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IN VIVO PROTEIN SYNTHESIS DETERMINATIONS IN HUMAN IMMUNE CELLS

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I should now be wiser than I was.
Yet I don't know whether I am wiser.

Czesław Miłosz

ABSTRACT

Intact immune responses are essential for defeating severe infections in individual patients. Insufficient function of the immune system contributes to a poor prognosis in these patients, in particular the ICU patients. Nevertheless, the immune system function is not easily monitored and evaluated. The ongoing metabolic activity of immune competent cells is reflected by their *in vivo* protein synthesis rate. The aim of this thesis was to apply *in vivo* protein synthesis measurement in cells of the immune system, in order to quantitatively characterise the state of their activation. Such measurement may add information on immune cells activation and serve as a tool for evaluation of immune competence in severely ill patients.

The *in vivo* fractional protein synthesis rate (FSR) was determined in the circulating peripheral blood cells: T lymphocytes, mononuclear cells and whole population of leukocytes, as well as in the stationary, unfractioned cells of palatine tonsils.

The FSR in the isolated T lymphocytes of healthy volunteers was 13.6 ± 0.9 %/24 h and was not affected by a 6-h cortisol infusion, either immediately after the end of the infusion or 18 h later. In contrast, a combined stress hormone infusion (cortisol, epinephrine, glucagon), as a human model of surgical trauma, given for 6 h to healthy volunteers decreased the *in vivo* protein synthesis rate in T lymphocytes by 34% from 13.0 ± 1.0 %/24 h to 8.6 ± 2.1 %/24 h. A more accentuated decrease by 53% was observed in the total mononuclear cells, from 13.3 ± 1.2 %/24 h to 6.3 ± 2.0 %/24 h. Following an endotoxin injection, a human model of the initial phase of sepsis, different patterns of the *in vivo* fractional protein synthesis rates were observed in the circulating blood cells of healthy volunteers. The isolated T lymphocytes responded with a 60% decrease of the protein synthesis rate from 9.4 ± 1.2 %/24 h to 3.8 ± 2.4 %/24 h, whereas the whole population of leukocytes showed an increase by 43% from 3.2 ± 1.2 %/24 h to 4.4 ± 1.1 %/24 h. A comparison of the *in vivo* fractional protein synthesis rate between the circulating and stationary immune cells of healthy subjects revealed that unfractioned tonsillar cells had a protein synthesis rate of 22.8 ± 5.7 %/24 h. This rate was higher compared with T lymphocytes, mononuclear cells and leukocytes separated from peripheral blood in these subjects, having *in vivo* protein synthesis rates of 10.7 ± 3.4 %/24 h, 10.8 ± 2.8 %/24 h and 3.2 ± %/24 h, respectively. Alterations in the protein synthesis rates were also observed during the early phase of the critical illness. In a pilot group of intensive care unit patients with a general systemic inflammatory activation, a distinct polarization of the protein synthesis responses was observed. The *in vivo* protein synthesis rate in the mononuclear cells and in the whole population of leukocytes was high, 21.6 ± 7.4 %/24 h and 8.9 ± 4.4 %/24 h, respectively, while that in T lymphocytes (12.5 ± 5.5 %/24 h) and tonsillar cells (27.9 ± 11.4 %/24 h) was not different from what was observed in healthy subjects.

In summary, uniform and characteristic changes of the *in vivo* rate of protein synthesis in response to exogenous stimuli in healthy volunteers and during the early phase of systemic inflammation in critically ill patients were described in the individual populations of immune cells. The *in vivo* protein synthesis determination in the immune cells reflects the state of activation of these cells. This measurement may be used as a tool to obtain additional information on the immune competence in studies concerning function of the human immune system.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-V.

I. **In vivo protein synthesis of circulating human T lymphocytes does not respond to a cortisol challenge within 24 hours.** Januszkiewicz A, Essén P, McNurlan MA, Ringdén O, Wernerman J, Garlick PJ. Acta Anaesthesiol Scand, 2000; 44: 202-209.

II. **Stress hormone infusion decreases protein synthesis of circulating human T lymphocytes.** Januszkiewicz A, Essén P, McNurlan MA, Ringdén O, Garlick PJ, Wernerman J. Metabolism, 2001; 11: 1308-1314.

III. **Response of in vivo protein synthesis in T lymphocytes and leukocytes to an endotoxin challenge in healthy volunteers.** Januszkiewicz A, Loré K, Essén P, Andersson B, McNurlan MA, Garlick PJ, Ringdén O, Andersson J, Wernerman J. Clin Exp Immunol, 2002; 130: 263-270.

IV. **Determination of in vivo protein synthesis in human palatine tonsil.** Januszkiewicz A, Klaude M, Loré K, Andersson J, Ringdén O, Rooyackers O, Wernerman J. Clin Sci, 2005; 108: 179-184.

V. **In vivo protein synthesis in immune cells of ICU patients – a pilot study.** Januszkiewicz A, Klaude M, Loré K, Andersson J, Ringdén O, Rooyackers O, Wernerman J. Manuscript.

Reprints of the above articles were made with the kind permission of the publishers: Blackwell Publishing (paper I and III), Elsevier Science (paper II) and Portland Press (paper IV).

CONTENTS

LIST OF ABBREVIATIONS

APACHE	acute physiology, age, chronic health evaluation
APE	atom percentage excess
BMI	body mass index
CARS	compensatory anti-inflammatory response syndrome
CD	cluster of differentiation
CRP	C-reactive protein
DC	dendritic cell
ENT	ear, nose and throat
FSR	fractional synthesis rate
GC-MS	gas chromatography-mass spectrometry
GM-CSF	granulocyte macrophage colony stimulating factor
HLA	human leukocyte antigen
ICU	intensive care unit
IFN	interferon
Ig	immunoglobulin
IL	interleukin
KIC	alpha-ketoisocaproic acid
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility complex
MNC	mononuclear cell
MODS	multiple organ dysfunction syndrome
MPE	mole percentage excess
NK cell	natural killer cell
PBS	phosphate buffered saline
PHA	phytohemagglutinin
SIRS	systemic inflammatory response syndrome
SRBC	sheep red blood cell
TCR	T-cell receptor
Th cell	T helper cell
TNF	tumor necrosis factor
tRNA	transfer ribonucleic acid
UPPP	uvulopalatopharyngoplasty
WBC	white blood cell

1 INTRODUCTION

Outcome in severe illness depends not only on adequate, goal-directed treatment, but also on the patient's response to the treatment. In particular, the state of immune system is crucial in cases of severe infections. Immune suppression, regardless of the underlying mechanism, is a factor adding to a poor prognosis in patients with severe infections. Existing scoring systems, designed to reflect organ failure and to give prognosis prediction for the patient, do not include any score for the status of the immune system. The reason for that is the absence of such a measure in the same way as it exists for respiration, circulation, coagulation, as well as for liver, kidney and mental function.
In vivo determination of protein synthesis rate in immune competent cells makes it possible to measure and to quantify the ongoing metabolic activity of these cells. Such measurement may add information on activation of various immune cells, making better estimates of immune competence possible. Application of *in vivo* protein synthesis measurements in cells of the immune system, in order to quantitatively characterise the state of their activation, is the main issue of this thesis.

1.1 IMMUNE SYSTEM

The word immune is coming from the Latin *immunis,* which means "freedom from public service" and refers to the exemption from legal prosecution offered to Roman senators during their tenures in office. In medical terms immune implies freedom from foreign agents.
We are surrounded by a range of potentially pathogenic bacteria, viruses, fungi and parasites. In order to protect ourselves, we have a complicated network of cells and molecules, called the immune system. The main task of the immune system is to discriminate between self and non-self (1-3). Recognition of antigens, which are natural constituents of the body, is leading to a state of tolerance, while recognition of foreign antigens mounts responses aimed at elimination of invading pathogens.

1.1.1 Innate and adaptive immunity

There are two different types of response to foreign antigens, the innate and the adaptive systems.
The innate immunity is always present and provides the first line of a non-specific defence against infections. It consists of anatomic, physiologic and inflammatory barriers, such as skin, epithelial cell surface, complement, lysosomes, Toll-like receptors, chemokines and other mediators of inflammation (4,5). The cellular components of the innate immunity comprise phagocyting macrophages and neutrophils, as well as cytotoxic natural killer (NK) cells.
The adaptive immunity is highly specific and provides memory, resulting in a quicker and more effective response following repeated exposures to the pathogen. The adaptive responses include cell-mediated immunity induced by T lymphocytes and humoral immunity comprising production of antibodies by B lymphocytes.

1.1.2 Cells of the immune system

Although the immune responses are mediated by a variety of cells, leukocytes derived from stem cells in bone marrow play a central role. Using histological stains and cell surface molecules (CD markers) identified by monoclonal antibodies, different cell subsets can be distinguished as leukocytes components (Fig. 1).

