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BRIEF REPORT

First Identification of H_4 Histamine Receptor in Healthy Salivary Glands and in Focal Sialadenitis in Sjögren's Syndrome

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Objective. **The conventional H_1 and H_2 histamine receptors have >10,000-fold lower avidity for histamine than H_4 histamine receptor, which has been implicated in autoimmune diseases. This study was undertaken to compare H_4 histamine receptor levels in the salivary glands (SGs) of healthy controls with those in the SGs of patients with primary Sjögren's syndrome (SS).**

Methods. **H_4 histamine receptor messenger RNA (mRNA) was analyzed using real-time quantitative polymerase chain reaction, and the receptor protein was examined using immunostaining. Effects of the H_4 histamine receptor agonist ST-1006 on cytokine synthesis by human SG (HSG) cells were analyzed using xMAP technology and enzyme-linked immunosorbent assay.**

Results. **Healthy SGs contained H_4 histamine receptor mRNA. The receptor protein was localized to the acinar and ductal epithelial cells. H_4 histamine receptor agonist stimulated HSG cells to produce the cytokines interleukin-8 and vascular endothelial growth factor. SS patients had low H_4 histamine receptor levels.**

Conclusion. **H_1 and H_2 histamine receptor antagonists are not effective in the treatment of autoimmune diseases. However, such antagonists do not affect the newly discovered H_4 histamine receptor. Dendritic cells and lymphocytes are nonprofessional histamine-producing cells, which produce histamine at 100–1,000-fold lower rates than mast cells do. Saliva contains only 0.31–12.4 ng/ml histamine, which is too low to stimulate H_1 or H_2 histamine receptor, but stimulates H_4 histamine receptor half maximally. Our findings show that H_4 histamine receptor is strongly expressed in tubuloacinar SG cells, which emphasizes the role of these cells in the pathogenesis of SS.**

Many autoimmune diseases, including primary Sjögren's syndrome (SS), are characterized by increased numbers of mast cells and increased histamine levels in serum and tissue fluids (1–3). However, in spite of some initial enthusiasm, the tentative role of mast cells and histamine in autoimmunity was not supported by trials with H_1 or H_2 histamine receptor antagonists (4,5).

Later it was found that in addition to the professional histamine-synthesizing, -storing, and -releasing cells, such as mast cells and basophils, many other cells, including dendritic cells (6) and T cells (7), can synthesize histamine, albeit at a 100–1,000-fold lower rate. These cells release histamine as they synthesize it, without intermediate storage or stimulated release. At first these observations raised little interest, but when the highly histamine-sensitive H_4 histamine receptor was discovered in 2000 (8), the potential role of histamine in

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autoimmune diseases became a hot topic. In fact, H_4 histamine receptor has a >10,000-fold higher affinity for histamine than H_1 histamine receptor (pK_i 8.3 versus pK_i 4.2) (9). Therapeutic concentrations of the traditional antihistamines and H_2 histamine receptor antagonists do not affect H_4 histamine receptor at all. Further, H_4 histamine receptor has been found in many hematopoietic cells, including dendritic cells and lymphocytes, and at lower levels in some peripheral tissue (8).

SS is sometimes referred to as autoimmune epitheliitis because the tubuloacinar epithelial cells are believed to form a source of autoantigens and alarmins and to function as nonprofessional antigen-presenting cells, equipped with class I major histocompatibility complex (MHC) and class II MHC antigens and with costimulatory molecules (10). Due to these exciting new developments, we decided to investigate whether H_4 histamine receptor is present in salivary glands (SGs) at the messenger RNA (mRNA), H_4 histamine receptor protein, and functional levels and to determine whether it is only constitutively expressed or is perhaps affected by autoimmunity in SS.

