate increased rates of TNF mRNA transcription or translation by tissues simply due to the provision of nutrients. In addition, TNF mRNA may be altered in tissues other than adipose tissue due to the provision of nutrients. One might also speculate that during illness or bacterial invasion, the anorexia and subsequent weight-loss might be a mechanism whereby the body regulates its own production of TNF and that the provision of nutrition support would disrupt this adaptive mechanism. If there were differential tissue production of TNF in response to nutrition under these circumstances, increased TNF production locally might provide some explanation for the observed effects.

7.4 ENTERAL VS PARENTERAL NUTRITION SUPPORT—THE PHYSIOLOGIC EFFECTS OF PROVIDING NUTRIENTS BY TPN

7.4.1 Nutrient Metabolism in Enteral and Parenteral Feeding

In these studies, nutrients were provided by two routes: orally or by central venous cannula. The provision of nutrients by total parenteral nutrition requires that nutrients be continuously infused at a slow rate in quantities determined to meet metabolic needs. This type of feeding is different from the more bolus-like feeding of our orally fed animals. It is therefore reasonable to question whether the differences observed in enterally fed and parenterally fed animals reflect differences in the metabolism of macronutrients and fuel use. Differences in fuel metabolism might contribute to the observed effects of TPN. The provision of enteral nutrients allows normal digestion by the gastrointestinal tract whereas the provision of nutrients by TPN releases these nutrients directly to the blood stream.

There is no evidence to suggest that parenterally provided nutrients are metabolized or
utilized differently than enterally provided nutrients. Amino acids are provided as crystalline amino acids and therefore do not require any cleavage of long peptide chains. These amino acids become readily available for protein synthesis by tissues. The only nutrient of question is the lipid emulsion as it does not undergo emulsification with bile acids in the intestine. However, commercial lipid preparations are based on soybean oil with the addition of egg yolk phospholipid which is used as an emulsifying agent. The phospholipid creates a mechanical barrier by rendering a negative surface charge (zeta potential) to the dispersed liquid phase. This maintains repulsive electrostatic forces between lipid particles, resulting in a stable emulsion. The addition of glycerol ensures that the lipid emulsion is isotonic. In addition, studies by Hallberg have shown that the elimination kinetics of intravenous lipids from the blood stream are similar to chylomicron elimination in humans. The droplet size and the interaction of the particle with lipoprotein lipase are also similar between lipid emulsions and dietary chylomicrons. Finally carbon labelling has allowed us to determine that these fat particles are oxidized with the subsequent release of $^{14}$CO$_2$. Therefore, it seems unlikely that differences in macronutrient metabolism between enterally and parenterally fed animals are responsible for any of the observed differences in TNF clearance or metabolism. In addition, our feeding studies were carried out in normal, non-septic animals, thereby preventing any confounding effects on nutrient metabolism due to the production of stress hormones. However, although nutrients seem to be metabolized in a similar fashion in enterally and parenterally fed animals, there are distinct differences associated with the provision of nutrients by TPN due to the physiological changes associated with by-passing the gastrointestinal tract. These differences will be examined in the following sections.

7.4.2. Evidence that TPN affects TNF Production

There is evidence that the provision of nutrients via a central venous catheter results in significant physiologic changes which may alter cytokine production and response to bacterial challenge, or in our case, to a bolus of TNF. Lowry et al. observed that parenterally fed patients exhibited an exaggerated cytokine response to an endotoxin challenge in comparison with enterally fed patients. In addition, enhanced cytokine production with TPN was also shown in rats provided with either a control chow diet or TPN for four or five days. Half of the TPN fed rats were killed after four days of TPN feeding while the remaining rats were
returned to chow food for one day and subsequently killed. TNF and IL-1 production in the blood and cerebrospinal fluid was analysed. After 4 days of TPN, the mean plasma concentration of TNF was significantly higher in the TPN rats in comparison with control rats. Levels of IL-1 were undetectable in either group. Levels of circulating TNF returned toward control levels one day after TPN was stopped resulting in circulating TNF levels which were not significantly different from control values. Therefore, in this study, simply providing nutrients by TPN was found to stimulate TNF production in these animals.

7.4.3. The Effect of TPN on the Immune System and Host Response to Injury

It has been suggested that the provision of parenteral nutrition support can affect the immune response of the host. Animal studies by Birkham and Renke have shown that TPN fails to maintain lymphocyte function when compared with enteral feeding. In addition, Kudsk et al. have shown that, when compared with parenteral feeding, enteral feeding in both malnourished and well-nourished rats improved survival to Escherichia coli hemoglobin peritonitis.