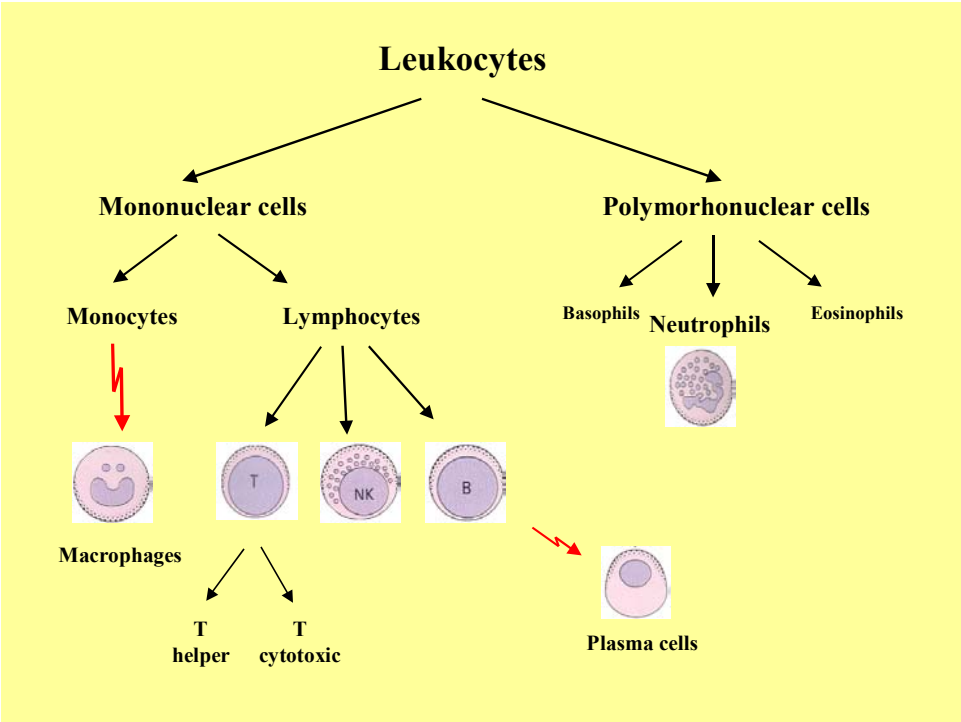

Fig. 1. *Major cell types involved in the immune responses. Upon activation, monocytes differentiate into macrophages, while B lymphocytes differentiate into plasma cells.*

1.1.2.1 T lymphocytes

T lymphocytes, called so as their cell maturation is taking place in the thymus gland, differentiate into two subtypes: helper and cytotoxic T cells. CD4+ helper T lymphocytes activate humoral and cellular responses, while CD8+ cytotoxic T lymphocytes are responsible for destruction of cells infected with viruses or other intracellular pathogens. Functionally, naïve CD4+ helper cells can be subdivided further into two populations based on the pattern of secreted cytokines. Th1 cells, secreting IL-2 and IFN-γ, mediate functions connected with cytotoxicity, delayed-type hypersensitivity and macrophage activation, while Th2 cells, secreting IL-4, IL-5, IL-6, and IL-10, induce humoral responses by stimulating B lymphocytes to antibody production. Both subpopulations, Th1 and Th2, inhibit each other, which enables only one type of response at a time. Common for all T lymphocytes is the presence of a T-cell receptor (TCR) on their surface, which specifically recognizes antigens associated with Major Histocompatibility Complex (MHC) molecules. CD8+ T lymphocytes are activated following recognition of MHC class I molecules, present on all nucleated cells, while CD4+ cells are activated by the antigen presenting cells via MHC II complexes present on monocytes, B cells and endothelial cells. Following activation, T

lymphocytes proliferate and differentiate into a smaller population of memory cells and a more abundant population of effector cells.

1.1.2.2 NK cells

NK cells are large granular lymphocytes, accounting for about 15% of all circulating lymphocytes (6). Capacity for early production of cytokines as well as cytotoxicity without prior stimulation makes them crucial components of the innate immune system. They play an important role in killing virus-infected cells and tumour cells, which lack MHC class I molecules.

1.1.2.3 B lymphocytes

B lymphocytes, representing about 5-15% of circulating lymphocytes, are responsible for the humoral immunity. Upon activation by helper T cells and recognition of the specific antigen, B lymphocytes proliferate and differentiate into plasma cells, producing large amounts of antibodies directed against the pathogen carrying this specific antigen. An interaction between the antibodies and the antigen is leading to activation of immune responses resulting in elimination of the pathogen.

1.1.2.4 Monocytes

Monocytes are circulating cells, which upon migration into the tissue differentiate into macrophages. As components of the mononuclear phagocyte system, they play an important role in phagocytosis and killing of microorganisms. Following activation, monocytes release cytokines and other mediators, starting a cascade of pro-inflammatory responses (7). They are professional antigen-presenting cells, which process antigens and present them to T or B lymphocytes via MHC class II receptors and thereby initiating the adaptive immune responses.

1.1.2.5 Neutrophils

Neutrophils are main components of polymorphonuclear granulocytes and the most abundant of the leukocytes. Responsible for the first line of defense, neutrophils migrate into the inflammatory site and kill the invading microorganisms by releasing proteolytic enzymes and oxygen radicals (8,9). The capacity to synthesize cytokines implies their role in regulating immune responses (10).

1.1.3 The lymphoid system

The lymphoid system comprises the cells involved in the immune responses, arranged as tissues and organs. The primary lymphoid organs include thymus and bone marrow, whereas the secondary lymphoid organs consist of spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT). MALT is a collection of non-capsulated lymphoid tissues present in respiratory, gastrointestinal and urogenital tract.

1.1.3.1 The palatine tonsils

The palatine tonsils are the pharyngeal components of the MALT. They consist of B lymphocytes as well as both phenotypes of T lymphocytes, CD4+ helper and CD8+ cytotoxic cells. As macrophages are present in a low number, dendritic cells (DCs) play a main role as antigen presenting cells.
Histologically there are three compartments with a typical composition of immune cells and distinct functions: lymphoepithelium, extrafollicular area and lymphoid follicles (11-13) (Fig.2). The tonsillar surface, formed by crypts and covered by lymphoepithelium, is a place of antigen uptake by M-(membrane) cells. In the extrafollicular area, populated mainly by T lymphocytes, the antigen is presented by DCs to T lymphocytes. The successful antigen recognition results in T cells activation, differentiation and proliferation. In the third compartment, lymphoid follicles, B lymphocytes upon activation by T lymphocytes proliferate and differentiate into memory cells and immunoglobulin-producing plasma cells. The presence of cytokines, secreted by the antigen presenting cells and T lymphocytes, as well as receptor-co-receptor interactions are necessary for the whole process of activation (14,15).

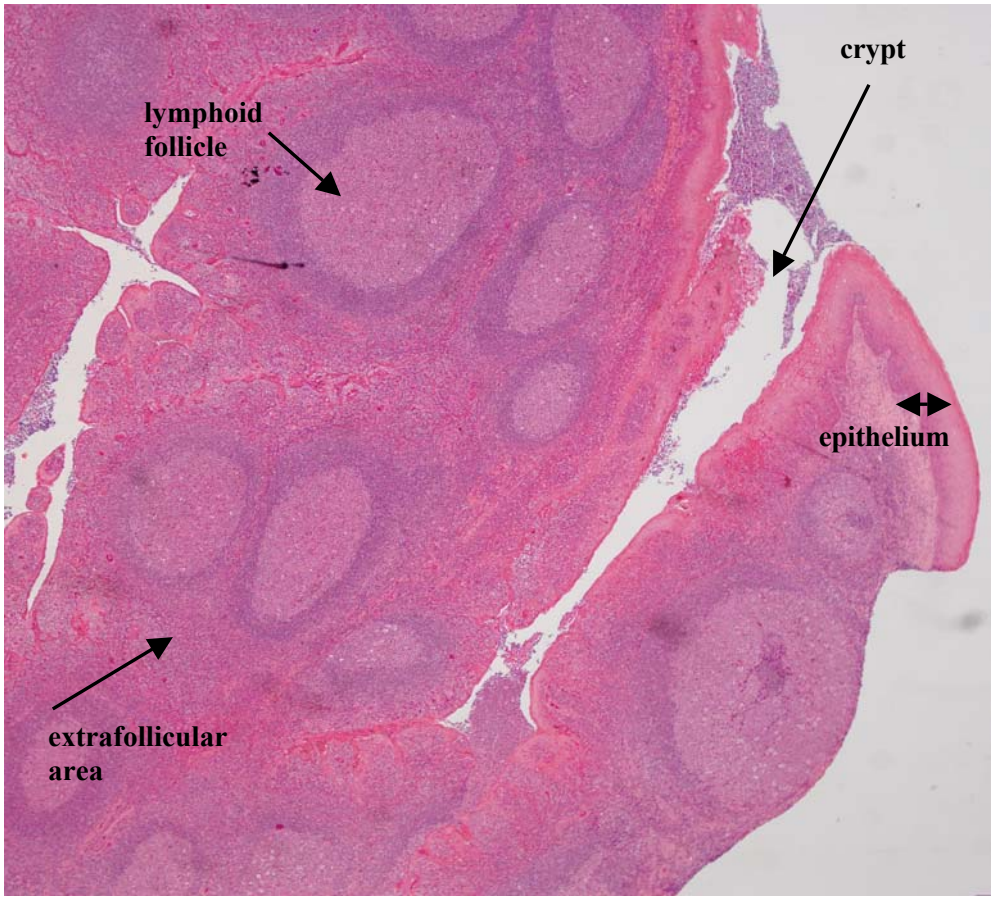

Fig. 2. *The microscopic picture of the human palatine tonsil demonstrating the characteristic compartments. Epithelium covering the crypt, being the place of antigen uptake, extrafollicular area populated mainly by T lymphocytes and lymphoid follicles consisting predominantly of B lymphocytes.*

1.2 IMMUNE RESPONSES

Injury is leading to activation of local responses, which are of balanced pro- and anti-inflammatory character (9,16). In most cases it results in resolution of inflammation and bringing back homeostasis. However, failure to restore a balance initiates a massive systemic reaction defined as a systemic inflammatory response syndrome (SIRS). This pro-inflammatory state is characterized by activation of the innate immune system and the release of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6 (17-19). SIRS is early counterbalanced by a compensatory anti-inflammatory response syndrome (CARS). In immunological terms CARS is characterized by upregulation of anti-inflammatory cytokines (IL-4, IL-10), impaired antigen presenting capacity of monocytes, T-cells anergy and diminished T-cells proliferation, all leading to suppressed immunity (19,20).