PATIENTS AND METHODS

Samples. The ethics committee of the Hospital District of Helsinki and Uusimaa approved the study (19/E5/03), and informed consent was obtained from all subjects. Labial SGs (LSGs) were obtained as part of routine diagnostic procedures when SS was suspected. For real-time quantitative polymerase chain reaction (qPCR), LSGs were snap-frozen in liquid nitrogen and stored at −80°C. For immunohistochemical analysis, LSGs were fixed in formalin and embedded in paraffin. The diagnosis or exclusion of primary SS was based on the American–European Consensus Group criteria (11). The SS patient group consisted of 1 man and 4 women, with a mean ± SD age of 48.8 ± 5.4 years, resting salivary flow of 0.5 ± 0.4 ml/15 minutes, and focus score of 1.7 ± 0.3. SS patients were positive for at least 4 of the 6 classification criteria, one of which was focal sialadenitis in all patients. Healthy controls included 1 man and 5 women, with a mean ± SD age of 47.1 ± 5.8 years and a resting salivary flow of 0.9 ± 0.3 ml/15 minutes. All controls had a focus score of <1, and none of them fulfilled the SS classification criteria.

SG cell culture and stimulation. Cells of the human submandibular gland–derived SG cell line were cultured in Dulbecco's modified Eagle's medium (DMEM)–Ham's F-12 nutrient mixture (Gibco BRL) supplemented with 2 m*M* glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS; HyClone). Stimulation experiments with 10 μ*M* H_4 histamine receptor–specific agonist ST-1006 ($C_{16}H_{20}Cl_2N_6 \cdot 2C_4H_4O_4$) ($H_4$ histamine receptor pK_i 7.94; 50% maximum response concentration in cell culture 6.9×10^{-7}*M*) (12) were performed in medium containing 2% FBS. After 24 hours, samples were collected and stored at −80°C.

Real-time qPCR. Tissue total RNA was isolated using a High Pure RNA Tissue kit (Roche). Complementary DNA (cDNA) was synthesized using a SuperScript First-Strand cDNA Synthesis System (Invitrogen), and real-time qPCR was performed using an iQ SYBR Green Supermix kit (Bio-Rad) and a iCycler iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Primer sequences, determined using the NCBI Entrez database, were as follows: for human H_4 histamine receptor, forward 5′-TGGAAGCGTGATCATCTCAG-3′ and reverse 5′-ATATGGAGCCCAGCAAACAG-3′; and for human β-actin housekeeping gene, forward 5′-TCACCCACACTGTGCCCATCTACGA-3′ and reverse 5′-CAGCGGAACCGCTCATTGCCAATGG-3′. A sequence similarity search was performed using the NCBI BLASTN program. Primers were synthesized in the core facilities of the University of Helsinki.

Immunohistochemical staining. LSG sections (4 μm thick) were deparaffinized and rehydrated. Antigen retrieval was performed by heating at 95°C in a microwave oven for 15 minutes in 10 m*M* sodium citrate buffer, pH 6.0, followed by cooling at 22°C for 30 minutes. Slides were rinsed in tap water and incubated in Dako REAL Peroxidase-Blocking Solution for 15 minutes and washed for 5 minutes in phosphate buffered saline (PBS), then incubated in 10% normal goat serum (Vector) for 1 hour, and then incubated in 0.5 μg/ml polyclonal peptide affinity-purified rabbit anti-human H_4 histamine receptor IgG (MBL International) overnight at 4°C. Rabbit anti-human H_4 histamine receptor IgG (0.5 μg/ml) derived from another source (13) was also used. Nonimmune rabbit IgG (0.5 μg/ml; R&D Systems) was used for negative control staining. Slides were washed in PBS 3 times for 5 minutes each time, then incubated in biotin-conjugated goat anti-rabbit IgG (1:200 in 1.25% bovine serum albumin–PBS; Vector) for 1 hour; then incubated in avidin–biotin–peroxidase complex (1:200 in H_2O; Vector) for 45 minutes; and then incubated in 0.006% H_2O_2 and 0.023% diaminobenzidine tetrahydrochloride for 7 minutes. Slides were counterstained in hematoxylin, dehydrated, and mounted for microscopy using a Leitz Diaplan microscope and a 5-megapixel Leica DFC420 digital camera (Leica Microsystems).

