Presence of the 55 kDa glycosylation inhibiting factor in human serum

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Abstract

An ELISA system for the human glycosylation inhibiting factor (GIF) was established using polyclonal antibodies against highly purified 13 kDa recombinant human GIF, and the concentration of GIF in the sera of healthy donors and patients with various diseases was determined. GIF was detected in the sera of most healthy individuals and its concentration tended to increase with age. It was also found that the serum GIF levels markedly increased in some patients with rheumatoid arthritis or malignant tumors. Analysis of serum samples by SDS–PAGE and immunoblotting revealed a 55 kDa protein that has both the GIF antigenic determinant and the TCR α chain determinant. A 13 kDa GIF was not detected in the sera. In view of our previous findings on antigen-specific GIF from murine suppressor T cell hybridomas indicating that the 55 kDa GIF is a post-translationally formed conjugate of a TCR α chain with 13 kDa GIF, we suspect that the 55 kDa GIF detected in human sera is a human homologue of the murine 55 kDa GIF.

Introduction

In the course of previous studies on regulation of the IgE antibody response in the mouse, we have described glycosylation inhibiting factor (GIF), a 13 kDa cytokine that is involved in the selective formation of IgE-suppressive factors (1). It was found that GIF inhibited N-glycosylation of IgE-binding factors and that the unglycosylated IgE-binding factors selectively suppressed IgE synthesis. The major cell source of GIF is antigen-specific suppressor T (T s) cells (2). It was also found that antigenic stimulation of murine T s hybridomas resulted in the formation of GIF having affinity for the specific antigen and that the antigen-specific GIF represented an antigen-specific T s factor (TsF) (3). In an attempt to biochemically identify the antigen-specific GIF from ovalbumin (OVA)-specific T s hybridoma and homologous transflectants of TCR α chain cDNA in the T s hybridoma, we identified a 55 kDa protein that is a subunit of TsF, and has both the GIF antigenic determinant and TCR α chain determinant (4,5). Evidence was also obtained that the 55 kDa protein is a post-translationally formed conjugate of the TCR α chain with the 13 kDa GIF.

Bioactive 13 kDa human GIF has been detected in culture supernatants of some human T cell hybridomas specific for bee venom phospholipase A2 (6). Upon stimulation with an anti-TCR αβ antibody, some of the GIF-producing T cell hybridomas released GIF having affinity for the specific antigen. However, the antigen-specific human GIF has not been biochemically identified. Molecular cloning of murine and human GIF indicated that GIF of both species are 13 kDa peptides of 115 amino acids, and that human GIF has 90% homology with murine GIF at the amino acid level (7). Unexpectedly, various cell lines, including Th hybridomas and B cell lines, contained mRNA that hybridized with GIF cDNA, and culture supernatants of these cell line cells contained a 13 kDa peptide that bound anti-GIF in immunoblotting. However, the peptide from these cell line cells lacked bioactivity, and only the peptide from T s hybridomas had the activity (8). Recombinant GIF expressed in Escherichia coli and COS1 cells were also inactive (7). Since the nucleotide sequence of the GIF cDNA from the T s hybridomas and B cell lines was identical to the GIF cDNA from T s cells, we speculated that the bioactivity of GIF peptides was generated...
by post-translational modifications of the peptide in Tα cells (8). Indeed, more recent studies have provided evidence that cysteinylaton of Cys60 in the inactive GIF, which causes conformational changes in the GIF molecules, is responsible for the generation of bioactive GIF (9,10).

In the present study, we obtained polyclonal antibodies against E. coli-derived recombinant human GIF and established an ELISA system for the detection of GIF in human sera. The results show that a measurable quantity of GIF is present in most human sera, and indicate that serum GIF in aged normal individuals and in patients with rheumatoid arthritis or malignant tumors represents a 55 kDa molecule having both the GIF determinant and the TCR α determinant.

**Methods**

**Recombinant human GIF, anti-GIF antibodies and anti-TCR α antibodies**

The rhGIF was expressed in E. coli. by the method previously described (9). Recombinant hGIF was purified from a solubilized fraction of the cells by the use of a CM-Sepharose Fast Flow (Pharmacia Biotech, Uppsala, Sweden) column with an NaCl step gradient and purified GIF was isolated by HPLC on a CM-5PW column with an NaCl linear gradient, followed by gel filtration on a Superdex 75 (Pharmacia Biotech) column (11). The final GIF preparation gave a single 13 kDa band in SDS–PAGE analysis.

Rabbits were immunized by i.m. injection of 100 µg of the rhGIF peptide included in complete Freund’s adjuvant, followed by seven booster injections of the peptide with incomplete Freund’s adjuvant. The IgG fraction of the antisera was employed to detect GIF by ELISA. The fraction was biotinylated using sulfocononimidyl N-[biotinyl]-6-aminoheptanoate (Dojindo, Kumamoto, Japan) following the manufacturer’s protocol.