In some patients an overwhelming and prolonged pro- or anti-inflammatory phase is observed. An excessive activation of pro-inflammatory cytokines, endothelium, complement as well as coagulation system leads to hypotension and inadequate organ perfusion, resulting in an early multiple organ dysfunction syndrome (MODS). On the contrary, a sustained anti-inflammatory response is associated with immune suppression and decreased resistance to nosocomial and opportunistic infections, leading to late MODS, often seen in long-term critically ill patients (16,21).

1.3 MONITORING OF THE IMMUNE SYSTEM

Monitoring of vital organs is a necessary tool for adequate treatment of ICU patients, since insufficient function of these organs is leading to multiple organ failure, with a high mortality rate despite all advances in the intensive therapy. While monitoring of organ function, such as lung, liver, kidney, etc. is well established, monitoring of the immune system function is insufficient (22). The balance of inflammatory responses can vary between pro- and anti-inflammatory phases leading to unexpected alterations in a patient's condition. In addition, despite failed clinical trials with the anti-inflammatory interventions directed against TNF-α and IL-1 (23,24), immunomodulatory therapies are still appealing, following a successful trial with pro-inflammatory mediators in immune-depressed sepsis patients (25,26). However, the choice of such an immunomodulatory therapy has to be adjusted to the inflammatory phase, which implies thorough immunomonitoring.

In clinical practice, white blood cell (WBC) count and C-reactive protein (CRP) are routinely used as markers of inflammation. Both rise in response to infections, but also due to non-infectious events, such as surgery, trauma, bleeding, stroke and myocardial infarction (27-30). On the other hand, no response or minor elevations of WBC or CRP are observed during some viral or chronic infections (29). Both leucopenia and leucocytosis are components of the APACHE II scoring system and their presence in the acute phase of disease is associated with poor outcome in critically ill patients (31).

Parameters clinically widely used to describe the immunological status include absolute cell counts, as well as proportion and absolute counts of cell populations and subpopulations. A decrease in T-cell subsets reported in response to trauma, infections, sepsis or burns (32-35) may reflect the transient migration of circulating cells into the site of injury and not necessarily an impaired function of these cells. Furthermore, there

is no correlation between the lymphocytes count and severity of illness or mortality rate (33). The lymphopenia and altered lymphocyte subset distribution is also observed in subjects not exposed to injury, such as patients with primary cancer or healthy volunteers in response to a short-term hyperglycemia (36,37). In contrast, a rise in monocyte count is observed in patients exposed to trauma, surgery or sepsis (38-40).
Cell activation can also be reflected by the increase or by the *de novo* appearance of activation markers, which are surface molecules expressed only on activated or dividing cells. One of the best characterized markers is HLA-DR (MHC class II), which is upregulated on the antigen presenting cells as well as expressed on a subpopulation of activated T cells. Persistent low HLA-DR expression on monocytes is proposed as a marker of CARS and a predictor of poor outcome in patients with severe sepsis (7,22,40-42). However, some investigators found contradictory results (43,44), questioning the value of HLA-DR as a single parameter characterizing the immunulogical status (45).
The pattern of secreted cytokines is another marker commonly used for the purpose of immunomonitoring. High concentrations of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6 as well as anti-inflammatory IL-10 are detected early following injury (17,19,46,47). However, interpretation of plasma cytokine levels can be difficult, because of their short half-life as well as the presence of soluble receptors or inhibitors (48,49). In addition, concentrations of cytokines may differ between the systemic circulation and the site of injury (50,51).
In vitro techniques have been widely used to measure responsiveness of the immune system. Skin tests or proliferative responses in unstimulated cells or cells stimulated with different mitogens are related to the impaired cell activity observed in patients following major surgery or in critically ill patients (52-55). However, a large intraindividual variation in response to mitogenic stimulation and a poor reproducibility are well-known drawbacks of these *in vitro* methods (56,57).

1.4 *IN VIVO* PROTEIN SYNTHESIS DETERMINATION AND IMMUNOMONITORING

An adequate function of the immune system requires rapid shifts from the ”stand-by” position into full activity. In the state of health, most immune cells are quiescent. However, the constant exposition to foreign antigens implies the need for continuous scanning in order to detect harmful signals and generate the immediate responses.
The defence mechanisms in the early phase of injury include activation of the innate immune system. The first phagocytic cells recruited into the site of injury are neutrophils, which produce and release reactive oxygen intermediates, a variety of proteolytic enzymes and immunoregulatory cytokines (10). In parallel, monocytes, which upon migration into the tissue transform into macrophages, synthesize cytokines and other mediators of inflammation. As professional antigen presenting cells, they present foreign antigens on the MHC II molecules to T lymphocytes. In addition, due to the ability to produce cytotoxic agents, they are involved in phagocytosis (3). Adaptive immunity, which is more specific, requires some days to become effective. Upon activation by antigen presenting cells, T lymphocytes proliferate and differentiate into effector cells. Depending on the nature of the triggering signal, differentiation results in cell-mediated immunity and/or in antibody production by activated B lymphocytes.

All immunologic events, such as synthesis of cytokines or other regulatory mediators, production of enzymes, receptors and immunoglobulins, cell differentiation and proliferation are protein demanding. In metabolic terms, this means varying activity in synthesis of both structural and export proteins and can be quantitatively determined by measuring the *in vivo* rate of protein synthesis.

The i*n vivo* fractional protein synthesis rate was for the first time quantitatively determined in human mononuclear cells of patients with metastasic colorectal cancer (58). The rate of protein synthesis is lower in these patients as compared with healthy control subjects. Following a 5-days treatment of the patients with recombinant IL-2, a three-fold increase in the *in vivo* fractional protein synthesis rate is observed. Also an increased *in vivo* fractional synthesis rate in the mononuclear cells is demonstrated 24 h after cholecystectomy in otherwise healthy patients (59). In contrast, in mononuclear cells of healthy volunteers exposed to a 6-h combined stress hormone infusion (epinephrine, cortisol and glucagon), as a model for surgical stress, a decrease is observed immediately after the end of the infusion, followed by normalisation at 18 h after cessation of the infusion (60).

The possibility to perform *in vivo* measurement of protein synthesis in cells of the immune system was appealing. It gave the opportunity to assess the *in vivo* metabolic activity, which may reflect the immune activity and competence. In these first studies changes in the metabolic activity of circulating mononuclear cells following immunostimulation and surgical stress were observed. This raised further questions about the activity of individual cell populations and the effects of other types of injury.

2 AIMS OF THE STUDY

The overall aim of the thesis was to further investigate the *in vivo* protein synthesis rate in cells of the immune system as a reflection of their activation. Initially the protein synthesis rate was studied in circulating blood cells. Henceforth, the *in vivo* protein synthesis determination was extended to lymphoid tissue, represented by the palatine tonsils.
The specific aims of the respective studies were:

1. to establish a method for separating the pure population of T lymphocytes convenient for the purposes of the *in vivo* protein synthesis determination (study I),

2. to determine the *in vivo* fractional protein synthesis rate in circulating T lymphocytes and mononuclear cells of healthy volunteers in response to an infusion of cortisol alone or as a part of a stress hormones cocktail, mimicking surgical trauma (study I and II),

3. to investigate the effect of endotoxin, as a human model for the initial phase of sepsis, on the *in vivo* fractional protein synthesis rate in T lymphocytes and in the whole population of leukocytes (study III),

4. to compare the rates of *in vivo* fractional protein synthesis between circulating peripheral blood cells (T lymphocytes, mononuclear cells, leukocytes) and cells of secondary lymphoid organs, represented by the palatine tonsil (study IV),

5. to determine the *in vivo* fractional protein synthesis rate in circulating as well as in stationary immune cells, in a pilot group of intensive care unit patients, well characterized by clinical and immunological parameters (study V).

3 MATERIALS AND METHODS

3.1 SUBJECTS

A total of 56 healthy volunteers participated in studies I-III. Patients (n=12) included in study IV were healthy apart from minor ear, nose or throat pathology, being the reason for the operation. In study V, patients (n=20) staying in the general intensive care unit (ICU) on ventilator treatment due to medical or surgical diseases, were investigated during their first week after admission. All subjects, except for the ICU patients, were studied in the morning after an overnight fast. Characteristics of all the participating subjects are given in Table 1. Detailed characteristics of the ICU patients are presented in paper V.

The purpose of the studies and possible risks were explained to all volunteers and patients (or their legal representatives if communication with the patient was not possible) before obtaining their consent. The research protocols were approved by the Ethical Committee of the Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden.