Multiplex assay and enzyme-linked immunosorbent assay (ELISA). Multiplex kits were used to screen 39 cytokines, chemokines, and/or growth factors (Millipore) after agonist stimulation. Standards or samples (50 μl) were pipetted in duplicate onto 96-well plates. Polystyrene beads (50 μl), internally labeled using different ratios of 2 spectrally distinct fluorophores, and coated with anti-human cytokine capture antibodies, were added. After 1 hour, wells were washed 3 times. Phycoerythrin-conjugated secondary detector antibodies were added for 45 minutes. Wells were washed twice, assay buffer was added, and samples were analyzed using a fluorescent bead–based flow cytometer equipped with 2 lasers (Bio-Plex 200 System; Bio-Rad).

The identification of 2 H_4 histamine receptor–regulated molecules, interleukin-8 (IL-8; CXCL8) and vascular endothelial growth factor (VEGF), by xMAP screening was validated using the more accurate ELISA (Quantikine; R&D Systems). The 96-well plates were precoated with capture monoclonal antibodies raised against recombinant human IL-8 or VEGF. Samples and standards, diluted in assay diluents (1:4 dilution for the test samples) were added for 2

hours, and then washed 4 times with buffer. Horseradish peroxidase–conjugated detector antibodies were added for 1–2 hours, followed by 3 washes and by addition of H_2O_2 substrate and tetramethylbenzidine chromogen solution for 20–30 minutes. Absorbance at 450 nm was measured within 30 minutes.

Statistical analysis. Results are presented as the mean ± SEM. Differences for skewed data (PCR results) were determined using the Mann-Whitney U test, and differences for normally distributed data (ELISA results) were determined using Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

H_4 histamine receptor in LSGs from healthy controls. H_4 histamine receptor mRNA was found in healthy LSGs (Figure 1). Strong H_4 histamine receptor immunoreactivity was seen in all serous demilunes of the acini (Figure 2A), and also in intercalated and striated ducts (results not shown), while H_4 histamine receptor in larger excretory ducts was localized to goblet cells (Figure 2A).

H_4 histamine receptor in LSGs from SS patients. H_4 histamine receptor mRNA levels were relatively low in LSGs from SS patients compared to those from healthy controls (Figure 1). Accordingly, when either the commercial rabbit anti-human H_4 histamine receptor

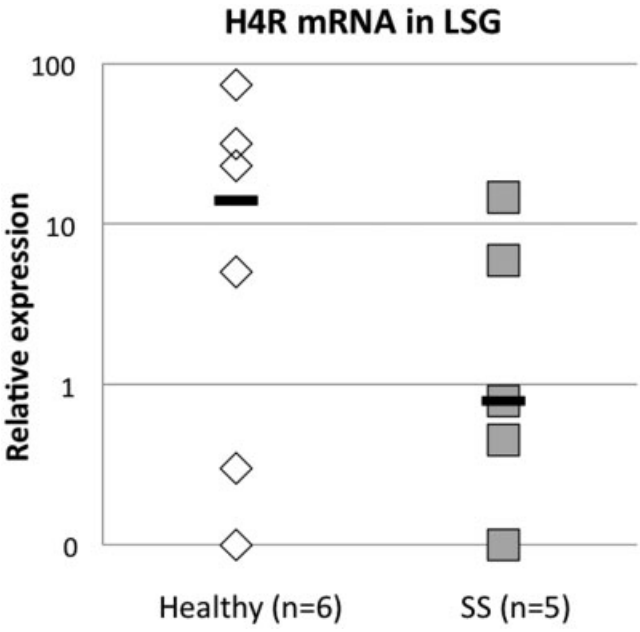

Figure 1. Relative expression of human H_4 histamine receptor (H4R) mRNA in labial salivary glands (LSGs) from healthy individuals and patients with Sjögren's syndrome (SS). Symbols represent individual patients; horizontal bars represent the mean. The difference in expression was not statistically significant ($P > 0.05$).

