Quantitative *in vivo* Protein Synthesis as a Measure of Immune Function

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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 the immune system is crucial in cases of severe infection. 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 similar to those existing for respiration, circulation, coagulation, as well as for liver, kidney and mental function.

In vivo determination of the rate of protein synthesis 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 the activation of various immune cells, allowing better estimates of immune competence. Application of *in vivo* protein synthesis measurements in cells of the immune system, in order to quantitatively characterize the state of their activation, is the main issue of a project within our working-group and here results are reported from a series of studies performed and published recently [1–5].

Background

Monitoring of the Immune System

Monitoring of vital organs is a necessary tool for adequate treatment of intensive care unit (ICU) patients, since insufficient function of these organs leads to multiple organ failure (MOF), with a high mortality rate despite all the advances in intensive therapy. While monitoring of organ function, such as lung, liver, kidney, etc., is well established, monitoring of immune system function is inadequate [6]. 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 anti-inflammatory interventions directed against tumor necrosis factor (TNF)-α and interleukin (IL)-1 [7], immunomodulatory therapies are still appealing, following a successful trial with pro-inflammatory mediators in immune-depressed sepsis patients [8]. However, the choice of such an immunomodulatory therapy has to be adjusted to the inflammatory phase, which necessitates 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. On the other hand, no response, or minor elevations in WBC or CRP, are observed during some viral or chronic infections. Both leukopenia and leukocytosis 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 [9].

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 [10] 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 lymphocyte count and severity of illness or mortality rate. 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 short-term hyperglycemia. In contrast, a rise in monocyte count is observed in patients exposed to trauma, surgery or sepsis [11].

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 human leukocyte antigen (HLA)-DR (MHC class II), which is upregulated on 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 compensatory anti-inflammatory response syndrome (CARS) and a predictor of poor outcome in patients with severe sepsis [6, 11]. However, some investigators have found contradictory results [12], questioning the value of HLA-DR as a single parameter to characterize the immunological status [13].

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. 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. In addition, concentrations of cytokines may differ between the systemic circulation and the site of injury.

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. However, large intraindividual variation in response to mitogenic stimulation and poor reproducibility are well-known drawbacks of these *in vitro* methods.

In vivo Protein Synthesis Determination and Immunomonitoring

An adequate function of the immune system requires rapid shifts from the 'standby' position into full activity. In health, most immune cells are quiescent. However, the constant exposure to foreign antigens implies the need for continuous scanning in order to detect harmful signals and generate 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 [14]. 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. 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 *in vivo* fractional protein synthesis rate was quantitatively determined for the first time in human mononuclear cells of patients with metastasic colorectal cancer [15]. The rate of protein synthesis is lower in these patients as compared with healthy control subjects. Following a 5-day 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 [16]. 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 normalization at 18 h after cessation of the infusion [17].

The possibility to perform *in vivo* measurement of protein synthesis in cells of the immune system is appealing. It provides the opportunity to assess *in vivo* metabolic activity, which may reflect 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.

Methods

Protocols

In three studies of healthy volunteers, the effects of a combined stress hormone infusion (n = 24) (epinephrine + cortisol + glucagon), a cortisol infusion (n = 18), and an endotoxin challenge (n = 18) on *in vivo* protein synthesis in circulating immune cells was elucidated [1–3]. The protocols were chosen to represent the acute effects of trauma and sepsis in a standardized way. A parallel aim of these studies was to validate and develop the techniques used to quantify the *in vivo* protein synthesis in circulating immune cells. In the patient studies we introduced tonsil biopsies to have a measurement also in stationary immune cells. The methodology for tonsils

was developed and validated in a group of ear, nose and throat (ENT) patients (n = 11) undergoing elective surgery [4]. Finally a group of ICU patients (n = 20) on ventilators in the pro-inflammatory phase with established or a high risk of developing MOF was studied in a pilot experiment [5]. The purpose was to establish how quantitative measurements of *in vivo* protein synthesis in immune cells appear in patients with generalized inflammation and severe infections.

The *in vivo* Fractional Protein Synthesis Rate

In vivo protein synthesis rates in human tissues can be quantified by incorporation of labeled amino acids into proteins. 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. In addition, the direction of changes depends on fasting or feeding conditions [18].