Table 1. *Characteristics of the healthy volunteers and patients participating in the studies on the in vivo protein synthesis rates in the cells of immune system. Means and SD. ENT, ear, nose and throat.*

	Study		Age (years)	Sex	Weight (kg)	Height (cm)	BMI (kg/m^2)
I	Cortisol	n=12	28 ± 7	12 M	75 ± 8	178 ± 7	23.5 ± 1.9
	Control	n=12	31 ± 9	12 M	75 ± 10	180 ± 9	23.2 ± 2.0
II	Stress hormones	n=8	34 ± 8	8 M	78 ± 15	182 ± 9	23.3 ± 4.2
	Control	n=8	28 ± 5	8 M	80 ± 10	181 ± 8	24.4 ± 1.8
III	Endotoxin	n=8	27 ± 1	8 M	83 ± 8	185 ± 6	24.4 ± 2.3
	Control	n=8	28 ± 4	8 M	73 ± 12	179 ± 8	22.7 ± 2.0
IV	ENT patients	n=12	44 ± 13	6M/6F	74 ± 12	171 ± 13	25.1 ± 3.0
V	ICU patients	n=20	60 ± 13	10M/10F	87 ± 15	173 ± 11	29.2 ± 4.5

3.2 MODELS OF SURGERY AND SEPSIS

A triple-hormone infusion and an endotoxin injection in healthy volunteers were used as human models of surgical trauma (study II) and sepsis (study III) respectively. These models were used in order to study the effects of the respective injury on *in vivo* protein synthesis in circulating immune cells.

A combined epinephrine, cortisol, and glucagon infusion in healthy volunteers is a well-established model of surgical trauma (60-62). This triple-hormone cocktail generates plasma hormone concentrations comparable to those found during major surgery and mimics metabolic changes observed after surgery or trauma (63-65). Given for 6 h, a combined stress hormones infusion is well tolerated by volunteers who, apart from the elevated heart rate per minute, do not experience any discomfort (study II).
Endotoxin, a component of the cell wall of Gram-negative bacteria, is an important factor in the pathogenesis of Gram-negative sepsis (66). Given in a low dose to healthy volunteers, endotoxin produces a reproducible systemic inflammatory response mimicking the initial phase of the acute response to sepsis (67). The subjects present the same time-course of subjective and objective symptoms following endotoxin administration, however, with a varying intensity. Headache, chills, nausea and muscle pain begin about 60 min after the endotoxin injection ebbing after 1-2 h. Fever appears at approximately 90 min and may last for some hours (68). These symptoms are accompanied by hemodynamic reactions including an increase in cardiac index and heart rate, a decrease in mean arterial pressure and systemic vascular resistance (69). Immunological responses include shifts in cell counts, activation of monocytes and neutrophils, changes in lymphocytes subsets toward a Th2 response, as well as an increase in both pro- and anti-inflammatory cytokines (68,70-73).

3.3 EXPERIMENTAL PROTOCOLS

Studies I-III were interventional studies in healthy male volunteers (n=24+16+16). They were randomised to receive: a cortisol (6 μg/kg/min) or saline infusion (study I), a combined stress hormone (epinephrine at 0.5 nmol/kg/min, cortisol at 6 μg/kg/min and glucagon at 3 ng/kg/min) or saline infusion (study II) and an endotoxin or saline injection (study III). Cortisol and stress hormones were given as a 6-h infusion, whereas endotoxin was administered as a single intravenous injection. All studies started with the 90-min determination of baseline protein synthesis and were followed by the pharmacological intervention. The second 90-min determination of protein synthesis was performed immediately after the end of the cortisol/saline infusion or 18 h later (study I), immediately after the 6-h stress hormone/saline infusion (study II) and 2.5 h after the endotoxin/saline injection (study III).
Studies IV and V were descriptive with a single determination of *in vivo* protein synthesis in circulating blood cells and in the tonsillar cells.

3.4 THE *IN VIVO* FRACTIONAL PROTEIN SYNTHESIS RATE

In vivo protein synthesis rates in human tissues can be measured by incorporation of labelled amino acids into the protein of interest. The method is based on the assumption that free amino acids enter the intracellular amino acid pool, which is the precursor reservoir for protein synthesis (Fig.3). The aminoacyl-tRNA, being an immediate precursor for polypeptide synthesis, may be supplied with amino acids not only from the intracellular pool, but also directly from protein degradation or directly from plasma. However, the amino acid trafficking within the cells is not equally distributed. It is speculated that amino acids from the protein degradation are preferentially directed to the tRNA pool, while amino acids from the plasma enter primarily the intracellular pool, which has been called a functional compartmentalization of amino acids pools

(74). In addition, the direction of changes is depending on the fasting or food conditions.
Administration of labelled amino acids makes them available as precursors for protein synthesis. Measuring the fraction of labelled amino acids incorporated into the protein of interest over time in relation to the fraction of labelled amino acids in the precursor pool enables calculation of the fractional synthesis rate. The constant infusion and the flooding techniques are two available approaches to administer labelled amino acids for the incorporation into proteins.

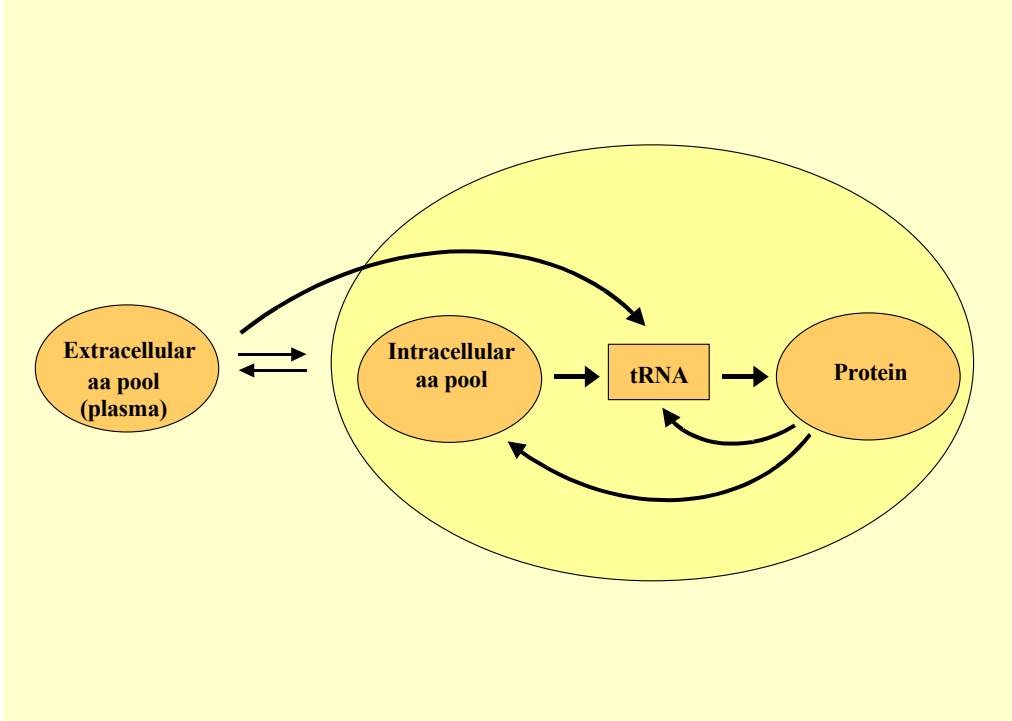

Fig. 3. *Free extracellular amino acids enter the intracellular amino acid pool, being the precursor reservoir for protein synthesis. The aminoacyl-tRNA, an immediate precursor of de novo synthesized proteins, may be also supplied with amino acids directly from protein degradation or directly from plasma.*

3.4.1 The constant infusion technique

The constant infusion technique has been extensively used for whole body protein turnover and for the tissue protein synthesis measurements in animal and human studies. With this method the labelled amino acid is given as a continuous intravenous infusion until a steady state is obtained in the precursor pool. In order to reduce the time of achieving an isotopic steady state, the constant infusion is often preceded by an intravenous priming dose of the tracer.
One of the main disadvantages of the constant infusion method is the difficulty to control the isotopic enrichment of the amino acids in the true precursor pool for protein synthesis. The optimal approach is to measure the isotopic enrichment in the

intracellular aminoacyl-tRNA. However, the very low concentration of tRNA and the high rate of turnover, makes this measurement technically difficult (75). Instead other precursor pools, such as the enrichment in plasma or enrichment of transamination products of the labelled amino acids, are used as surrogate measures of the true precursor pool. However, it has been shown that enrichments of leucine and α-ketoisocaproic acid (KIC, the product of deamination of leucine) in plasma are higher than those of aminoacyl-tRNA and tissue free leucine in skeletal muscle (74). In consequence, using plasma leucine or KIC enrichments for protein synthesis calculations underestimates the protein synthesis rates. The tissue intracellular free amino acid pool enrichment is very similar to aminoacyl-tRNA enrichment and technically reasonably easy to measure. Therefore tissue free amino acid pool is the best substitute for the aminoacyl-tRNA when applying the constant infusion technique. A particular difficulty is that the relation between plasma and tissue enrichments is variable, due to physiological fluctuations or interventions, and not predictable.
Another drawback of the constant infusion method is that a relatively long study period is needed to allow an isotopic steady state to be reached. As the method requires not only isotopic, but also metabolic steady state during the whole study, the prolonged study time may be a limitation in studies involving critically ill patients with unstable conditions or during surgical procedures. The prolonged study time can potentially also give a problem with recycling of the tracer. During the incorporation period protein degradation releasing the tracer again occurs, leading to the reappearance of the labelled tracer amino acid in the precursor pool. In addition, in the tissues with high protein turnover, protein synthesis rates may be underestimated because of the escape of export proteins during the labelling period.