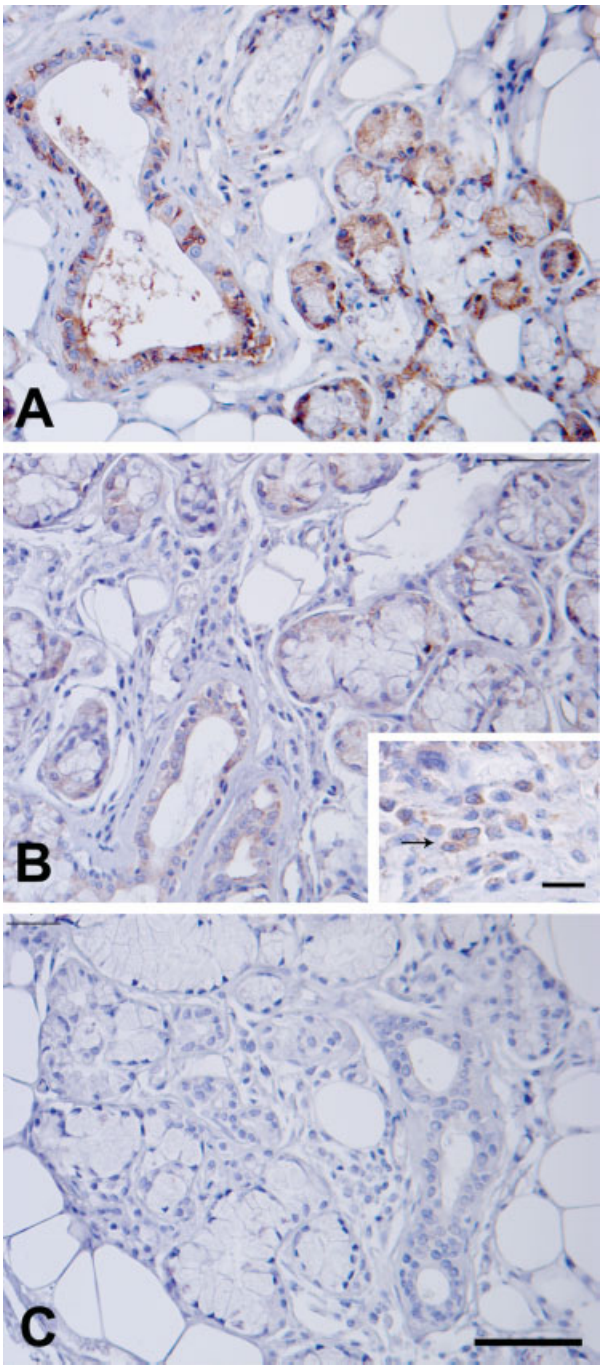

Figure 2. Immunolocalization of H_4 histamine receptor in human labial salivary glands (SGs). **A,** Staining of a sample from a healthy control subject. **B,** Staining of a sample from a patient with Sjögren's syndrome. **Inset** is a higher-magnification view showing much weaker H_4 histamine receptor staining on leukocytes infiltrating SGs. **Arrow** indicates H_4 histamine receptor–positive leukocyte infiltrate. Bar = 50 μm in **C**; 20 μm in **inset**. Original magnification × 20 in **A–C**; × 40 in **inset**. **C,** Staining of a sample from a healthy control subject with nonimmune rabbit IgG (negative control).