Administration of labeled amino acids makes them available as precursors for protein synthesis. Measuring the fraction of labeled amino acids incorporated into the protein of interest over time in relation to the fraction of labeled amino acids in the precursor pool enables calculation of the fractional synthesis rate. Constant infusion and the flooding technique are the two available approaches to administer labeled amino acids for incorporation into proteins.

The Constant Infusion Method. The constant infusion technique has been extensively used for whole body protein turnover and for tissue protein synthesis measurements in animal and human studies. With this method, the labeled 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 to achieve 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. The very low concentration of tRNA and the high rate of turnover, make this measurement technically difficult [19]. Instead other precursor pools, such as the enrichment in plasma or enrichment of transamination products of the labeled 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 [18]. In consequence, using plasma leucine or KIC enrichments for protein synthesis calculations falsely 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, the 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 labeled tracer amino acid in the precursor pool. In addition, in tissues with high protein turnover, protein synthesis rates may be underestimated because of the escape of export proteins during the labeling period.

The Flooding Method. 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 unlabeled and labeled amino acid is administered intravenously as a bolus over a short period of time [20]. Due to the overabundance of the given amino acid, all existing free amino acid pools are reached and equilibrated rapidly, including the true precursor pool for protein synthesis, aminoacyl-tRNA, and an isotopic equilibration is established. Indeed, the assumption on equilibration between plasma, the tissue free amino acid pool and aminoacyl-tRNA has been confirmed, which allows the plasma to be used as a valid substitute for the true precursor pool [21, 22]. 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 needed, 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 labeled 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. The main remaining problem with the flooding technique is that the labeled and incorporated amino acids do not fulfil the criteria for being a true tracer. 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 [23]. In particular a flood of leucine has been suggested to stimulate the protein synthesis rate in human skeletal muscle [24].

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 fractional synthesis rate in human skeletal muscle measured by flooding with leucine or phenylalanine, shows similar rates of protein synthesis [25]. Furthermore, comparison between the two techniques, when the precursor pool has been adequately equilibrated during a constant infusion, shows identical results [26].

Determination of Fractional Synthesis Rate by the Flooding Technique. In the studies reported here, 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 of 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, labeled 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. How-

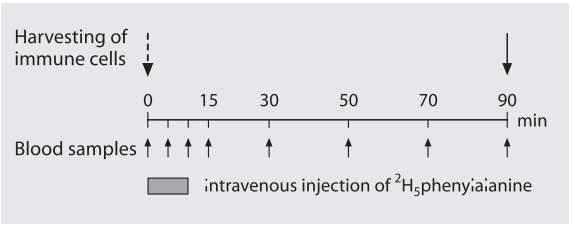

Fig. 1. Schematic study protocol for the flooding technique for the determination of the fractional protein synthesis rate in immune competent cells

ever, 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.

In vivo protein synthesis of human lymphoid tissue represented by the palatine tonsil was actually determined for the first time. 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 [27]. The general protocol for sampling of immune cells and blood for the purpose of determination of in *vivo protein* synthesis rates is presented in Figure 1.

Cell Separation

Available methods for T lymphocyte isolation were tested and validated. The aim was to find a method that 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 disturb mass spectrometry. In addition, the presence of antibody proteins interfered 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 [28]. The method is based on the presence of receptors for sheep erythrocytes on the surface of human T lymphocytes. 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.

Tonsil Biopsies

Tonsil biopsy is a well-documented technique, which relatively easily enables access to lymphoid tissue even on an outpatient basis [29]. This technique was applied in order to compare the *in vivo* fractional synthesis rates in circulating cells of the peripheral blood with those of stationary cells of the lymphoid tissue. The biopsies were taken with a punch forceps and no complications due to bleeding were ob-

served. The procedure of tonsil biopsy in ICU patients turned out to be technically more difficult compared to that in healthy subjects. One of the explanations was the fact that ICU patients, although receiving sedatives and analgesics, were not muscle relaxed. Besides, one of the features of the early phase of critical illness is general edema, due to capillary leakage as well as fluid supply, which is a part of the intensive treatment. Owing to all these factors, visualization of the palatine tonsils was much more difficult in the ICU patients.