3.4.2 The flooding technique

To overcome the problems with the precursor pool enrichment and long study times, the flooding technique has been developed. In this approach a large dose of both unlabelled and labelled amino acid is administered intravenously as a bolus over a short period of time (76). Due to that overabundance of the given amino acid, all possible amino acid pools are reached and equilibrated rapidly, including the true precursor pool, aminoacyl-tRNA, and an isotopical equilibration is established. Indeed, the assumption on equilibration between plasma, the tissue free amino acid pool and aminoacyl-tRNA has been confirmed, which enables using the plasma as a valid substitute of the true precursor pool (77,78). Thus, using the flooding method, the problem with measuring the true precursor pool for protein synthesis is avoided. Another advantage of the flooding approach is a relatively short study time, making determination of protein synthesis possible within 30-90 min. Consequently, the method is more suitable for studies in unstable conditions, as well as in tissues with a high secretory activity. The short study period also minimizes the problem of recycling of the labelled amino acids.

The drawback of the flooding method is that the large dose of the amino acid gives an elevated concentration of the amino acid in plasma and tissues. This may interfere with protein synthesis rates. It has been pointed out that fractional synthesis rates measured with the flooding technique were higher when compared with the constant infusion technique (79). In particular a flood of leucine has been suggested to stimulate the protein synthesis rate in human skeletal muscle (80). However, the discrepancies were mainly attributable to poor control of the precursor pool in the early studies employing the constant infusion technique. A comparison of the FSR in human skeletal muscle measured by flooding with leucine or phenylalanine, shows similar rates of protein synthesis (81). Furthermore the comparison between the two techniques, when the precursor pool has been adequately equilibrated during a constant infusion, shows identical results (82). The main remaining problem with the flooding technique is that the labelled and incorporated amino acids are not fulfilling the criteria for being a true tracer.

3.4.3 Determination of the FSR by the flooding technique

In studies I-V the flooding technique was applied to determine *in vivo* fractional protein synthesis rates in immune competent cells. The main reason for choosing this approach, instead of the constant infusion, is a short study time. Cells of the immune system are capable to rapid changes in their immunological activity. By measuring protein synthesis with the flooding method and avoiding the problems with recycling of amino acids and disappearance of secreted proteins, we are more likely to capture the corresponding changes in metabolic activity of immune cells. Furthermore, labelled amino acids in plasma can be used as a reliable estimate of the true precursor pool. With the constant infusion technique, the intracellular amino acid pool would be the most correct surrogate of the aminoacyl-tRNA. However, to measure the intracellular amino acid pool in immune cells is technically difficult due to the limitations in both the volume of blood samples and size of tonsil biopsies.

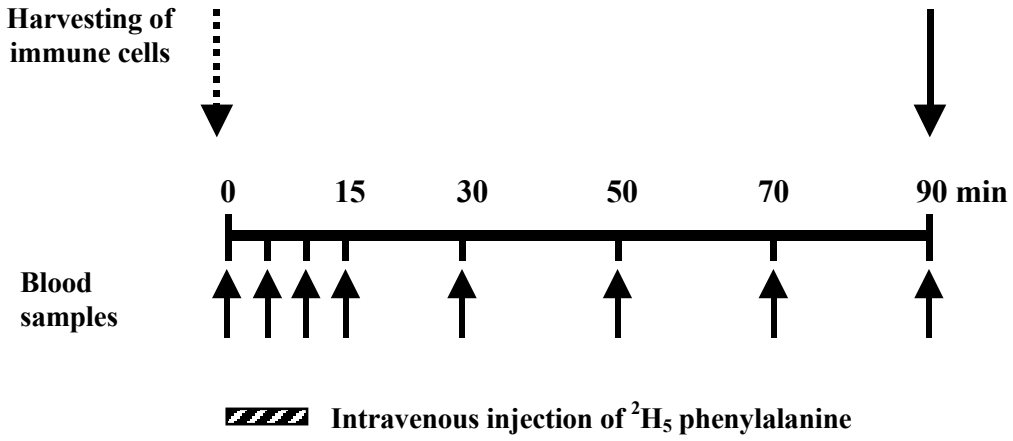

Fig. 4. *Schematic study protocol for the flooding technique for the determination of the fractional protein synthesis rate in immune competent cells.*

In vivo protein synthesis of human lymphoid tissue represented by the palatine tonsil was for the first time determined in studies IV and V. Preparation of the tonsillar specimens for mass spectrometry analysis for protein synthesis determination was similar to that of muscle tissue, including freeze-drying enabling removal of connective tissue and blood.
The general protocol for sampling of immune cells and blood for the purpose of determination of in *vivo protein* synthesis rates is presented in Fig. 4, whereas the detailed descriptions are presented in papers I-IV.

3.5 CELL SEPARATION

Preceding study I, available methods for T lymphocyte isolation were tested and validated. The aim was to find a method, which would give a high purity of T cells, without plasma protein contamination and without interference with the mass-spectrometry analysis. The magnetic cell separation methods: MACS Microbeads and Dynal® were evaluated. Unfortunately, in both cases difficulties in removing the beads coated with antibodies resulted in the presence of magnetic particles in the samples, which would disturb mass spectrometry. In addition, the presence of antibody protein would interfere with protein synthesis calculations. Besides, the cells were isolated from a relatively large blood volume, which made both methods time-consuming and unpractical. For the same reasons the separation technique with the fluorescence-activated cell sorter was not chosen.
Isolation of T lymphocytes by rosetting with sheep erythrocytes is an old and well-established technique (83). The method is based on the presence of receptors for sheep erythrocytes on the surface of human T lymphocytes (84). Lymphocytes become surrounded (rosetted) with the red cells and can then be isolated by density gradient centrifugation. In our hands, the purity of the T lymphocyte population separated with the rosette method is 90-95% as verified by flow cytometry.
Mononuclear cells (MNC) were obtained by density gradient centrifugation, whereas leukocytes were isolated by lysing erythrocytes from the whole blood samples.
The details on T lymphocyte, MNC and leukocyte separation procedures are presented in papers I-IV.

3.6 TONSIL BIOPSIES

Tonsil biopsy is a well-documented technique, which relatively easily enables access to lymphoid tissue even on an outpatient basis (85). This technique was applied in study IV and V in order to compare the *in vivo* fractional synthesis rates in circulating cells of the peripheral blood with that of stationary cells of the lymphoid tissue. The biopsies were taken with a punch forceps and no complications due to bleeding were observed. The procedure of tonsil biopsy in ICU patients turned out to be technically more difficult as compared to that in healthy patients, despite help from colleagues from the ENT Department. One of the explanations was the fact that ICU patients, although receiving sedatives and analgesics, were not muscle relaxed compared with the subjects in study IV. Besides, one of the features of the early phase of illness is a general edema, due to capillary leakage as well as fluid supply, which is a part of the intensive treatment. Owing to all those factors visualization of the palatine tonsils was much more difficult in the ICU patients.

3.7 PHENOTYPIC CHARACTERIZATION OF CELLS

In order to characterize the immunological status of the studied subjects, flow cytometric analysis was performed in studies II, III, IV and V. Cell surface expression for various characteristic receptors exclusively expressed on the different immune competent cell types in peripheral blood and in palatine tonsils was determined. Also expression of markers associated with differentiation or activation stage on these cells was studied. The detailed method descriptions are presented in the respective papers. To find out if there was a relationship between the phenotypic and metabolic manifestations of activation, the expressions of activation markers were related to the *in vivo* fractional protein synthesis rates.

3.8 PLASMA CYTOKINES

Plasma cytokine concentrations were determined in studies III and V as part of the characterization of the immunological status in the investigated subjects. In study III the sandwich enzyme-linked immunosorbent assay (ELISA) was used. In study V plasma cytokines were analysed using the multiplex bead array assay. This relatively new method permits simultaneous flow cytometric quantitation of multiple cytokines by capturing them onto the beads labeled with fluorophores and coated with antibodies, specific for the cytokines of interest (86). The detailed descriptions of the plasma cytokines analyses are presented in papers III and V.

3.9 *IN VITRO* PROLIFERATIVE RESPONSES

In study II the effect of the stress hormone infusion on the *in vivo* fractional synthesis rate in circulating cells of healthy volunteers was investigated. The question was addressed if there was a relationship between the *in vitro* proliferative responses and *in vivo* determination of the protein synthesis rate. *In vitro* proliferative responsiveness was determined in unstimulated (spontaneous proliferation) as well as phytohemagglutinin stimulated (mitogenic proliferation) mononuclear cells. Detailed description of these *in vitro* methods is presented in paper II. Technical problems with the isolation and the culturing of the mononuclear cells, resulted in exclusion of 6 out of 16 subjects. In the unstimulated group, the combined stress hormone infusion induced an enhanced proliferative response in some subjects, but the change was not uniform. Following the phytohemagglutinin stimulation, the tendency to a decreased mitogenic proliferation was observed in both groups. Taken together, the *in vitro* results were not conclusive. Rather discrete changes in the *in vitro* proliferative responses did not correspond to the 50% decrease of *in vivo* protein synthesis in mononuclear cells following the stress hormones challenge.

3.10 INTRACELLULAR CYTOKINES

In study III healthy volunteers were exposed to an endotoxin injection stimulating, among other effects, the release of cytokines. In order to investigate if there is a relationship between the *in vivo* protein synthesis in circulating peripheral blood cells and the production of cytokines, we used the immunocytochemical staining, which enables detection of intracellular cytokine expression (87). The method is described in

detail in paper III. The cytokine-expressing cells were analysed in the whole population of peripheral blood leukocytes without previous stimulation *in vitro*. We found undetectable expression or very few expressing cells (<1/10 000 cells) for the studied cytokines irrespective of saline or endotoxin injection. A common approach for detection of cytokine producing cells is to culture the cells *in vitro* with Brefeldin A or Monensin in addition to the stimulus. This step is necessary in order to inhibit secretion of cytokines and instead allow for accumulation of cytokines within the cytoplasm of the producing cells. However, this approach is not possible to use *in vivo*. Therefore the amount of cytokines within producing cells may have been too low for detection by immunochemistry in study III. However, the more likely explanation for our lack of finding cytokine-expressing cells among the leukocytes in the subjects who received endotoxin, is that all activated cells had left the circulation and migrated into the site of injury or to the draining lymph nodes.