In another study, the effects of TPN on immune function and pulmonary alveolar macrophage response to a bacterial challenge was studied in rats fed either a chow diet or TPN for 7 days. TPN and chow fed animals were exposed to an intratracheal bacterial load. Three days after bacterial exposure, these animals were killed and the lungs were removed aseptically. Phagocytosis of Candida albicans by pulmonary macrophages was significantly lower in the TPN group than in the chow fed group. Three days after inoculation with E. coli, pulmonary residual bacteria were significantly higher in the TPN group in comparison with the chow fed group. Therefore, pulmonary bacterial clearance was decreased significantly in TPN fed rats in comparison with control rats. In addition, after bacterial challenge, the TPN rats had subjectively more severe symptoms than those in the chow fed group. Finally, chow fed animals demonstrated improved survival rates in comparison with TPN fed animals (92% vs 55%). This study demonstrated decreased killing of Candida albicans by pulmonary macrophages as well as delayed bacterial clearance in animals fed by TPN. TPN also significantly increased mortality in rats receiving intratracheal E. coli. Therefore, this study demonstrates for the first time, that TPN results in a significant impairment of pulmonary macrophage antimicrobial functions and that these defects are associated with increased mortality in response to a septic challenge. It may be that the
provision of nutrients by TPN results in significant structural and physiologic changes resulting in gut atrophy, intraluminal bacterial overgrowth and bacterial translocation.

A study by Meyer et al. looked at the effect of the route of feeding on neutrophil activation in humans. Circulating neutrophil counts, neutrophil migration to LTB4, and generation of LTB4, a leukotriene that acts as a potent neutrophil chemotaxin and is thought to amplify the inflammatory response, were measured before and after infusion of endotoxin. Volunteers were provided with either an enteral diet or TPN at a sufficient quantity to maintain body weight for one week. Differences were observed in circulating neutrophil counts between the two feeding groups early in the study with the TPN fed group having a baseline neutrophil count which was almost double that of the enterally fed group. In addition, there were significant differences in neutrophil migration of LTB4 in volunteers receiving oral feeding in comparison with parenterally fed volunteers. The oral fed group had increased neutrophil chemotaxis, which may represent an important early host response to gram-negative infection, which could be altered by the route of feeding. The enhanced response may be the result of neutrophil stimulation by chemotactic factors from complement activation or from other mediators. Therefore, the TPN fed volunteers manifested different neutrophil kinetics with increased baseline neutrophil counts, less marked neutrophilia and a reduction in baseline LTB4 chemotactic responsiveness in comparison with enterally fed volunteers. In addition, higher LTB4 levels were generated by neutrophils in the parenterally fed group than in the enteral group. The authors concluded that their results supported previous animal studies which showed differences in the host response to infection due to the provision of enteral or parenteral nutrition.

The provision of TPN in humans has previously been associated with decreased survival and increased rates of infectious complications. Kudsk et al. did a prospective randomized trial of 98 patients who suffered severe abdominal trauma. The patients fed via TPN had significantly higher rates of infectious complications, especially pneumonia, in comparison with those fed enterally. Finally, Moore et al. analyzed 8 prospective randomized clinical trials using meta-analysis and found that enterally fed patients had a significantly lower incidence of infectious complications when compared with parenterally fed surgical and trauma patients. The mechanism by which the provision of TPN results in poorer outcome in these clinical trials is unclear. However, potential explanations have focused on the changes associated with total parenteral nutrition on mucosal structure, permeability to
bacteria. gut immunology and bacterial colonization.

### 7.4.4. The Effect of TPN on Gut Mucosal Atrophy and Bacterial Translocation

The provision of nutrients by total parenteral nutrition has been criticized due to the resulting lack of stimulation and atrophy of the gastrointestinal tract. Lack of gut stimulation can result in the loss of its barrier function thereby allowing the escape of bacteria or luminal toxins into the portal or systemic circulation. If this occurs, immunologically competent cells will respond by the production of inflammatory mediators including cytokines in an effort to combat these toxins.

The effect of antecedent bowel rest and TPN on the response to endotoxin was assessed in a study of 12 normal human volunteers. The volunteers were divided so that 6 subjects remained on enteral feedings of sustacal (‡ Mead Johnson Inc., Ottawa, Canada), a liquid nutritional supplement, and six subjects were provided with isocaloric feedings by TPN for one week prior to a challenge with endotoxin. Peak circulating levels of stress hormones glucagon and epinephrine were measured. The peak circulating levels of these hormones were significantly higher in the TPN fed group than in the enterally fed group. There were no significant changes in insulin response between groups. Levels of circulating TNF were also significantly higher in TPN fed individuals than in the enterally fed group. The TPN group did not show alterations in circulating glucose levels nor in glucose balance after endotoxin challenge but did show a significant induction of lactate production compared with enterally fed subjects.