Anti-human TCR α antibody (H-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The preparation is affinity-purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of TCR α of human origin.

**Measurement of the serum GIF level by ELISA**

The serum concentration of GIF was measured using a sandwich ELISA system as follows. Each well of a 96-well microtiter plate (Maxi Sorp; Nunc, Roskilde, Denmark) was coated with 100 µl of the IgG fraction of rabbit anti-rhGIF at 1 µg/ml in 50 mM sodium bicarbonate buffer (pH 9.0) and left overnight at 4°C. The plate was washed 4 times with PBS containing 0.05% Tween 20 (Sigma, St Louis, MO) (PBST) and the wells were rinsed with PBS. The plate was washed between each of the following steps. The wells were blocked with Super Block (Pierce, Rockford, IL) at 4°C overnight. Serum samples were diluted 1/10 with PBS, and these samples (100 µl) were added to the wells and incubated at 4°C overnight. Recombinant hGIF diluted with PBS containing 0.5% BSA was used as a standard. Then, 100 µl of 0.5 µg/ml biotinylated anti-rhGIF in PBST was added and incubated at 4°C overnight. The plates were then incubated with 100 µl of 1/5000 dilution of horseradish peroxidase (HRP)-streptavidin (Zymed, San Francisco, CA) for 2 h at room temperature. TMB solution (Dako, Carpinteria, CA) was added to each well to develop the ELISA signals and after incubation for 30 min at room temperature, 1 N HCl/3 N H₂SO₄ was added to each well to stop the reaction. ELISA signals were determined by absorption at 450 nm and GIF concentration was calculated from a standard curve drawn by using rhGIF of known concentrations.

**Subjects**

The subjects employed in this study were 362 healthy donors (71 males and 291 females, aged 20–99 years), 26 patients with allergic diseases (seven males and 19 females; aged 20–85 years; median age, 32 years), 39 patients with malignant tumor (20 males and 19 females; aged 20–100 years; median age, 76 years) and 47 patients with rheumatoid arthritis (eight males and 39 females; aged 19–74 years; median age, 51 years).

Among the 362 adult healthy donors, most of the donors aged 20–69 years were staff workers of hospitals, nursing homes and laboratories, and most of the aged subjects (over 70 years old) were residents of old-age homes. All healthy donors were free of acute infection and immunological diseases. Many of the aged subjects were receiving medication for hypertension, hyperlipidemia, sleeplessness, osteoporosis or angina pectoris. Among the 26 patients with allergic diseases, 14 suffered from allergic rhinitis, 13 suffered from allergic dermatitis and six suffered from bronchial asthma (some patients had two or more symptoms.). Diagnosis of rheumatoid arthritis was made using the 1987 criteria of the American College of Rheumatology (12). Patients were clinically active and did not meet the criteria for clinical remission of rheumatoid arthritis (13). The mean duration of the disease was 6 years ± 1 month (range, 3 months to 28 years). To assess the radiological bone damage in the patients with rheumatoid arthritis, Larsen’s damage score, which indicates the degree of bone erosion of 20 joints of the fingers, was employed (14). The rates of progression in Larsen’s damage score were calculated by dividing the difference in the damage scores obtained at two different times by the number of months between the scoring. The 39 malignant tumor cases included seven cases of stomach cancer, four cases of lung cancer, four cases of hepatocellular carcinoma, four cases of colorectal cancer, three cases of multiple myeloma, two cases of malignant lymphoma, two cases of pancreas cancer, two cases of cholangiocarcinoma, two cases of renal cell carcinoma, two cases of prostate cancer, one case of esophageal cancer, one case of breast cancer, one case of skin cancer, one case of thyroid cancer, one case of metastatic liver cancer of unknown origin, one case of lung cancer with hepatocellular carcinoma and one case of acute leukemia. All of these cases were in the advanced stage.

**Identification of serum GIF by immunoblotting**

Serum GIF were analyzed by SDS–PAGE and immunoblotting. Serum samples were diluted 1/50 with PBS and 25 µl of each sample, mixed with the same volume of sample buffer, was electrophoresed in an 18% polyacrylamide slab gel under reducing conditions at 20 mA for 4–5 h. The peptides were then transferred to a PVDF membrane (Immobilon; Millipore,
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Fig. 1. Detection of GIF protein by ELISA. The insert graph shows absorption at low GIF levels (<0.4 ng/ml). To confirm the specificity, a portion of the biotinylated IgG fraction of anti-GIF antiserum was absorbed with a rhGIF-coupled Sepharose column. As a detection antibody in ELISA, biotinylated anti-GIF (O) or the flow-through fraction of the antibody from the rhGIF-column (C) was used. Absorption at 0 ng/ml of rhGIF was subtracted from each data, and the results are expressed as means ± SEM.