Phenotypic Characterization of Cells

In order to characterize the immunological status of the studied subjects, flow cytometric analysis was performed. 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. Expression of markers associated with differentiation or activation stage on these cells was also studied. To find out if there was a relationship between the phenotypic and metabolic manifestations of activation, the expressions of activity markers were related to the *in vivo* fractional protein synthesis rates.

Plasma Cytokines

Plasma cytokine concentrations were determined as part of the characterization of the immunological status in the investigated subjects. In the healthy volunteers subjected to endotoxin, the sandwich enzyme-linked immunosorbent assay (ELISA) was used. In the ICU patients, plasma cytokines were analyzed using the multiplex bead array assay. This relatively new method permits simultaneous flow cytometric quantitation of multiple cytokines by capturing them onto beads labeled with fluorophores and coated with antibodies, specific for the cytokines of interest [30].

Results

The *in vivo* Fractional Synthesis Rate in Circulating Cells

Determination of protein synthesis was performed in circulating peripheral blood cells, purified T lymphocytes, total mononuclear cells, and the whole population of leukocytes. The use of peripheral blood cells in studies on function and activity of the immune system has been criticized. Indeed, circulating lymphocytes represent only about 2% of the total lymphocyte pool in the normal, human body. 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.

Fractional Synthesis Rate in T Lymphocytes. 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 the subjects who received a combined stress hormone infusion and in subjects exposed to an endotoxin injection (Fig. 2).

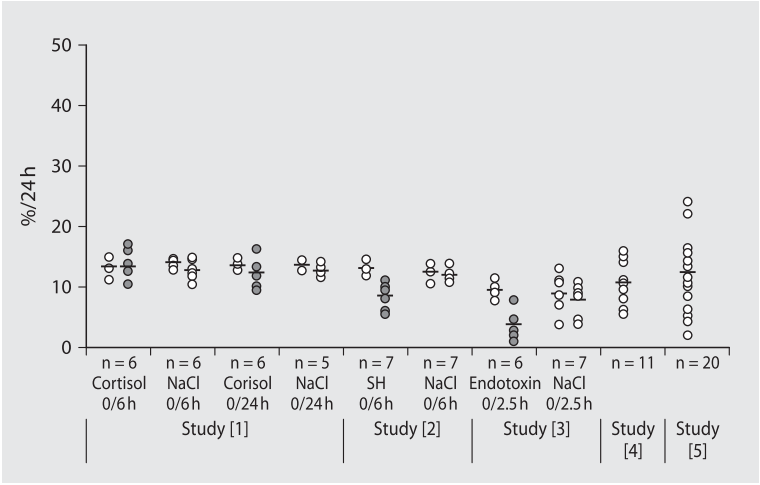

Fig. 2. The fractional protein synthesis rate in the isolated population of T lymphocytes determined in healthy volunteers [1–3], non-infected ear, nose and throat patients [4] and ICU patients [5]. Circles represent individual values, horizontal lines represent means. Open circles represent basal values, filled circles represent values following an intervention (cortisol or stress hormone (SH) infusion or endotoxin injection)

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 [31] and the human liver, producing both stationary and export proteins, which has a fractional synthesis rate of approximately 24%/24 h [32]. An *in vivo* fractional synthesis rate comparable with our results was reported in a human study where lymphocytes were separated from monocytes using iron particles [33]. 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 [34]. 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 tissues [35]. 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 fractional synthesis rates in T lymphocytes was relatively low in healthy subjects, but was higher in the investigated patients. This was not surprising in the ICU group, being heterogeneous in terms of diagnosis, age, APACHE II, type of infections, etc. A possible explanation for the greater scatter in patients with minor ENT pathology might be that the age distribution was wider and that the median age was higher compared to the healthy volunteers. Known age-associated alterations include changes in immune cell composition, accompanied by varying, both diminished and enhanced, functional activity of the immune system [36]. 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 patients were included. Gender differences in the innate

and adaptive immune system have been reported in humans [37]. However, the *in vivo* protein synthesis rates in the T lymphocytes were not different between the men and women participating in the study.