The route of nutrition support may influence the course of disease through its effects on intestinal mucosal integrity. Enteral feeding has been hypothesized to be trophic for the gut resulting in enhanced mucosal integrity in comparison with parenteral feeding which results in bowel rest and gut mucosal atrophy. In addition, total parenteral nutrition and bowel rest have been associated with increased bacterial translocation across the mucosal barrier. This study demonstrated that the provision of antecedent TPN significantly enhanced the systemic responses of endotoxemia with enhanced cytokine response including increased TNF production. Since TPN has been associated with increased bacterial translocation in animal models and has been linked to the overgrowth of bacteria in the intestinal lumen, the authors hypothesized that the increased response to bacterial endotoxin challenge in TPN fed volunteers was the result of increased splanchnic and systemic reticuloendothelial
responsiveness. This priming effect of TPN could result in down-regulation of TNF receptors and therefore an impaired ability of the host to respond to repeat bacterial challenge. In our experiments, the TPN fed weight-gaining animals may have had impaired ability to respond to the bolus of labelled and unlabelled TNF due to prior exposure to humoral mediators as a result of the antecedent TPN.

Enhanced reticuloendothelial responsiveness in TPN fed animals is hypothesized to be the result of exposure to bacterial endotoxin or other humoral factors as a result of gut bacterial overgrowth or bacterial translocation as a result of gut atrophy. A study by Pappo et. al. was designed to investigate whether the provision of nutrients via TPN resulted in an overgrowth of intestinal gram negative bacteria. Five groups of rats were studied - group I was maintained on oral food alone while groups II to V received TPN. In addition, groups III to V were also treated with oral antibiotics of varying combinations. The free feeding rats were found to have significantly lower concentrations of gram negative bacteria in the cecum in comparison with TPN treated animals. The antibiotic treated rats had significantly less cecal bacteria than either the free fed or the TPN fed animals. The rate of bacterial translocation to the mesenteric lymph nodes was not different between feeding groups. Therefore, bacterial translocation cannot explain differences in orally and parenterally fed animals. Peritoneal macrophages were collected and their spontaneous production of TNF was measured. TNF production was significantly higher in the macrophages of TPN treated rats than control rats or rats who received TPN plus antimicrobial therapy. There were no differences in circulating plasma TNF levels. The authors hypothesized that it is an increase in lactose-positive and lactose negative organisms that could release a variety of hepatotoxic substances such as LPS or TNF that cause injury and that the decrease in TNF production from macrophages in polymixin B (antibiotic) treated rats might support this hypothesis. Another explanation would be that TPN fed animals displayed a heightened cytokine and metabolic response due to a more responsive reticuloendothelial system due to increased exposure to gram negative bacteria as suggested previously.

Shou et al. conducted animal studies in order to determine whether intraluminal bacterial overgrowth would result in changes in the host immune system and specifically would affect macrophage functioning. Rats were provided with an iso-nitrogenous diet orally or by TPN for seven days after which peritoneal macrophages were harvested. In subsequent experiments, the diet of the TPN fed rats was adjusted to include 10 or 20% of the total intake
as chow. In contrast to the previous study, animals that received nutrients solely by TPN had a significantly increased percentage of bacteria-positive mesenteric lymph nodes in comparison with those fed orally (77% vs 17%). In addition, TPN fed animals had decreased peritoneal macrophage superoxide production and *Candida albicans* phagocytosis in comparison with orally fed animals. Provision of some oral nutrients (20% of intake) resulted in a normal superoxide production by peritoneal macrophages as well as enhanced *C. albicans* phagocytosis and a lower percentage of bacteria positive mesenteric lymph nodes (44%). This study demonstrated that the provision of TPN resulted in increased bacterial translocation. Increased bacterial translocation provides one explanation for the observed changes in immune function. A small amount of enteral feeding was shown to reverse the observed effects of TPN on the immune system, strongly supporting the importance of gut stimulation.

### 7.4.5. The Effect of TPN on Soluble TNF Receptor Production

The lack of intestinal nutrient stimulation that occurs with total parenteral nutrition appears to also regulate the response to inflammatory challenge in humans. Prolonged bowel rest has been implicated in having an amplifying effect on the metabolic and systemic response to endotoxin challenge resulting in an exaggerated acute phase response, enhanced counter-regulatory protein production, splanchnic cytokine release and increased neutrophil activity. TNF itself or a dysfunction of the TNF receptor system has been hypothesized to play a role in the exaggerated response noted in parenterally fed individuals after exposure to endotoxin. Therefore, TNF receptor expression and soluble TNF receptor appearance were studied in order to explain the enhancement of metabolic effects associated with TPN and bowel rest. The subjects included 17 volunteers who were randomized to receive parenteral nutrition or enteral nutrition (sustacal) for 4 days. Intravenous lipids were not provided. The diets were isocaloric. The subjects underwent a challenge with bacterial endotoxin at the end of the feeding protocol and levels of cytokines and soluble and cell surface TNF receptors were measured. The parenterally fed group demonstrated an enhanced systemic response to endotoxin by showing significantly increased core temperature and a greater decline in mean arterial pressure. They also showed higher baseline neutrophil counts in comparison with enterally fed volunteers. Baseline TNF bioactivity was similar between groups and although the TPN fed group did have higher circulating levels of TNF in response to endotoxin, the results did not reach statistical significance. IL-6 levels were also found to
be significantly altered in the parenterally fed group over the enterally fed group. Peak levels of IL-6 in parenterally fed volunteers were double those of the enterally fed volunteers. These investigators were unable to show that TPN alone induced shedding of TNF receptors. However, shedding of TNF receptors was noted after challenge with endotoxin. A different pattern of receptor release was seen in enterally and parenterally fed subjects. For the p75, the increase in shedding was immediate with TPN fed volunteers having higher levels of soluble p75 by 30 minutes post endotoxin administration and statistically significant differences being present at 1 hour post injection. These differences were still significant at 3 hours post injection. The same result was seen for the p55 receptor but the difference took longer to occur and statistical significance was not reached until 1.5 hours post endotoxin administration. Differences in soluble TNF receptor shedding resulting in differences in cell associated TNF receptors may provide one explanation for our observed findings.