3.11 STATISTICAL METHODS

Data are presented as means and standard deviations (SD) (studies I-IV) or medians and ranges (studies IV, V). Fractional synthesis rates in the circulating cells within and between the groups were compared using Student's t-test for paired and unpaired samples respectively (studies II-III) or ANOVA (study I). Other multiple comparisons within the group were tested with one-way ANOVA and between the groups with two-way ANOVA (study II, III), using Bonferroni's correction in study III. In study IV comparative statistics of CD markers between the tonsillar and blood cells were performed using the non-parametric Wilcoxon rank sum test. Correlations were calculated using a regression analysis or Spearman's test.

4 DISCUSSION OF RESULTS

4.1 THE *IN VIVO* FRACTIONAL PROTEIN SYNTHESIS RATE IN THE CIRCULATING CELLS

Determination of protein synthesis was performed in circulating peripheral blood cells: purified T lymphocytes (studies I-V), total mononuclear cells (studies II, IV, V) and the whole population of leukocytes (studies III-V). The use of peripheral blood cells in studies on function and activity of the immune system has been critisized. Indeed, circulating lymphocytes represent only about 2% of the total lymphocyte pool in the normal, human body (88). However, in human studies the possibility to sample material from the lymphoid organs is rather limited. Thus, despite the fact that circulating cells may not reflect alterations in the whole lymphoid tissue, blood samples still remain the main source of information on the function of the human immune system.

4.1.1 FSR in T lymphocytes

For the first time the *in vivo* rate of protein synthesis was determined in circulating human T lymphocytes in a total of 50 healthy male volunteers (studies I-III) and in 31 patients (Fig. 5). Out of the 31 patients, 11 were healthy adults (study IV), whereas the subjects investigated in study V (n=20) were ICU patients.

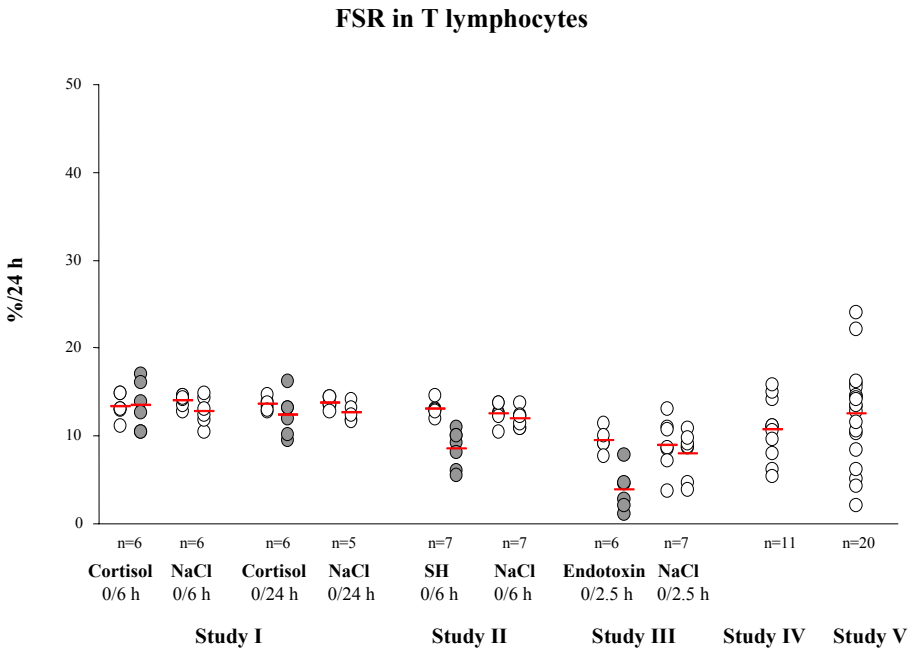

Fig. 5. *The fractional protein synthesis rate in the isolated population of T lymphocytes determined in healthy volunteers (studies I-III), healthy ENT patients (study IV) and ICU patients (study V). Circles represent individual values, horizontal lines represent means. Open circles represent basal values, filled circles represent values following an intervention (cortisol or stress hormone (SH) infusion or endotoxin injection). ENT, ear, nose and throat.*

The results of the *in vivo* protein synthesis determination in T lymphocytes showed that the metabolic activity of this cell population was similar in healthy volunteers and in patients. In contrast, distinct decreases in the rate of *in vivo* protein synthesis rate were observed in two of the interventions groups, e.g. in subjects who received a 6-h stress hormone infusion (study II) and in subjects exposed to an endotoxin injection (study III).

The mean value of the *in vivo* fractional synthesis rate in circulating T lymphocytes was approximately 12%/24 h. This is a relatively high rate, compared with that of human skeletal muscle having a fractional rate of protein synthesis of only 2%/24 h (89) and the human liver, producing both stationary and export proteins, which has a fractional synthesis rate of approximately 24%/24 h (90). *In vivo* fractional synthesis rate comparable with our results is reported in a study where lymphocytes were separated from monocytes using iron particles (91). T lymphocytes play an important role in maintaining the homeostasis of the immune system. Memory T cells continuously migrate via blood to lymphoid and non-lymphoid organs scanning for foreign antigens and alerted to immediate responses in case of recognition of a non-self antigen (92). A number of mediators and chemokines are necessary to enable the different steps of this T lymphocytes traffic, such as adhesion to endothelium or transmigration into the tissue (93). Production of these factors may be a considerable contribution to the protein synthesis rate observed in T lymphocytes under physiological conditions.

The scatter in the rates of FSR in T lymphocytes was relatively low in healthy subjects (studies I-III), but was higher in the investigated patients. This was not suprising in the ICU group (study V), being heterogeneous considering diagnoses, age, APACHE II, type of infections, etc. A possible explanation for the greater scatter in patients with minor ear, nose, throat pathology might be that the age distribution was wider and that the median age was higher compared to the subjects in studies I-III. Known age-associated alterations include changes in immune cells composition, accompanied by varying, both diminished and enhanced, functional activity of the immune system (94). Thus we cannot exclude that ageing is associated with alterations in the rate of *in vivo* protein synthesis in circulating T lymphocytes. Another explanation could be the difference in gender, as both male and female were included into study IV. Gender differences in the innate and adaptive immune system have been reported in humans (95). However, the *in vivo* protein synthesis rates in the T lymphocytes were not different between the men and females participating in study IV.

4.1.2 FSR in MNC

The *in vivo* fractional rate of protein synthesis in the total population of mononuclear cells was determined in a total of 45 subjects (Fig. 6). The results showed that FSR was similar in volunteers and in healthy ENT patients (studies II, IV). In the group of ICU patients (study V) the rates of protein synthesis were consistently higher, with the lowest value corresponding to the mean value in the healthy subjects. A 6-h stress hormone infusion resulted in a decrease of the mononuclear cell *in vivo* protein synthesis rate (study II).

The population of total mononuclear cells was isolated by gradient centrifugation and in healthy subjects it consisted of approximately 60-70% T lymphocytes, 10% B lymphocytes and 20-30% monocytes (96). The protein synthesis rate was comparable to that in the circulating T lymphocytes, suggesting that protein synthesis in both

monocytes and B lymphocytes was of the same magnitude as in the T cells. There is no data available on the *in vivo* protein synthesis rate in B cells, but considering the different functions of T and B lymphocytes, it cannot be excluded that the metabolic activity of B lymphocytes in unstimulated healthy subjects is lower compared with the T cells. In contrast, monocytes, as antigen presenting cells, play an important imunnoregulatory role together with T lymphocytes, which may explain a relatively high protein synthesis rate in this cell population in basic physiological conditions. The cell distribution in the ICU patients following the density gradient separation might have been different, depending on the altered composition of white blood cells. The flow cytometric analysis showed a high proportion of cells expressing surface markers for monocytes and concurrently a low proportion of cells expressing surface markers for T lymphocytes. It is well known that monocytes are activated during an early phase of injury, releasing large amounts of cytokines and other pro-inflammatory mediators (7). Thus the high rate of the *in vivo* fractional protein synthesis rate observed in the mononuclear cells of the ICU patients may reflect an enhanced protein synthesis rate in monocytes.