Fractional Synthesis Rates in Mononuclear Cells. The *in vivo* fractional rate of protein synthesis in the total population of mononuclear cells was determined in a total of 45 subjects (Fig. 3). The results showed that the fractional synthesis rate was similar in volunteers and in patients undergoing elective ENT surgery. In ICU patients, 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.

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 [38]. 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 are 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 immunoregulatory 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 WBCs. 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 proinflammatory mediators. Thus the high rate of the *in vivo* frac-

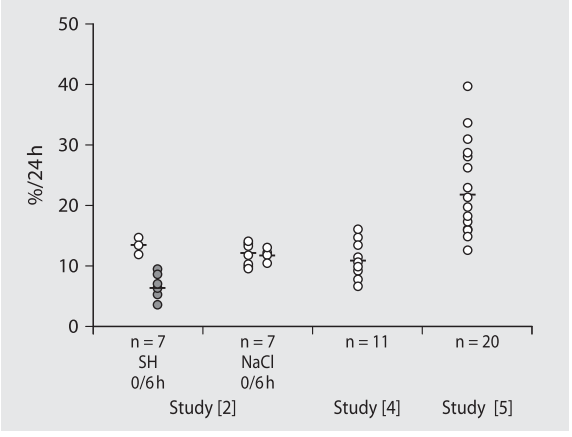

Fig. 3. The fractional protein synthesis rate in the total population of mononuclear cells determined in healthy volunteers [2], non-infected ear, nose and throat patients [4] and ICU patients [5]. Circles represent individual values, horizontal lines represent means. Open circles represent basal values, filled circles represent values following an intervention (SH, stress hormone infusion)

tional protein synthesis observed in the mononuclear cells of the ICU patients may reflect an enhanced protein synthesis rate in monocytes.

The *in vivo* rate of protein synthesis in the total mononuclear cell population has been determined previously. In surgical patients with metastatic cancer a 5-day immunostimulatory treatment with IL-2 results in an increase in the fractional synthesis rate of mononuclear cells [15]. Also, surgical trauma *per se* leads to an enhanced *in vivo* rate of protein synthesis [16]. On the other hand, a biphasic metabolic response of mononuclear cells is observed following a 6-h combined stress hormone infusion in healthy volunteers [17]. 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 pre-infusion levels 18 h later. The protein synthesis rates determined in the mononuclear cells in that study were lower compared with those in our study of healthy subjects. The fact that the procedure of preparing samples for the gradient centrifugation was slightly different and that samples were analyzed at another laboratory, may explain the differences between the fractional synthesis rate 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 [39]. In that study also the results were approximately 50% lower compared with those in our present study.

The *in vivo* fractional protein synthesis rate in the total mononuclear cells of healthy volunteers has also been determined with the constant infusion technique [40], showing lower values compared with our results in study II determined with the flooding approach [2]. The discrepancy may partly depend on the problem with recycling of labeled amino acids, and partly on secretion of export proteins during the 4 h incorporation period when applying the constant infusion technique.

Fractional Synthesis Rate 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 (Fig. 4). The mean values were similar and the scatter was low. Following endotoxin administration an increase in the rate of protein synthesis was observed. 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. 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 [41], 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 phagocytosing 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 ICU patients.

The results of the fractional synthesis rates in healthy subjects in our studies were comparable to those 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 [40].

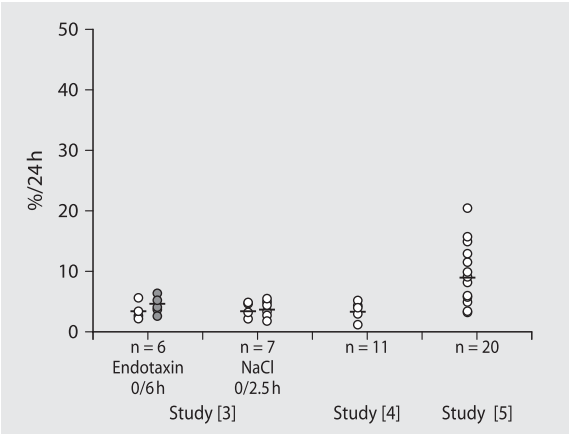

Fig. 4. The fractional protein synthesis rate in the whole population of leukocytes determined in healthy volunteers [3], non-infected ear, nose and throat patients [4] and ICU patients [5]. Circles represent individual values, horizontal lines represent means. Open circles represent basal values, filled circles represent values following an intervention (endotoxin injection)