Porteau et al. reported that the p55 receptor is preferentially internalized whereas the p75 receptor is shed into the circulation. The data in this study supports this earlier finding as they observed a significant increase in soluble p75 with a less pronounced release of p55. This group also demonstrated that although both soluble receptors were released in response to endotoxin, the p75 was significantly higher in the TPN subjects than in the enterally fed subjects after LPS infusion. The enterally fed subjects released more p55 over the first period post-LPS than the TPN group. Therefore, TPN was seen to augment post-endotoxin inflammatory mediator production. Soluble TNF receptors may modulate the systemic response to TNF by competing with cell surface receptors thereby preventing ligand interactions. In addition, soluble receptor-TNF complexes have been shown to circulate, even in the absence of detectable levels of free TNF. Lower sTNF-receptor levels have been correlated with improved prognosis in critically ill septic patients and provision of soluble TNF receptors in septic patients lead to increased mortality.

In a very recent randomized multi-centered clinical trial, escalating doses of soluble TNF fusion protein were given to critically-ill patients in an effort to inhibit TNF binding to cell-surface receptors and therefore, reduce its toxic effects. Unfortunately, this trial had to be stopped early as there was a significant increase in mortality in the patients receiving the fusion protein over those receiving placebo. In addition, there was a dose response relationship between the dose of fusion protein and mortality. The authors hypothesized that one reason this could have happened is that TNF soluble receptors or fusion proteins are
acting as carriers of TNF in the circulation and therefore, are prolonging the presence of bioactive TNF, thereby prolonging the inflammatory reaction. This finding supports our hypothesis that an increase in circulating soluble TNF receptors in TPN fed weight-gain animals could enhance the metabolic response to TNF. Less soluble TNF receptor might therefore, represent a lesser degree of pro-inflammatory signal. In addition, there may be an effect of antecedent nutrition support on the regulation of TNF receptors and the endotoxin induced appearance of soluble TNF receptors.

In a study by Jansen, infusion of TNF resulted in an almost instantaneous shedding of cell surface receptors with peak levels being measured 15 minutes post-TNF injection. Therefore, differences in the shedding of TNF cell surface receptors may also occur in response to prior feeding by TPN and result in the observed differences in TNF distribution seen in our study. Changes in soluble TNF receptor production either before the provision of the bolus of labelled TNF or in response to the labelled TNF is one of the most reasonable hypotheses for our observed differences in TNF clearance kinetics as it would explain the expansion of the volume of distribution as well as the observed impairment of organ uptake.

7.5 SUMMARY OF THE EFFECTS OF NUTRIENTS AND TPN ON TNF CLEARANCE

It is clear, at least in animals, that the provision of nutrients by total parenteral nutrition results in physiologic changes, primarily to the intestinal mucosa, which may influence host immune responses to bacterial invasion. It is less clear whether these TPN associated changes result in alterations in TNF clearance. The provision of TPN has been associated with impaired gut barrier function as well as bacterial overgrowth and translocation to the mesenteric lymph nodes. Several authors have speculated that it is exposure to these bacteria or their products that results in a priming of the host immune system with the subsequent production of humoral mediators and cytokines. Enhanced production of cytokines as a result of TPN has been shown in one animal study. Stimulation of the cascade of endogenous mediators involved in the response to such a bacterial invasion prior to our clearance study, could result in down-regulation of TNF receptors and resistance to a subsequent challenge, such as our bolus of TNF. In addition, TPN has been associated with impaired macrophage function, decreased clearance of a bacterial load and decreased survival in comparison with enteral feeding. Therefore, changes in the host immune response
could effect the ability of the host to clear TNF, resulting in higher circulating levels and potentially explaining our observed potentiation of the metabolic effects of TNF with TPN. The normalization of pulmonary macrophage function seen in TPN fed animals receiving some oral nutrition strongly supports the importance of enteral nutrition in the maintenance of immune system integrity.

