Determination of in vivo protein synthesis in human palatine tonsil

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ABSTRACT

The palatine tonsils are constantly exposed to ingested or inhaled antigens which, in turn, lead to a permanent activation of tonsillar immune cells, even in a basic physiological state. The aim of the present study was to investigate if the immunological activation of the human palatine tonsil is reflected by a high metabolic activity, as determined by in vivo measurement of protein synthesis. The protein synthesis rate of the tonsil was also compared with that of the circulating T-lymphocytes, the total blood mononuclear cells and the whole population of blood leucocytes. Phenotypic characterization of immune-competent cells in tonsil tissue and blood was performed by flow cytometry. Pinch tonsil biopsies were taken after induction of anaesthesia in healthy adult patients (n = 12) scheduled for ear surgery, uvulopalatopharyngoplasty or nose surgery. Protein synthesis was quantitatively determined during a 90-min period by a flooding-dose technique. The in vivo protein synthesis rate in the palatine tonsils was 22.8 ± 5.7 %/24 h (mean ± S.D.), whereas protein synthesis in the circulating T-lymphocytes was 10.7 ± 3.4 %/24 h, in mononuclear cells was 10.8 ± 2.8 %/24 h and in leucocytes was 3.2 ± 1.2 %/24 h. CD3+ lymphocytes were the most abundant cell population in the tonsil. The in vivo protein synthesis rate in human tonsils was higher compared with the circulating immune cells. This high metabolic rate may reflect the permanent immunological activity present in human tonsils, although cell phenotypes and activity markers do not explain the differences.

INTRODUCTION

The immune system is a dynamic network of cells, tissues and molecules, whose main task is to distinguish between self and non-self. This is an active process, which leads to tolerance of own cells of the body and protection from invading pathogens. The basic state of immune activity shifts rapidly following recognition of harmful antigens or molecules, which initiates immune responses aimed at elimination of the invaders. This is reflected by changes in the functional activity of immune-competent cells, such as synthesis of cytokines or other regulatory factors, production of enzymes, receptors and immunoglobulins, cell differentiation and proliferation. In metabolic terms, this means varying activity in synthesis of both structural and export proteins. Therefore determination of in vivo protein synthesis in immune-competent cells enables an assessment of their functional activity and may lead to a...
greater understanding of the balance between health and disease.

We have demonstrated previously [1] that the in vivo rate of protein synthesis decreases in circulating peripheral blood T-lymphocytes in healthy volunteers in response to a 6-h infusion of stress hormones as a model for surgical stress. The decrease is even more pronounced after intravenous exposure to endotoxin, serving as a human model of the initial phase of the acute response to sepsis [2]. In contrast, a 6-h infusion of cortisol alone does not change protein synthesis in circulating T-lymphocytes of healthy volunteers either immediately or 18 h after the infusion period [3].

However, under normal conditions, circulating lymphocytes represent only 2% of the total lymphocyte pool in the body and therefore they may not reflect alterations in the whole lymphoid tissue [4]. The palatine tonsils belong to the MALT (mucosa-associated lymphoid tissue) and play an important role in protecting both the gastrointestinal and upper respiratory tract from invading antigens and molecules. They consist of specialized compartments with a typical composition of immune cells [5–8]. Lymphoepithelium covering the tonsillar crypts is the place of antigen uptake by DCs (dendritic cells), whereas, in the extrafollicular area populated mainly by T-lymphocytes, migrated DCs present the antigens to CD4+ T-lymphocytes, resulting in their activation, proliferation and differentiation. The third compartment, lymphoid follicles, consists predominantly of B-lymphocytes which, upon activation by T-cells, proliferate and differentiate into memory cells and immunoglobulin-producing plasma cells. All these events are regulated by receptor–receptor interactions as well as by cytokines secreted by the antigen-presenting cells and T-lymphocytes [6,9]. Even in a physiological state, healthy palatine tonsils are exposed continuously to antigens and stimulated, which often is considered as a permanent activation [10].

Thus the objective of the present study was to quantitatively determine in vivo protein synthesis in the human palatine tonsil as a reflection of the immunological activation under normal conditions. The metabolic activity of immune cells from the tonsils was compared with that of circulating peripheral cells [T-lymphocytes, total blood MNCs (mononuclear cells) and the whole population of blood leucocytes]. In order to characterize the distribution of immune-competent cells in tonsil tissue versus peripheral blood, flow cytometry analysis was performed.