The *in vivo* Fractional Synthesis Rate in the Palatine Tonsils

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 patients undergoing elective ENT surgery and of ICU patients. 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. 5). 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 [42]. Thus, the high *in vivo* metabolic activity, corresponding to that seen in the human liver [32] is not surprising. The scatter in the ICU patients was larger, as expected. Interestingly 9 out of the 19 ICU patients studied showed a high *in vivo* protein synthesis rate, outside the range of the healthy subjects.

Discussion

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.

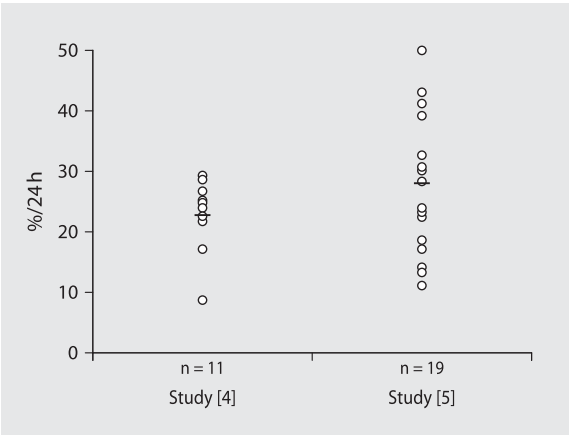

Fig. 5. The fractional protein synthesis rate in the unfractionated cells of the palatine tonsil determined in non-infected ear, nose and throat patients [4] and ICU patients [5]. Circles represent individual values, horizontal lines represent means

The importance of thorough monitoring of the immunological status, as an integral part of the surveillance in the ICU patients, has been highlighted recently [6, 7]. 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 basal activity. 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 cell proliferation. The metabolic reflection of these changes in immunological activity is an alteration in the ongoing protein turnover, which may be quantified by determination of the *in vivo* protein synthesis rate in immune competent cells. Quantifying the fraction of *de novo* synthesized proteins in basic, physiological conditions estimates the level of 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. Following the combined stress hormone infusion 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 [17]. 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, 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. 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 *in vivo* endotoxin administration [43]. On the other hand, the whole population of leukocytes showed an enhanced *in vivo* metabolic activity, which fits well with an increased expression of leukocyte activation markers reported previously [44]. Taken together the results from our studies in human models of trauma and sepsis made it obvious that the metabolic response of immune cells to different types of injury is not uniform, but varies between individual populations of circulating peripheral blood cells.

The key question is whether 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 our pilot study, a group of 20 ICU patients during the initial phase of multiple organ failure (MOF) was characterized by means of clinical and immunological parameters, completed with the metabolic measurements of immune competent cells. Although heterogeneous regarding their 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 metabolic activity, a distinct polarization of responses was observed. The *in vivo* fractional protein synthesis rates in the total circulating 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 those 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. However, this enhanced metabolic reaction probably reflects an early inflammatory, non-specific immune response, as not all ICU patients had a sepsis diagnosis. 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 [17]. This discrepancy may be due to the different time points of protein synthesis determination, immediately versus 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 [31] 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 cells other than T lymphocytes. It can be speculated as to whether T lymphocytes had maintained their basal metabolic activity or whether they had successively increased their activity following an initial suppression. The distinct decrease in the fractional synthesis rate 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 whether 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 cell anergy being correlated to mortality in abdominal sepsis [45]. We also found negative correlations between the protein synthesis rate in leukocytes and the platelet 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 with the CRP level and with the sequential organ failure assessment (SOFA) score on the study day ($p = 0.01$), which together suggest a relationship between the metabolic activity of leukocytes and the severity of 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.

Conclusion

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. Our results encourage future studies, in order to characterize the alterations in the *in vivo* metabolic activity in immune competent cells in later phases of the ICU stay, characterized by a general anti-inflammatory activity and decreased resistance to opportunistic infections.

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