While there are distinct differences between the provision of nutrients enterally or parenterally, there is significantly less information regarding the effects of the quantity of nutrients infused on TNF production or clearance. In animal studies, both low energy and low protein diets were shown to provide a survival advantage after endotoxin challenge \(^{200, 201}\). This finding supports our basic hypothesis that the anorexia observed in orally fed animals given TNF is an adaptive mechanism which somehow provides a survival advantage. There has also been speculation that intravenous lipids may compete for clearance at the liver with alpha\(_2\)-macroglobulin:TNF complexes. If this is true, then a hypocaloric diet would contain less lipid thereby resulting in less competition and enhanced clearance of alpha\(_2\)-macroglobulin:TNF complexes. Since our diets used identical lipid emulsions it is unlikely that the type of fat played a role in the production or subsequent clearance of TNF. An additional possibility is that during weight-gain, animals manifest different patterns of TNF mRNA as demonstrated in obese and lean humans. These changes in mRNA might lead to increased TNF production in response to the bolus of TNF given. Differences in TNF mRNA were examined primarily in adipose tissue but may differ in other sites, even resulting in localized areas of increased TNF production and subsequently might explain differences in organ uptake of TNF in weight-gain animals. However, there would also have to be a compounding effect of TPN additional to these changes in TNF production as otherwise orally fed animals would also be expected to manifest the same changes in TNF mRNA as TPN fed weight-gain animals.

One of the most intriguing possibilities is that the provision of nutrients by TPN causes physiologic changes that result in a shedding of soluble TNF receptors. Prior exposure to TNF, due to the presence of intestinal bacteria or their toxins, may not be sufficient to result in elevated circulating levels of TNF but may be sufficient to stimulate the release of soluble TNF receptors. In addition, the release of soluble TNF receptors could reflect changes in cell surface receptor numbers. A change in the distribution of receptors to favour increased soluble TNF production in TPN fed weight-gaining rats would result in a retention of TNF in the circulation and impaired uptake by tissues. This alteration in receptor balance may reflect
differences in immune system activation or reflect an enhanced responsiveness in these animals due to the provision of TPN. These effects must be augmented by the fact that nutrition support is provided in amounts that are sufficient to allow weight-gain and that the provision of minimal nutrition support would not result in the same alterations as full nutrition support. Differences in TNF receptor distribution are under preliminary study in our lab and results indicate that there are indeed fewer cell-associated TNF receptors in tissues from weight-gaining TPN fed animals.

7.5.1. Application of the Model to the Clinical Setting

We have previously found that a slow continuous infusion of TNF into rats results in the development of the characteristic wasting, anorexia, anemia, and redistribution of body proteins seen in critical illness and chronic disease in humans. When circulating TNF was present, the concurrent provision of TPN resulted in a potentiation of the metabolic responses to TNF with a significant increase in mortality and morbidity. This was not seen in orally fed wasted animals. Therefore, nutrition support, aimed at the prevention of malnutrition and muscle wasting resulted in a significant potentiation of the metabolic effects of TNF. It seems also reasonable to extend this to the clinical setting. Since both the critically-ill and the chronically ill patient have been shown to have elevated circulating TNF levels and since many of these patients are also provided with nutrition support, could we not be enhancing the metabolic effects of TNF in the clinical setting by our aggressive approach to nutrition support in an effort to prevent malnutrition? This thesis reports on two mechanistic studies designed to answer the question of whether TPN given in amounts sufficient to prevent malnutrition (weight-gain) affects TNF metabolism and clearance. Impaired TNF clearance would result in increased and prolonged circulating levels of TNF which could be linked to the potentiation of the metabolic effects of TNF.

We have demonstrated that the route of nutrition support as well as the nutrient density of that support can affect the clearance, distribution and degradation of TNF. Therefore, these studies indicate that TNF metabolism can be modulated simply due to the type of nutrition support provided. The provision of hypo-caloric TPN resulted in an improved pattern of TNF clearance kinetics and organ uptake which was similar to orally fed animals. Therefore, these studies should be considered in light of our feeding practices in the intensive care units where circulating levels of TNF may already be elevated.
REFERENCES


67. van der Poll T, van Deventer JH: Tumor necrosis factor α: A common mediator of the spectrum of diverse changes in sepsis.


69. Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H, Stanley KK: Surface location and high affinity for calcium of a related 500 kd liver membrane protein closely related to the LDL receptor suggest a physiological role as lipoprotein receptor. EMBO 7:4119-4127, 1988.

70. Kristensen T, Moestrup SK, Gliemann J, Bendtsen L, Sand O, Sottrup-Jensen L: Evidence that the newly cloned low-density lipoprotein receptor related protein (LRP) is the α₂-macroglobulin receptor. FEBS 276:151-155. 1990.