**MATERIALS AND METHODS**

**Materials**

L-[\(^{2}\)H\(_5\)]Phenylalanine (99 atom %; Cambridge Isotope Laboratory, Andover, MA, U.S.A.) was dissolved in sterile water together with unlabelled phenylalanine (Ajinomoto Company, Tokyo, Japan) to a concentration of 20 g/l and an appropriate isotopic enrichment. The solutions were prepared, heat-sterilized and stored in sterile containers.

**Subjects and experimental protocol**

Twelve healthy patients, scheduled for elective ear surgery, UPPP (uvulopalatopharyngoplasty) or nose surgery, were included in the present study (Table 1). The patients were studied in the morning after an overnight fast. In the operating room, two venous lines were inserted contralaterally into the forearm and antecubital veins and a glucose solution (Glucos Baxter 25 mg/ml buffrad; Baxter Medical AB, Kista, Sweden) was started at 100 ml/h. An intravenous injection of L-[\(^{2}\)H\(_5\)]phenylalanine (45 mg/kg; 10 or 20 mole % excess) was given over 10 min. Venous blood samples were taken from the opposite arm before (time 0), and at 5, 10, 15, 30, 50, 70 and 90 min after injection, for measurement of the isotopic enrichment of phenylalanine in plasma. For the measurement of the enrichment of L-[\(^{2}\)H\(_5\)]phenylalanine in the protein of T-lymphocytes, MNCs and leucocytes, 60 ml of blood was drawn at 90 min after the start of the isotope injection. All patients were operated on under general anaesthetic, which was induced with intravenous agents and maintained with volatile anaesthetics. Within 12 min (mean; range 5–20 min) after induction of anaesthesia and intubation and at 90 min after the start of the isotope injection, two tonsil biopsies were performed using punch forceps. The first biopsy specimen was frozen immediately in liquid nitrogen and then stored at −80 °C until analysis. The second specimen was put on ice and then transported for the flow cytometry analysis, which was performed within 1 h. In the patients operated for UPPP (n = 4), the whole tonsil was obtained, as a tonsillectomy was an initial part of the operative procedure. One patient was excluded due to an early postoperative infection.

The nature, purpose of the study and possible risks were explained to all patients before obtaining their voluntary consent. The research protocol was approved by the Ethical Committee of the Karolinska Institutet, Huddinge University Hospital, Stockholm, Sweden.

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**Table 1** Demographic data of subjects studied

<table>
<thead>
<tr>
<th>Variable</th>
<th>Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female/male)</td>
<td>6/6</td>
</tr>
<tr>
<td>Age (years)</td>
<td>46 (24–60)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>75 (54–90)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.71 (1.50–1.90)</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>25 (19–29)</td>
</tr>
</tbody>
</table>

Values are medians (range). BMI, body mass index.
Preparation of tonsil biopsies

After freeze-drying the tissue, biopsies were homogenized in 4% (w/v) SSA (5-sulphosalicylic acid dihydrate). The homogenates were centrifuged at 16 000 g for 10 min. Pellets were resuspended and washed twice in 4% (w/v) SSA. The pellets were then dissolved in 0.3 M NaOH and the concentration of alkali-soluble proteins was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with BSA as a standard. Proteins were precipitated again and washed twice in 4% (w/v) SSA. To the final pellet, 1 ml of 6 M HCl was added and proteins were hydrolysed at 110 °C for 24 h. Samples were cooled and then dried by vacuum centrifugation.

Separation of T-lymphocytes and MNCs

Separation of T-lymphocytes was as described previously [3]. In brief, 55 ml of blood (5 ml for MNC isolation and 50 ml for further separation of T-lymphocytes) was decanted into six heparinized 10 ml tubes containing cycloheximide (0.5 mM; Sigma, St. Louis, MO, U.S.A.) in order to inhibit protein synthesis. Blood was diluted 1/1 with PBS (pH 7.4; Unimedic, Matfors, Sweden) and centrifuged at 800 × g for 20 min at room temperature on a Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway). MNCs were harvested from the interface and washed three times with PBS [11]. At this step, the cell pellet from one tube was retained for determination of protein synthesis in MNCs and was stored at −80 °C, whereas the pellets collected from the other five tubes were processed further. T-lymphocytes were separated from MNCs by the rosette method with 1% 2-aminoethylisothiuronium bromide-treated SRBCs (sheep red blood cells). SRBC rosette-forming cells were separated on a Lymphoprep™ [12]. SRBCs were lysed with 1% ammonium oxalate for 5 min at room temperature. The cells were washed three times with PBS and stored at −80 °C.