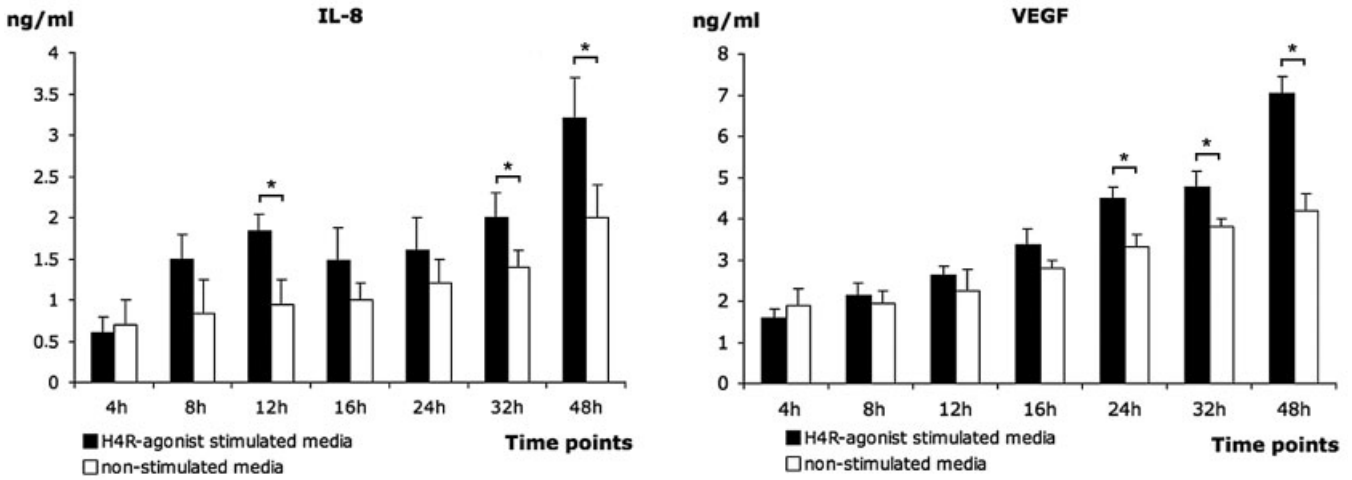

Figure 3. Interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) concentrations over time in human submandibular gland–derived salivary gland cell culture media left unstimulated or stimulated with the H_4 histamine receptor (H4R) agonist ST-1006, as determined by enzyme-linked immunosorbent assay. Bars show the mean ± SEM. $* = P < 0.05$.

antibody or a rabbit anti-human H_4 histamine receptor antibody that was produced in house was used, H_4 histamine receptor immunoreactivity was much weaker in the acini and salivary ducts of SS patients (Figure 2B) than in those from controls (Figure 2A). Infiltrating lymphocytes stained relatively weakly in both healthy controls and SS patients (inset in Figure 2B) compared to tubuloacinar structures (Figures 2A and B).

Functional H_4 histamine receptor in SG cells. According to xMAP screening and ELISA validation of histamine H_4 agonist–stimulated cells of the human submandibular gland–derived SG cell line, H_4 histamine receptor was functionally active in these cells and regulated at least IL-8 and VEGF (Figure 3).

DISCUSSION

Due to previous observations of increased mast cell numbers in tissue and high histamine levels in body fluids in various autoimmune diseases (1–3), histamine has been thought to play a role in autoimmunity. New findings show that so-called nonprofessional histamine-synthesizing cells produce small quantities of histamine and that histamine in relatively low concentrations can stimulate the more recently discovered high-avidity H_4 histamine receptor (6,7). Therefore, we analyzed the presence of H_4 histamine receptor mRNA in healthy HSGs. H_4 histamine receptor mRNA was found, which suggests local production by some SG cells in human LSGs.

In the present study, immunohistochemical staining was performed to localize the corresponding H_4 histamine receptor protein. This is relevant because bone marrow–derived hematopoietic cells contain H_4 histamine receptor (8,9). Intravascular and tissue leukocytes could account for the H_4 histamine receptor mRNA that was detected in HSGs by PCR. However, immunostaining showed that H_4 histamine receptor was mostly localized to the resident acinar and ductal epithelial cells. Some H_4 histamine receptor immunoreactive leukocytes were seen intravascularly and in the interstitial tissue. Immunostaining, using antibodies specifically produced against human H_4 histamine receptor, confirmed and extended the mRNA-level data.

The effect of H_4 histamine receptor stimulation on human submandibular gland–derived SG cells was studied using the H_4 histamine receptor–specific agonist ST-1006 (12). Cultured HSG cells were stimulated with an effective concentration of ST-1006, and the cell culture supernatant was screened for 39 different cytokines using xMAP technology and multiplex kits. This approach is useful for screening, but its interassay variation is too high for accurate measurements. Therefore, the results were validated by measuring the 2 cytokines that appeared to be the most affected, IL-8 (also known as angiogenic factor or CXCL8) and VEGF (VEGF-A), using ELISA.