194. Raina N. Cameron RG. Jeejeebhoy KN: Gastrointestinal, hepatic and metabolic effects of enteral and parenteral nutrition in rats infused with Tumor Necrosis Factor. Submitted.


Determining values for the rate constants in any two-compartment model

We consider the two-compartment model represented in Figures 4.1-4.3. The system contains no tracer prior to $t=0$, at which time, a bolus of tracer is injected into compartment $A$. The differential equations governing this model are of the form

\[ \frac{dC_A}{dt} = \lambda_1 C_A + \lambda_2 C_B \]  

(A1)

\[ \frac{dC_B}{dt} = \lambda_3 C_A + \lambda_4 C_B \]

where $C_A^*$ and $C_B^*$ are the concentrations or tracer in compartments $A$ and $B$ respectively. Solutions to these differential equations are always given by biexponential functions of the form

\[ C_A(t) = C_1 e^{\beta_1 t} + C_2 e^{\beta_2 t} \]

\[ C_B(t) = -C_3 e^{\beta_1 t} + C_4 e^{\beta_2 t} \]  

(A2)

where the $C_i$ are constant, $\beta_1 > \beta_2 > 0$ are real constants, and $C_3 > 0$. We define $\Lambda$ to be the matrix

\[ \begin{bmatrix} \lambda_1 & \lambda_2 \\ \lambda_3 & \lambda_4 \end{bmatrix} \]

$C$ to be the matrix

\[ \begin{bmatrix} C_1 & C_2 \\ -C_3 & C_3 \end{bmatrix} \]

and $\beta$ to be the matrix

\[ \begin{bmatrix} -\beta_1 & 0 \\ 0 & -\beta_2 \end{bmatrix} \]

As shown by Norwich (169, p122), the above matrices are related by
the equation

\[ \Lambda = C\beta C^{-1} \]  \hspace{1cm} (A3)

where \( C^{-1} \) is the inverse matrix of \( C \). The remainder is straightforward algebra.

Referring now to Figures 4.1-4.3, we can write the particular form of Equations (A1) that refer to this model:

\[ \frac{dC_A}{dt} = -(K_{{BA}} + K_{{OA}})C_A + K_{{AB}}C_B \]  \hspace{1cm} (A4)

\[ \frac{dC_B}{dt} = K_{{BA}}C_A - K_{{AB}}C_B \]

The explicit form of the matrix \( \Lambda \) is now

\[ \Lambda = \begin{bmatrix} -K_{{BA}} & -K_{{OA}} & K_{{AB}} \\ K_{{BA}} & -K_{{AB}} \end{bmatrix} \]  \hspace{1cm} (A5)

The values of \( \beta_1 \) and \( \beta_2 \) are obtained by curve-fitting the measured \( C_A^* \) values to the first of equations (A2), thus giving us the matrix \( \beta \) in numerical form. The same curve-fitting process provides values for the coefficients \( C_1 \) and \( C_2 \), so that two of the four elements of the matrix \( C \) are numerically known. Equation (A3) then defines four scalar equations, one for each element of the matrix \( \Lambda \). Solving the four scalar equations then provides values for the three rate constants \( K_{{BA}}, K_{{AB}}, K_{{OA}} \) as well as for the unknown quantity, \( C_3 \), (which we do not expressly require).

\[ K_{{BA}} = \frac{C_1C_2(\beta_1 - \beta_2)^2}{(C_1 + C_2)(\beta_1C_2 + \beta_2C_1)} \]

\[ K_{{AB}} = \frac{\beta_1C_2 + \beta_2C_1}{C_1 + C_2} \]
\[ K_{Od} = \frac{\beta_1 \beta_2 (C_1 + C_2)}{\beta_1 C_2 + \beta_2 C_1} \]
Masses of Cachectin in Steady State

From equation (A4) Appendix 1

\[
\frac{dC_B}{dt} = K_{BA}C_A - K_{AB}C_B
\]

As steady state is approached, \( \frac{dC_B}{dt} \rightarrow 0 \), so that,

\[
\frac{C_B}{C_A} = \frac{K_{BA}}{K_{AB}} = \text{BARATIO}
\]

Then

\[
V_A C_A = \frac{V_A C_B}{\text{BARATIO}}
\]

\[
= \frac{V_A}{V_B} \times \frac{V_B C_B}{\text{BARATIO}}
\]

\[
M_A = \frac{V_A}{V_B} \times \frac{1}{\text{BARATIO}} \times M_B
\]

\[
\frac{M_A}{M_B} = \frac{V_A}{V_B} \times \frac{1}{\text{BARATIO}}
\]