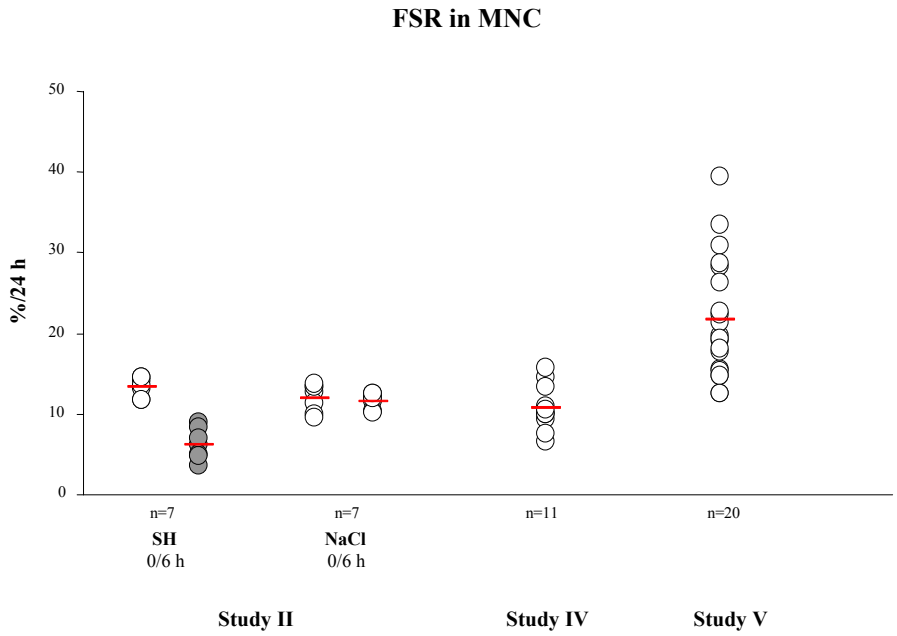

Fig. 6. *The fractional protein synthesis rate in the total population of mononuclear cells determined in healthy volunteers (study II), healthy ENT patients (study IV) and ICU patients (study V). Circles represent individual values, horizontal lines represent means. Open circles represent basal values, filled circles represent values following an intervention (SH, stress hormone infusion). ENT, ear, nose and throat.*

The *in vivo* rate of protein synthesis in the total mononuclear cells has been determined previously. In surgical patients with a metastatic cancer a 5-days immunostimulatory treatment with IL-2 results in an increase in the FSR of mononuclear cells (58). Also surgical trauma per se leads to an enhanced *in vivo* rate of protein synthesis (59). On the other hand, a biphasic metabolic response of mononuclear cells is observed following a

6-h combined stress hormone infusion in healthy volunteers (60). The immediate decrease of the *in vivo* fractional protein synthesis rate in the mononuclear cells at the end of the infusion is followed by return to preinfusion levels 18 h later. The protein synthesis rates determined in the mononuclear cells in that study were lower compared with those in study II. The facts that the procedure of preparing samples for the gradient centrifugation was slightly different and that samples were analysed at another laboratory, may explain the differences between the FSR values. However, the magnitude of change in response to the stress hormone challenge was the same in both studies. The *in vivo* fractional protein synthesis rate has also been studied before in the total mononuclear cells of 15 ICU patients with both surgical and medical diagnoses on days 2-30 after ICU admission (97). Also in that study the results were approximately 50% lower compared with those in study V.

The *in vivo* fractional protein synthesis rate in the total mononuclear cells of healthy volunteers has also been determined with the constant infusion technique (98), showing lower values of the FSR compared with our results in study II determined with the flooding approach. The discrepancy may partly depend on the problem with recycling of labelled amino acids, and partly on secretion of export proteins during the 4 h incorporation period when applying the constant infusion technique.

4.1.3 FSR in leukocytes

A striking feature of the *in vivo* fractional protein synthesis rates in the whole population of human blood leukocytes was the agreement between the volunteers and the healthy ENT patients (studies III, IV) (Fig. 7). The mean values were similar and the scatter was low. Following endotoxin administration an increase in the rate of protein synthesis was observed (study III). The whole population of leukocytes in the ICU patients showed high protein synthesis rates, with the lowest values corresponding to the mean values measured in the healthy subjects (study V). This was similar to the results for the mononuclear cells.

In normal, healthy subjects, whole blood leukocytes consist of 50-70% of neutrophils. In the acute phase of injury, the proportion of neutrophils in ICU patients was higher, up to 90%, as calculated from the WBC and differential counts in these patients. Neutrophils are unable to proliferate and are inactive in basic, physiological conditions (99), which fits well with their low *in vivo* metabolic activity. However, in the acute phase of injury, the neutrophils provide the first line of defense by phagocyting the invading microorganisms. This is accomplished by the production and secretion of proteolytic enzymes, oxygen radicals, and regulatory cytokines, which may explain the high rate of protein synthesis observed in the whole population of blood leukocytes in the early phase of illness in the ICU patients.

The results of the FSR in healthy subjects in study III and IV were comparable to the FSR determined in the neutrophils of healthy volunteers when using the constant infusion technique and the intracellular free amino acids in neutrophils as the precursor pool (98).

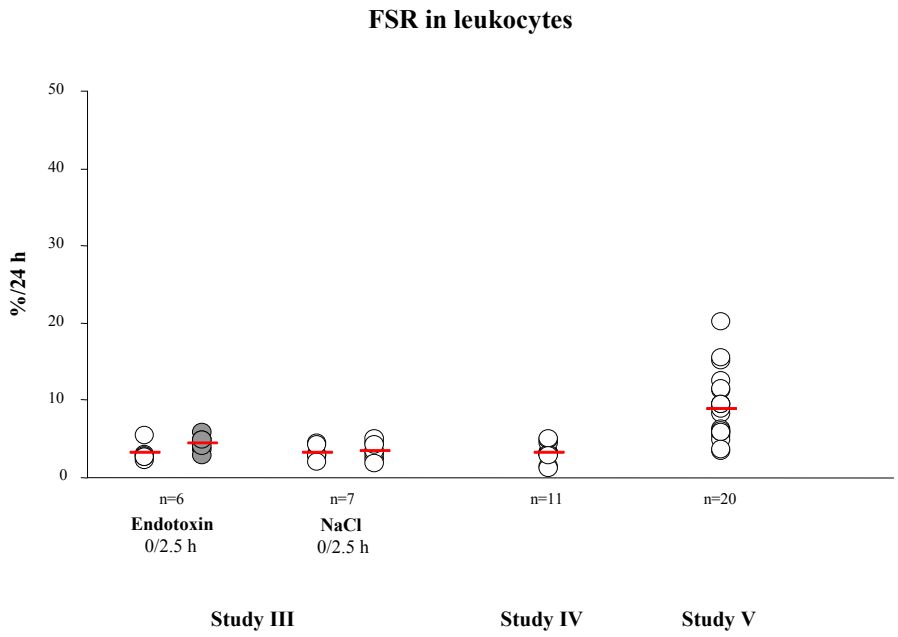

Fig. 7. *The fractional protein synthesis rate in the whole population of leukocytes determined in healthy volunteers (study III), healthy ENT patients (study IV) and ICU patients (study V). Circles represent individual values, horizontal lines represent means. Open circles represent basal values, filled circles represent values following an intervention (endotoxin injection). ENT, ear, nose and throat.*

4.2 THE *IN VIVO* FRACTIONAL PROTEIN SYNTHESIS RATE IN THE PALATINE TONSIL

In order to compare the metabolic activity of circulating blood cells with the activity of cells in lymphoid organs, for the first time the *in vivo* protein synthesis rate was determined in the palatine tonsils of healthy subjects and ICU patients (studies IV, V). The palatine tonsils constitute the pharyngeal part of the mucosa-associated lymphoid tissue. Due to their location and specific functions, the palatine tonsils might not be representative for the responses during the acute phase of injury taking place in the other components of the lymphoid system, such as lymph nodes or spleen. However, they are relatively easily accessible and may add information about the functional activity of the human immune system.

The *in vivo* protein synthesis rates in the unfractioned cells of the palatine tonsils were similar in both groups of investigated subjects, and they were consistently higher compared with circulating blood cells (Fig. 8). Due to the location, healthy palatine tonsils are continuously exposed to antigens and stimulated even in a basic, physiological state, which often is considered as a permanent activation (100). Thus, the high *in vivo* metabolic activity, corresponding to that seen in the human liver (90) is not surprising. The scatter in the ICU patients was larger, as expected. Interestingly 9 out of the 19 ICU patients showed a high *in vivo* protein synthesis rate, outside the range of the healthy subjects.

Due to the problems with visualization of the palatine tonsils in study V, particularly the second biopsy, aimed for the flow cytometric analysis, was more difficult. The phenotypic characterization did not confirm the presence of pure lymphoid tissue in seven cases. The *in vivo* protein synthesis rate was determined in the first biopsy specimen. As both the mean and median values of the FSR were almost identical also after exclusion of these seven patients, all available data of protein synthesis in the palatine tonsil of ICU patients were presented.

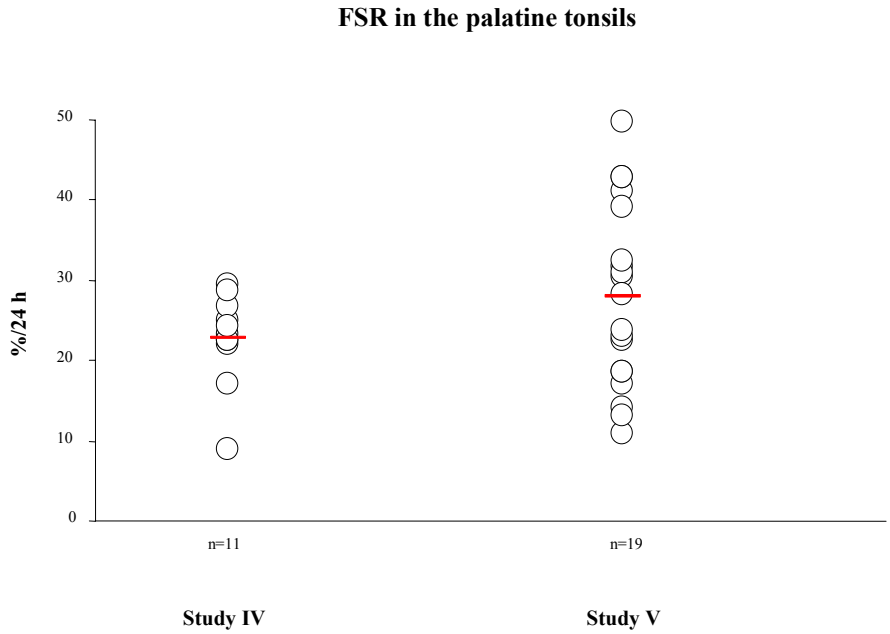

Fig. 8. *The fractional protein synthesis rate in the unfractioned cells of the palatine tonsil determined in healthy ENT patients (study IV) and ICU patients (study V). Circles represent individual values, horizontal lines represent means.ENT, ear, nose and throat.*

5 GENERAL DISCUSSION

In this thesis the *in vivo* fractional protein synthesis rate in immune competent cells was determined as a measure of their functional activity. Furthermore, the possible application of the protein synthesis measurement as an instrument for immunomonitoring was addressed.