Isolation of leucocytes

A portion (5 ml) of blood was decanted into a heparinized tube containing cycloheximide. Erythrocytes were lysed twice with a lysing buffer consisting of 1.5 M NH₄Cl, 0.1 M KHCO₃ and 1 mM Na-EDTA (pH 7.5) for 10 min in 37 °C. The cells were washed with PBS and stored at −80 °C.

GC–MS analysis of protein-bound phenylalanine

Samples were prepared for GC–MS analysis largely as described previously [13]. The dried protein hydrolysates were dissolved in 400 μl of 0.5 M sodium citrate buffer (pH 6.3) and were filtered through a 0.22 μm centrifugal filter device. Phenylalanine standards containing 0–0.25 APE (atom percentage excess) of L-[2H₅]phenylalanine were dissolved in 200 μl of citrate buffer. A suspension (100 μl) of L-tyrosine decarboxylase (0.7 units plus 0.25 mg of pyridoxal phosphate) was added to 200 μl of the samples and standards to convert phenylalanine into phenylethylamine. After incubation at 50 °C overnight, 100 μl of 6 M NaOH was added, samples were mixed and centrifuged at 16 000 g for 10 min. The supernatant was transferred to an Eppendorf tube and extracted with 500 μl of ether. The bottom layer was frozen in ethanol/dry-ice and the ether layer transferred to a new tube containing 100 μl of 0.1 M HCl. After shaking the tubes, the bottom layer was frozen and the ether layer discarded. Samples were transferred into GC–MS vials and dried by vacuum centrifugation and were then derivatized in MTBSTFA [N-methyl-N-(t-butyldimethylsilyl)tri-fluoroacetamide]/ethyl acetate (1/1, v/v) at 60 °C for 1 h. The amount of MTBSTFA was varied depending on the sample size, which was estimated from the protein concentration and dry weight. All standards were derivatized in 50 μl and the tonsil samples in 25–70 μl, so that samples and standards gave approximately the same peak abundance in the GC–MS analysis.

The ratio of [2H₅]-phenylethylamine and phenylethylamine was determined by GC–MS (Agilent 5973n; Agilent Technologies, Stockholm, Sweden). GC was performed on a HP-5MS column (30 m, 0.25 mm inner diameter, 1 μm film; J&W Scientific, Agilent Technologies). Approx. 500 ng of decarboxylated phenylalanine of the standards and samples was injected. Ions m/z 180 (m + 2) and m/z 183 (m + 5) were analysed.

Calculations

The enrichment of protein-bound [2H₅]phenylalanine in plasma was determined by monitoring the ions m/z 336 and 341.

GC–MS analysis of plasma free phenylalanine

Plasma free phenylalanine was prepared and analysed by GC–MS as described previously [14]. The enrichment of [2H₅]phenylalanine in plasma was determined by monitoring the ions m/z 336 and 341.

Phenotypic characterization of cells

The isolated blood leucocytes and cell suspensions of tonsil tissue were washed in PBS supplemented with 0.5% BSA in polystyrene round-bottom tubes and then the cell surface was stained. Antibodies used for phenotypic analysis were combinations of directly conjugated anti-CD45, anti-CD4, anti-CD8, anti-CD3, anti-CD16, anti-CD19, anti-HLA-DR (Becton Dickinson, San Jose, CA, U.S.A.), anti-CD14, anti-CD15, anti-CD56, anti-CD25 and anti-CD54 (DakoCytomation, Glostrup, Denmark).
FSRs in the circulating immune cells of peripheral blood (T-lymphocytes, MNCs and leucocytes) and in the tonsils of healthy subjects (n = 11)