Bedford, MA). After blocking with 2% BSA in PBS for 1 h at room temperature, the membrane was incubated with 1 μg/ml of the IgG fraction of anti-rhGIF antiserum in PBST at 4°C overnight. After washing, the membrane was incubated with a 1/4000 dilution of HRP-conjugated anti-rabbit Ig (Amersham, Little Chalfont, UK) and HRP–streptavidin (Zymed), and the specific signals were visualized using an enhanced chemiluminescence Western blot detection system (Amersham).

The same method was used for the detection of peptides with the TCR α chain determinant except for the use of goat polyclonal anti-human TCR α chain antibody and HRP-conjugated anti-goat Ig (Dako).

Affinity chromatography was employed to confirm the presence of peptides with the TCR α chain determinant in human serum. Affinity-purified anti-human TCR α chain antibody was coupled to HiTrap NHS-activated Sepharose (Pharmacia Biotech) following the manufacturer’s protocol. A serum sample (5 ml) was diluted 1/10 in PBS and applied to 1 ml of a TCR α chain-coupled column and the flow-through fraction was circulated at 4°C overnight through the column. After washing with a 30 column volume of PBS, proteins retained in the column were eluted with 0.1 M glycine–HCl buffer (pH 3.0). After adjusting pH to 7.0, the fraction was added with 0.1% SDS and dialyzed against PBS. The sample was then analyzed by SDS–PAGE and immunoblotting with anti-rhGIF antibody.

Results

Serum GIF levels in healthy adult donors

We established a sandwich ELISA system for human GIF protein using the IgG fraction of polyclonal anti-GIF serum. As shown in the insert of Fig. 1, rhGIF at a concentration of 0.2 ng/ml or higher could be quantitatively measured.

To see the specificity of the ELISA system, biotinylated anti-

GIF was absorbed with rhGIF-coupled HiTrap NHS-activated Sepharose and the flow-through fraction was used in place of biotinylated anti-GIF antibody for the detection of rhGIF. As shown in Fig. 1, no increase in the ELISA signal was detected with the flow-through fraction.

We speculated that GIF might be present in normal human sera and measured the GIF levels in the sera of the 362 healthy adult donors. As shown in Fig. 2, GIF was detectable in many of the normal serum samples. It was also found that the GIF level in normal serum tends to increase with advancing age. Although no significant differences were found among the 20s, 30s, 40s, and 50s age groups or among the 60s, 70s, 80s and 90s age groups, GIF levels in the 20s and 30s age groups were significantly lower than those in the 60s and older age groups.

Increase in serum GIF levels in patients with rheumatoid arthritis and those with malignant tumor

Considering possible role of GIF in the regulation of IgE synthesis, we measured serum GIF levels of patients with allergic disease such as bronchial asthma, allergic rhinitis and atopic dermatitis. Sixteen cases out of 26 showed IgE levels that exceeded the normal range and 10 cases showed IgE levels within the normal range. We also measured serum GIF levels of age-matched healthy donors. We could not obtain a significant difference between the two groups, because 21 out of 26 patients with allergic diseases and 15 out of 26 healthy donors showed lower serum GIF levels than significant level (2 ng/ml).

However, we found that the serum GIF levels had markedly increased in some patients with rheumatoid arthritis. Measurement of the levels of GIF in sera from 47 patients with rheumatoid arthritis indicated that the patients had significantly higher serum GIF levels (18.96 ± 2.19 ng/ml) than the age-matched controls of healthy individuals (4.53 ± 0.53 ng/ml (Fig. 3).

Serum GIF levels in patients with rheumatoid arthritis may have some clinical significance. We therefore compared the
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Fig. 5. The specificity of the measurement of serum GIF by ELISA. GIF in human serum samples was measured by the ELISA system using biotinylated anti-GIF (Ab) or the flow-through fraction of the antibody preparation from a rhGIF column (Absorbed Ab) as a detection antibody (cf. Fig. 1).

Biochemical identification of serum GIF

We attempted to identify the GIF peptide in the serum samples obtained from patients with rheumatoid arthritis and those with malignant tumors by immunoblotting. Normal sera with undetectable levels of serum GIF were used as controls. Analysis of the samples by SDS-PAGE under reducing conditions and immunoblotting with anti-GIF antibody revealed a 55 kDa band, but no 13 kDa band, in the sera of patients with rheumatoid arthritis or malignant tumors (Fig. 6A). As expected, no GIF band was detectable in the control sera.