The importance of a thorough monitoring of the immunological status, as an integral part of the surveillance in the ICU patients, has been recently highlighted (22,25,101). However, the parameters recommended as markers of different phases of the inflammatory responses, have their limitations. The measurement of the *in vivo* metabolic activity in cells of the immune system may add information on their functional status.

Although components of the immune system are in a stand-by position in basic, physiological conditions, they have a varying level of basic activity (9,102). The continuous confrontation with foreign antigens implies a constant process of scanning to distinguish harmless signals from those, which are dangerous and have to be disarmed. The recognition of pathogens induces rapid shifts in the activity of immune competent cells including the immediate production of multiple mediators, proteolytic substances, cell receptors, as well as cells proliferation. The metabolic reflection of these changes in the immunological activity is an alteration in the ongoing protein turnover, which may be quantified by the *in vivo* protein synthesis rate determination in immune competent cells. Quantifying the fraction of *de novo* synthesised proteins in basic, physiological conditions estimates the level of the metabolic turnover, corresponding to maintenance of the basic, immunological activity. Following injury, alterations in the *in vivo* fractional protein synthesis rates manifest enhanced or diminished immunological activity.

To elucidate specific effects of different types of injury on the activity of immune competent cells, the *in vivo* fractional protein synthesis rate was studied in human models of surgical trauma and sepsis (studies II, III). Following the combined stress hormone infusion (study II) a decrease in the protein synthesis rate in the total population of mononuclear cells in healthy volunteers was observed, which reproduced the results reported previously (60). In addition, the isolated population of circulating T cells showed the same type of alteration with a diminished *in vivo* metabolic activity. Immunomodulatory, mainly immunosuppressive effects of the neuroendocrine system on the immune system are well known. These effects include modulation of cytokine expression, suppression of immune cell maturation, differentiation and proliferation, reduction of cell trafficking and diminished expression of adhesion molecules (103), which is in accord with a decreased *in vivo* metabolic activity. Intravenously administered endotoxin, as a well-established human model for the early course of sepsis, affected the metabolic responses of immune competent cells in several ways (study III). Circulating T lymphocytes responded with an immediate decrease of the *in vivo* protein synthesis rate, suggesting suppression of their function. This is in line with a suppressed *in vitro* proliferative responsiveness of T lymphocytes to mitogen stimulation in healthy volunteers following the *in vivo* endotoxin administration (104). On the other hand, the whole population of leukocytes showed an enhanced *in vivo* metabolic activity, which fits well with an increased expression of leucocyte activation

markers reported previously (71). Taken together the results from studies II and III made it obvious that the metabolic response of immune cells to different types of injury is not uniform, but vary between individual populations of circulating peripheral blood cells.

The key question is if the alterations in the *in vivo* protein synthesis rates of immune competent cells reflect the state of activation of the immune system in severely ill patients. In a pilot study, a group of 20 ICU patients during the initial phase of multiple organ failure was characterized by means of clinical and immunological parameters, completed with the metabolic measurements of immune competent cells. The metabolic determinations included the *in vivo* fractional protein synthesis rates in different populations of circulating blood cells and tonsillar cells, representing lymphoid tissue. Although heterogeneous regarding the diagnoses, the ICU patients presented a uniform, general activation of immune responses. This activation was reflected by a decrease in the number of circulating T lymphocytes and an increase in monocyte count, an enhanced activity of adhesion molecules as well as elevated levels of selected pro- and anti-inflammatory cytokines. With regard to the metabolic activity, a distinct polarization of responses was observed. The *in vivo* fractional protein synthesis rates in the circulating total mononuclear cells and in the whole population of leukocytes were high, whereas the protein synthesis rates in the circulating T lymphocytes and in the tonsillar cells were not different from that observed in healthy subjects.

The metabolic activation of leukocytes in the ICU patients is in agreement with an increased *in vivo* protein synthesis rate seen in leukocytes of healthy volunteers exposed to an endotoxin injection, as a human model of sepsis (study III). However, this enhanced metabolic reaction probably reflects an early inflammatory, non-specific immune response, as only part of the ICU patients had sepsis diagnosed. Furthermore, the total mononuclear cells of the ICU patients had a high metabolic rate, which is in contrast to the results observed in the human model of surgical trauma, showing a drop in the protein synthesis rate (study II), (60). This discrepancy may be due to the different time points of protein synthesis determination, immediately vs some days after the onset of the injury. An increase in the *in vivo* fractional protein synthesis rate in the total population of mononuclear cells 24 h after uncomplicated elective surgery (89) supports the biphasic time course of metabolic responses. The isolated population of circulating T lymphocytes in the ICU patients had an *in vivo* protein synthesis rate comparable to that observed in healthy subjects, suggesting that the elevated metabolic activity in the leukocytes and mononuclear cells was represented by the cells other than T lymphocytes. It can be speculated if T lymphocytes had maintained their basic metabolic activity or if they had successively increased their activity following an initial suppression. The distinct decrease of the FSR in T cells in the human sepsis model supports the later explanation, indicating a dynamic time-course of the immune responses to injury.

To address the question if determination of metabolic activity in immune cells may be useful for evaluation of the immune status of the ICU patients, the possible relationships between the *in vivo* protein synthesis rates and relevant clinical parameters were tested post hoc. We found a negative correlation between the *in vivo* protein synthesis rate of T lymphocytes and the plasma CRP concentration both on the first day of ICU admission ($p=0.009$) and on the study day ($p=0.01$), suggesting low metabolic activity of T cells in cases of pronounced inflammation. Although not statistically

significant (p=0.056), the negative correlation between the protein synthesis rate in T lymphocytes and ICU survival raises the question whether suppression of T cell activity is associated with poor outcome, which would be in accord with the report on T cells anergy, being correlated to mortality in the abdominal sepsis (55). We also found negative correlations between the protein synthesis rate in leukocytes and the platelets count (p=0.002) as well as the plasma albumin concentration (p=0.03). On the other hand the fractional synthesis rate of leukocytes correlated positively to CRP level (p=0.02) and to the SOFA score on the study day (p=0.01), which together suggest a relationship between the metabolic activity of leukocytes and the severity of the disease. There were also strong statistical correlations between the metabolic activity of leukocytes and IL-6 (p=0.000006), IL-8 (p=0.000002) and IL-10 (p=0.00008) plasma concentrations, indicating that a high rate of protein synthesis in leukocytes was seen in patients with more pronounced inflammatory responses.

The presence of correlations between the *in vivo* rates of protein synthesis and relevant clinical parameters suggests that determination of the ongoing metabolic activity in immune competent cells reflects changes in the functional activity of the immune system, being of importance for the severity and time course of the critical illness. The results of studies I-V encourage future studies, in order to characterize the alterations in the *in vivo* metabolic activity in immune competent cells in later phases of ICU stay, characterized by a general anti-inflammatory activity and decreased resistance to opportunistic infections.

6 CONCLUSIONS

1. The *in vivo* fractional protein synthesis determination, as a reflection of the ongoing metabolic activity in immune competent cells, has been established and further developed. For the fist time the *in vivo* fractional protein synthesis rate was determined in T lymphocytes separated by rosette technique. Repeated measurements of protein synthesis have shown good reproducibility.

2. The *in vivo* fractional protein synthesis rates in circulating immune cells of healthy volunteers in response to an infusion of cortisol alone or as a part of the combined stress hormones infusion, as a human surgical trauma model, were determined. The stress hormone cocktail, but not cortisol alone, decreased the *in vivo* fractional protein synthesis rate in circulating T lymphocytes and in mononuclear cells.

3. The effect of endotoxin, as a human model of the initial phase of sepsis, on the *in vivo* fractional protein synthesis rates in circulating T lymphocytes and in the whole population of leukocytes was characterized, showing an increase of the protein synthesis rate in the whole population of leukocytes, but a decrease in T lymphocytes.

4. For the first time protein synthesis was determined in stationary cells of the palatine tonsil. The *in vivo* fractional protein synthesis rate in the tonsillar cells was high compared with the circulating peripheral blood cells: T lymphocytes, mononuclear cells and leukocytes in healthy subjects.

5. A pilot group of intensive care unit patients were characterized with clinical and immunological parameters. The *in vivo* fractional protein synthesis rates in circulating mononuclear cells and leukocytes were high, while the protein synthesis rates in circulating T lymphocytes and in unfractioned cells of the palatine tonsil were not different from that of healthy subjects.

The results of the thesis show that the *in vivo* fractional protein synthesis rate in immune competent cells reflects the functional activity of these cells. It remains to be shown how the functional activity reflects the functional capacity and how it relates to patients outcome. *In vivo* protein synthesis determinations may become a part of immunomonitoring in the clinical studies concerning function of the human immune system.

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9 PAPERS