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>T-lymphocytes</th>
<th>MNCs</th>
<th>Leucocytes</th>
<th>Tonsil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.2</td>
<td>9.3</td>
<td>1.5</td>
<td>22.0</td>
</tr>
<tr>
<td>2</td>
<td>14.1</td>
<td>14.5</td>
<td>3.3</td>
<td>22.6</td>
</tr>
<tr>
<td>3</td>
<td>5.4</td>
<td>9.9</td>
<td>1.3</td>
<td>23.2</td>
</tr>
<tr>
<td>4</td>
<td>8.0</td>
<td>6.6</td>
<td>3.2</td>
<td>24.9</td>
</tr>
<tr>
<td>5</td>
<td>15.0</td>
<td>13.4</td>
<td>3.0</td>
<td>17.1</td>
</tr>
<tr>
<td>6</td>
<td>11.1</td>
<td>7.6</td>
<td>2.9</td>
<td>29.4</td>
</tr>
<tr>
<td>7</td>
<td>11.0</td>
<td>10.2</td>
<td>4.5</td>
<td>26.7</td>
</tr>
<tr>
<td>8</td>
<td>11.2</td>
<td>11.0</td>
<td>4.8</td>
<td>22.5</td>
</tr>
<tr>
<td>9</td>
<td>10.5</td>
<td>9.9</td>
<td>3.0</td>
<td>28.8</td>
</tr>
<tr>
<td>10</td>
<td>15.8</td>
<td>15.7</td>
<td>5.1</td>
<td>8.9</td>
</tr>
<tr>
<td>11</td>
<td>9.6</td>
<td>10.6</td>
<td>2.9</td>
<td>24.2</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>10.7 ± 3.4</td>
<td>10.8 ± 2.8</td>
<td>3.2 ± 1.2</td>
<td>22.8 ± 5.7</td>
</tr>
</tbody>
</table>

Table 2

Statistical analysis

Data are presented as means ± S.D., or medians (ranges) when appropriate. Comparative statistics of CD markers between the tonsillar and blood cells were performed using the non-parametric Wilcoxon rank sum test. Correlation between the protein synthesis rate and the expression of CD19+ B-lymphocytes in the tonsils was tested by Spearman’s test.

RESULTS

In vivo protein synthesis in blood and tonsils in healthy adult patients was determined quantitatively during a 90 min period by intravenously injecting L-[3H]phenylalanine and then measuring its enrichment in immune-competent cells. The in vivo FSR in the palatine tonsils was 22.8 ± 5.7%/24 h (Table 2). This protein synthesis rate was found to be higher compared with peripheral blood. In peripheral blood cells, the in vivo FSR was 3.2 ± 1.2%/24 h in the whole leucocyte population (Table 2). In total MNCs and sorted T-lymphocytes from peripheral blood, the FSRs were 10.8 ± 2.8 and 10.7 ± 3.4%/24 h respectively (Table 2).

The phenotypic analysis of the tonsillar and blood cell subsets by flow cytometry is shown in Table 3. All markers were calculated from the combined lymphocyte and monocyte gate. CD15+ cells in blood were also calculated from the granulocyte gate. CD3+ T-lymphocytes were the most abundant cell populations in both the tonsils and blood. The ratio of CD4+ T-lymphocytes to CD3+ T-lymphocytes was higher among tonsillar cells compared with peripheral blood cells (P < 0.01). A higher proportion of CD19+ B-lymphocytes was observed in the tonsil as compared to blood (P < 0.01). In contrast, CD3+/CD4+ (P < 0.01) and CD14+ monocytes (P < 0.01), CD15+ granulocytes (P < 0.01), CD16+ monocyte subpopulation and natural killer lymphocytes (P < 0.01) and CD56+ natural killer lymphocytes (P < 0.01) were present at low levels in tonsils compared with blood. CD19+ B-lymphocytes did not correlate with the FSR in the tonsils (r = 0.53, P = 0.09).

HLA-DR+ was expressed in 30% of the cells and no differences in the distribution between blood and tonsils were observed. Another activity marker, CD25+, was detectable at very low levels in both the tonsillar and circulating blood cells. The expression of the adhesion molecule CD54 was higher in blood than in the tonsil (P < 0.01).

DISCUSSION

For the first time, the present study has quantified in vivo protein synthesis in human lymphoid tissue represented by the palatine tonsil. The rate of protein synthesis in the tonsil was twice as high as that in the circulating T-lymphocytes and MNCs. CD3+ lymphocytes were the most abundant cell population in the tonsil.