We can now calculate the ratio of the masses of labeled cachectin in compartments A and B using the BARATIO, \( V_A \) and \( V_B \) which can be found in Table 4.1. Therefore for each experimental group:
Wt. Gaining: \[ \frac{M_A}{M_B} = \frac{12.571}{9.256} \times \frac{1}{0.718} = 1.892 \]

Wt. Losing: \[ \frac{M_A}{M_B} = \frac{6.973}{10.437} \times \frac{1}{2.094} = 0.319 \]

Oral Controls: \[ \frac{M_A}{M_B} = \frac{8.179}{8.119} \times \frac{1}{1.89} = 0.533 \]
Figure 1 - THE ELUTION PROFILE OF BLUE DEXTRAN - The elution volume of blue dextran was used to determine the void volume of a G-200 Sephadex column. The void volume was 14.8 ml.
Figure 2 - ELUTION PROFILE OF ALBUMIN AND CHYMOTRYPSINOGEN A MARKERS - The elution profile of two low molecular weight markers: Kav Albumin = 0.483, Kav Chymotrypsinogen A = 0.789.

Figure 3. ELUTION PROFILE OF OVALBUMIN AND RIBONUCLEASE A MARKERS - The elution profile of two low molecular weight markers: Kav ovalbumin = 0.448, Kav ribonuclease A = 0.956.
<table>
<thead>
<tr>
<th>Column</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Total Organ Uptake of TNF</td>
<td>192</td>
</tr>
<tr>
<td>2.0</td>
<td>Uptake of TNF per Gram Organ</td>
<td>192</td>
</tr>
<tr>
<td>3.0</td>
<td>TNF Uptake by the EDL</td>
<td>193</td>
</tr>
<tr>
<td>4.0</td>
<td>TNF Uptake per Gram EDL</td>
<td>193</td>
</tr>
<tr>
<td>5.0</td>
<td>TNF Uptake by the Soleus</td>
<td>194</td>
</tr>
<tr>
<td>6.0</td>
<td>TNF Uptake per Gram Soleus</td>
<td>194</td>
</tr>
<tr>
<td>7.0</td>
<td>TNF Uptake by the Diaphragm</td>
<td>195</td>
</tr>
<tr>
<td>8.0</td>
<td>TNF Uptake per Gram Diaphragm</td>
<td>195</td>
</tr>
<tr>
<td>9.0</td>
<td>TNF Uptake by the Heart</td>
<td>196</td>
</tr>
<tr>
<td>10.0</td>
<td>TNF Uptake per Gram Heart</td>
<td>196</td>
</tr>
<tr>
<td>11.0</td>
<td>TNF Uptake by the Spleen</td>
<td>197</td>
</tr>
<tr>
<td>12.0</td>
<td>TNF Uptake per Gram Spleen</td>
<td>197</td>
</tr>
<tr>
<td>13.0</td>
<td>TNF Uptake by the Intestine</td>
<td>198</td>
</tr>
<tr>
<td>14.0</td>
<td>TNF Uptake per Gram Intestine</td>
<td>198</td>
</tr>
<tr>
<td>15.0</td>
<td>TNF Uptake by the Stomach</td>
<td>199</td>
</tr>
<tr>
<td>16.0</td>
<td>TNF Uptake per Gram Stomach</td>
<td>199</td>
</tr>
<tr>
<td>17.0</td>
<td>TNF Uptake by the Lung</td>
<td>200</td>
</tr>
<tr>
<td>18.0</td>
<td>TNF Uptake per Gram Lung</td>
<td>200</td>
</tr>
<tr>
<td>19.0</td>
<td>TNF Uptake per Gram Carcass</td>
<td>201</td>
</tr>
</tbody>
</table>
Figure 1 TOTAL ORGAN UPTAKE OF TNF - The mean uptake of 125I-TNF by all organs and tissues in each of the feeding groups over the experimental period.

Figure 2 UPTAKE OF TNF PER GRAM ORGAN - The mean uptake of 125I-TNF on a per gram dry weight of all organs in each feeding groups over the experimental period.
Figure 3  TNF UPTAKE BY THE EDL - The mean uptake of 125I-TNF by the EDL in the three feeding groups over the experimental period.

Figure 4  TNF UPTAKE PER GRAM EDL - The mean uptake of 125I-TNF per gram EDL in the three feeding groups over the experimental period.
AREA UNDER UPTAKE CURVE

TPN WT LOSE  8.3 + 1.4
TPN WT GAIN   6.1 + 1.7
ORAL FED      4.9 + 0.7

TIME POST TNF INJECTION (minutes)

TPN WT LOSE  TPN WT GAIN  ORAL FED

Figure 5  TNF UPTAKE BY THE SOLEUS - The mean uptake of 125I-TNF by the soleus in the three feeding groups over the experimental period.