Compared to healthy control glands, the proportion of IL-8–immunoreactive cells was increased in the acinar and ductal epithelial cells in glands from SS patients. Salivary IL-8 concentration is higher in SS

patients than in healthy controls. VEGF has been determined to play a harmful role in SS due to a strong relationship between pathologic neovascularization and impaired secretory function. In SS patients, histamine derived from the activated mast cells and nonprofessional histamine-producing dendritic cells and lymphocytes may excessively stimulate tubuloacinar cells via H_4 histamine receptor to produce IL-8 and VEGF. This may lead to down-regulation of H_4 histamine receptor at the mRNA and receptor protein levels in SS (see below).

Next, we compared H_4 histamine receptor levels in SGs from healthy controls to those in SGs from patients with SS. Although mRNA levels were slightly higher in healthy controls, in this small sample the difference was not statistically significant. However, immunostaining showed strong H_4 histamine receptor staining in healthy glands but very weak H_4 histamine receptor staining in glands from SS patients. This is consistent with our hypothesis that the histamine-producing cells stimulate H_4 histamine receptor–positive tubuloacinar cells so that their H_4 histamine receptor protein expression is down-regulated, e.g., via clathrin-mediated endocytosis and subsequent degradation of H_4 histamine receptor in secondary lysosomes. SGs from SS patients were relatively heavily infiltrated by hematopoietic cells, which represent the best recognized cellular source of H_4 histamine receptor (8,9). In spite of this, the H_4 histamine receptor levels were lower in glands from SS patients than in glands from healthy controls.

Measurements of histamine in saliva, using optical beam deflection fluorometry after high-performance liquid chromatography and ELISA, show that salivary histamine levels range from 0.31 to 12.4 ng/ml (2.8–112.7 nmoles [1 nmole = 0.11 ng/ml]) (14). These salivary histamine values are all too low to affect H_1 histamine receptor (pK_i 4.2) or H_2 histamine receptor (pK_i 4.3), but they do stimulate H_4 histamine receptor, because $10^{-8.1}M$ histamine will half-maximally activate this receptor (H_4 histamine receptor pK_i 8.1) (9). This suggests that H_4 histamine receptor may play some constitutive role in the maintenance of healthy salivary epithelium, a function which, based on changes in local histamine synthesis and/or greatly diminished H_4 histamine receptor levels, is disturbed in SS.

H_4 histamine receptor exists in both active and inactive forms, which undergo spontaneous isomerization. One of the important consequences is that H_4 histamine receptor, even in vivo, is constitutively (physiologically and dynamically)—without any ligand—almost half-maximally activated (15). Histamine agonists bind to the active H_4 histamine receptor isomer, stabilizing it and, thus, enhance H_4 histamine receptor activity. Many H_4 histamine receptor antagonists are inverse agonists, which bind to the inactive H_4 histamine receptor isomer, stabilizing it. This decreases H_4 histamine receptor activity. Neutral antagonists bind to both the active and inactive H_4 histamine receptor isomers, without discrimination, and do not alter H_4 histamine receptor activity, but they interfere with the subsequent binding of agonists or inverse agonists. This is consistent with the role of H_4 histamine receptor in the maintenance of the healthy tubuloacinar epithelium, with or without its natural histamine ligand in saliva. Further, H_4 histamine receptor is internalized after ligand binding or spontaneously (Stegaev V, et al: unpublished observations), leading to desensitization, but also to an ability to rapidly translocate to the cell surface via endocytotic receptor recirculation. This phenomenon may also affect the H_4 histamine receptor staining pattern.

Due to the clinical importance and commercial success of the H_1 and H_2 histamine receptor blockbuster drugs, there is great interest in H_4 histamine receptor antagonists, inverse agonists, and agonists in the pharmaceutical industry. A role for mast cells and histamine in autoimmune diseases has long been suspected. The discovery of the nonprofessional histamine-synthesizing cells, and later, at the turn of the millennium, of H_4 histamine receptor, has revived these expectations. Many H_4 histamine receptor–selective small molecular weight chemical compounds are undergoing intense investigation by major drug companies (15). They might in the future provide alternatives to relatively expensive and widely used biologic agents for the treatment of autoimmune diseases, including SS.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Konttinen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Stegaev, Sillat, Mackiewicz, Chazot, Konttinen.

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Analysis and interpretation of data. Stegaev, Sillat, Falus, Buzás, Mackiewicz, Chazot, Konttinen.

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