Tonsil biopsy is a well-documented technique, which enables access to the lymphoreticular system relatively...
easily even on an outpatient basis [15]. In our present study, all biopsies were performed in anaesthetized patients after induction of anaesthesia, but before the start of the operation. The biopsies were taken with punch forceps from the upper part of the tonsil (except for four patients scheduled for UPPP, where tonsillectomy was a part of the surgical procedure) and no complications due to the bleeding were observed. The time from induction of anaesthesia until tonsil biopsies was 12 min (mean; range 5–20 min). As the total incorporation time was 90 min, 70–85 min of the incorporation period was in the awake state before induction of anaesthesia. Separate biopsies were taken for determination of protein synthesis and for phenotypic characterization. All biopsies used for the latter purpose contained tonsillar tissue. Although the palatine tonsils consist of three different compartments with specific anatomy and functions, immunologically active cells are present in all three compartments. The relatively low scatter of the in vivo protein synthesis rate in the palatine tonsil, being of similar magnitude to that of the circulating cells, suggests that the biopsy specimens were representative for the tonsil tissue.

The in vivo FSR in human palatine tonsil was 23 %/24 h, which is a relatively high value, probably reflecting the continuous process of antigen scanning, which involves production of regulatory factors and cell interactions, as well as cellular activation and proliferation. This protein turnover is in the same range as that seen in the human liver, known for its high metabolic activity, which has an FSR of total (both stationary and export) proteins of approx. 25 %/24 h [16]. In the human intestinal mucosa, a twice as high FSR is reported, mainly due to a rapid cell turnover [17,18]. Also, in rats a similar pattern to that demonstrated in human subjects is reported with a lower FSR in the peripheral blood MNCs compared with lymphoid organs, such as bone marrow, thymus and spleen [19,20].

It can be speculated that the high protein turnover seen in the tonsil reflects a more pronounced cell proliferation taking place in lymphoid organs. In vitro studies have shown an increased thymidine and uridine incorporation in the tonsillar cells compared with the blood lymphocytes [21]. On the other hand, the proliferation rates in lymph nodes are not higher than proliferation rates in the T-lymphocyte subsets in an ex vivo study using the Ki67 antigen as a proliferation marker [22]. As there are no data available on the in vivo proliferation rates in human tonsils, the high protein synthesis rate observed in our study still may be related to an enhanced proliferation of immune-competent cells.

In vivo protein synthesis of the circulating peripheral blood cells was determined in parallel with that of the tonsils. Both T-lymphocytes and MNCs had a similar in vivo FSR of approx. 11 %/24 h, which is consistent with our previous studies performed in healthy volunteers [1–3]. The protein synthesis rate, although 50 % lower compared with the lymphoid tissue, still indicates a high metabolic activity of these cells, even in the resting state. This can be explained by the fact that T-lymphocytes, as well as monocytes, play an important immunoregulatory role, including production of receptors, cytokines and other regulatory factors. The low FSR of approx. 3 %/24 h seen in the total population of leukocytes in the peripheral blood is also in agreement with an earlier study in healthy volunteers [2]. This probably reflects the fact that neutrophils, which are the most abundant cell population in peripheral blood, are not active in the absence of stimulation [23].

In order to characterize cell populations in the tonsil and to compare them with the populations of peripheral blood, flow cytometry was performed. Our results showed that immune-competent cells were distributed differently in the lymphoid organs and blood, which is in accordance with a previous report [24]. CD3+ T-lymphocytes were the dominant cell population, present in the same proportions in both pools investigated. In contrast with blood, the percentage of B-lymphocytes was three times higher in the palatine tonsil, as shown previously [25]. It is known that lymphoid organs are the location where B-cells mature and differentiate and the question can be raised whether the high metabolic activity observed in tonsils may be related to the activity of B-lymphocytes rather than T-lymphocytes. However, there was no correlation between the protein synthesis rate in the tonsil and the expression of CD19+ B-lymphocytes in the tonsillar cells.

The expression of HLA-DR+ and CD25+ is often used to characterize the state of activation of immune cells. Despite the higher protein synthesis in tonsillar tissue, HLA-DR+ (MHC class II), which is up-regulated on monocytes, DCs and B-cells during antigen presentation as well as expressed on a subpopulation of activated T-cells, was equally distributed between blood and tonsils. Similarly, CD25+, up-regulated transiently mainly on activated T-lymphocytes [26] and expressed on very few cells, showed no difference in the distribution between the tonsil and blood.

In summary, the present study has shown that the in vivo protein synthesis rate in the human palatine tonsils is higher compared with circulating peripheral blood cells. This high protein turnover may reflect the continuous state of immunological activation seen in the human tonsil. Nevertheless, the differences seen in the rates of protein synthesis between the tonsils and blood cannot be explained by activity markers and subpopulations in immune-competent cells.

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