AREA UNDER UPTAKE CURVE

TPN WT LOSE  215 + 33
TPN WT GAIN   175 + 48
ORAL FED      130 + 21

TIME POST TNF INJECTION (minutes)

TPN WT LOSE  TPN WT GAIN  ORAL FED

Figure 6  TNF UPTAKE PER GRAM SOLEUS - The mean uptake of 125I-TNF per gram of soleus in the three feeding groups over the experimental period.
Figure 7  TNF UPTAKE BY THE DIAPHRAGM - The uptake of 125I-TNF by the diaphragm in the three feeding groups over the experimental period.

Figure 8  TNF UPTAKE PER GRAM OF DIAPHRAGM - The uptake of 125I-TNF per gram dry weight of diaphragm in each of the three feeding groups over the experimental period.
Figure 9 TNF UPTAKE BY THE HEART - The mean uptake of 125I-TNF by the heart in the three feeding groups over the experimental period.

Figure 10 TNF UPTAKE PER GRAM HEART - The mean uptake of 125I-TNF per gram of heart in the three feeding groups over the experimental period.
Figure 11  TNF UPTAKE BY THE SPLEEN - The uptake of 125I-TNF by the spleen in the three feeding groups over the experimental period.

Figure 12  TNF UPTAKE PER GRAM SPLEEN - The mean uptake of 125I-TNF per gram of spleen in the three feeding groups over the experimental period.
Figure 13  TNF UPTAKE BY THE INTESTINE - The mean uptake of 125I-TNF by the intestine in the three feeding groups over the experimental period.

Figure 14  TNF UPTAKE PER GRAM INTESTINE - The mean uptake of 125I-TNF per gram of intestine in the three feeding groups over the experimental period.
Figure 15  TNF UPTAKE BY THE STOMACH - The uptake of 125I-TNF by the stomach in each of the feeding groups over the three hour experimental period.

Figure 16  TNF UPTAKE PER GRAM STOMACH - The uptake of 125I-TNF per gram dry weight of stomach in each of the three feeding groups over the three hour experimental period.
Figure 17  TNF UPTAKE BY THE LUNG - The mean uptake of 125I-TNF by the lung in the three feeding groups over the experimental period.

Figure 18  TNF UPTAKE PER GRAM LUNG - The mean uptake of 125I-TNF per gram of lung in the three feeding groups over the experimental period.
Figure 19  TNF UPTAKE PER GRAM OF CARCASS - The uptake of 125I-TNF per gram dry weight of carcass in the three feeding groups over the experimental period.
APPENDIX 5 SUMMARY OF HUMAN TNF STUDY
ST. MICHAEL'S HOSPITAL

Background: Cachectin/tumor necrosis factor-alpha (TNF) is a 17kDa polypeptide that is widely accepted to be the prime mediator of the host response to infection and inflammation. While much is known regarding the effect of TNF on the metabolism of nutrients little is known regarding the effect of nutrients on TNF metabolism.

Previous Work: We have previously observed a potentiation of the metabolic response to cachectin by total parenteral nutrition (TPN) but not in anorexic orally fed animals. In addition, TPN fed animals with the most severe metabolic abnormalities had higher circulating levels of TNF. These findings indicated that nutrition support might affect the clearance kinetics of TNF. In our most recent work, we have demonstrated that rats receiving nutrients sufficient to allow growth by TPN had altered TNF clearance kinetics as well as impaired organ uptake of TNF. These weight-gaining TPN fed rats (WGR) had an increased proportion of the total TNF mass in the plasma suggesting a "pooling" of TNF in the plasma. One explanation for our observations would be that the WGR had higher levels of soluble TNF receptors which were binding TNF and retaining it in the plasma. We wish to run a parallel study in humans to examine whether the provision of nutrition support does influence the quantity of circulating soluble TNF receptors.

Hypothesis: 1) That the provision of nutrition support sufficient to meet nutrient requirements will result in the shedding of soluble TNF receptors leading to increased circulating levels of these receptors and decreased TNF cytotoxicity.
2) That the ratio of bound to free TNF will change depending on the route and the nutrient density of the nutrition support.

General Plan: In this prospective study we will use 20 patients identified at the ICU of St. Michael's Hospital as being septic, SIRS, or non-inflammatory. Baseline blood will be drawn from each patient upon entrance into the ICU (non-fed) and on two other occasions once enteral or parenteral feeding has been initiated for measurement of TNF-alpha as well as soluble TNF receptors using commercially available ELISA kits. Ratios of bound to free TNF will be determined by subtracting the soluble TNF receptor concentration from the results of the TNF-alpha ELISA (measures bound and free TNF). Cytotoxicity assays will also be performed on serum samples. Nutrition support will be classified as sub-optimal (<50% of needs) or optimal (>50% of needs). Nutrient needs will be assessed using indirect calorimetry with adjustment for illness. The results will allow both within subject and between subject analysis of the effect of nutrition support on the levels of soluble TNF receptors. The information obtained will be valuable in defining the role of nutritional support and energy intake in groups of patients with critical illness.