In view of our previous results on the products of GIF-producing murine T cell hybridomas showing that the 55 kDa murine GIF bound both anti-GIF antibodies and monoclonal anti-TCR α chain antibodies in immunoblotting (4,5), we investigated whether the 55 kDa GIF in the same sera has the TCR α determinant. As shown in Fig. 6B, the 55 kDa protein bound not only anti-GIF but also the antibodies specific for the human TCR α chain. In addition to the 55 kDa band, a weak 27 kDa band was detected in serum samples in which the concentration of the 55 kDa GIF was relatively high (Fig. 6B). The 27 kDa protein was usually undetectable when anti-GIF was employed for immunoblotting (cf. Fig. 6A). In some serum samples, however, a faint 27 kDa band was detected even with anti-GIF. The nature of the 27 kDa protein is unknown, but we suspect that this protein is a degraded product of the 55 kDa GIF.

We also analyzed several serum samples obtained from healthy aged individuals by SDS-PAGE and immunoblotting. In all of the serum samples with a GIF level of 10 ng/ml or higher, the 55 kDa protein with both the GIF determinant and the TCR α chain determinant was detected, but the 13 kDa GIF was not detected in any of the samples analyzed (results

Clinical features of 10 cases with high serum GIF levels (≥25 ng/ml) and 37 cases with low serum GIF levels (≤25 ng/ml) (Fig. 3). No significant difference between these two groups was found in serum CRP level, classification of functional status, radiological staging of bone damage or number of swelling joints. However, a positive correlation was found between serum GIF level and rate of progression in Larsen's damage score (cf. Methods and H. Takahashi et al., submitted).

We also measured the serum GIF concentrations in serum samples from patients with malignant tumor. As shown in Fig. 4, the serum GIF levels in the patients with malignant tumor were higher (13.61 ± 2.93 ng/ml) than those in age-matched healthy donors (5.07 ± 0.56 ng/ml). Four patients with extremely high levels of serum GIF suffered from invasion of cancer and/or metastasis. Indeed, among the 17 patients with a serum GIF level of 10 ng/ml or higher, 11 had metastasis, while only one of the 22 patients with a serum GIF level of less than 10 ng/ml had metastasis.

To confirm the specificity of the measurement of GIF in the sera of patients with rheumatoid arthritis or malignant tumors, ELISA was performed on representative cases using the biotinylated IgG fraction of anti-GIF and that absorbed with rhGIF immunosorbent. The results shown in Fig. 5 indicated that the anti-GIF antibodies in the IgG fraction were responsible for giving ELISA signals in the serum samples.

Fig. 3. Serum GIF levels in the patients with rheumatoid arthritis. Serum GIF levels in the 47 patients with rheumatoid arthritis were measured by ELISA and compared with those in age-matched healthy donors (n = 40). The results were analyzed using the unpaired t-test. *P < 0.01 compared with healthy donors.

Fig. 4. Serum GIF levels in the patients with malignant tumors. Serum GIF levels in the 39 patients with malignant tumors were measured by ELISA and compared with those in age-matched healthy donors (n = 50). The results were analyzed using the unpaired t-test. *P < 0.01 compared with healthy donors.
Fig. 6. Identification of serum GIF by immunoblotting. Immunoblotting of sera obtained from patients with various diseases with the anti-rhGIF antibody (A) or with the anti-human TCR α chain antibody (B). Lanes 1 and 2, healthy donors (serum GIF level: <2 ng/ml); lanes 3 and 4, patients with rheumatoid arthritis (serum GIF levels: 69.5 and 42.5 ng/ml respectively); lanes 5, 6 and 7, patients with malignant tumors (serum GIF levels: 75, 44.5 and 63 ng/ml respectively); lane 8, rhGIF.

not shown). We did not pursue the experiments to detect the 55 kDa GIF in the serum samples containing less than 10 ng/ml GIF by ELISA, because non-specific bands appeared in immunoblotting by using a 1:20 dilution of serum samples (see Methods).

To confirm that the 55 kDa GIF in human serum binds to anti-TCR α chain antibody, we absorbed a serum of a patient with breast cancer, that had a GIF level of 98 ng/ml, with an anti-TCR α chain antibody-coupled Sepharose column, and the acid eluate of the column was analyzed by SDS-PAGE and immunoblotting with anti-GIF. As shown in Fig. 7, the 55 kDa protein with the GIF determinant was detected in the acid eluate, indicating that the protein possessed both the GIF antigenic determinant and the TCR α chain determinant.

Discussion

In the present study, we established a sandwich ELISA method for the measurement of GIF, and detected a protein with the GIF determinant(s) in the sera of healthy aged individuals and patients with rheumatoid arthritis or malignant tumors. As the anti-GIF antibodies employed for the ELISA were obtained by immunization of rabbits with a highly purified recombinant GIF having a mol. wt of 12,346 (9), we anticipated that GIF detected in human sera would be the 13 kDa GIF. Unexpectedly, we detected a 55 kDa GIF protein, which also possessed the TCR α determinant, rather than 13 kDa GIF. Indeed, the 55 kDa GIF bound to the anti-TCR α chain antibody-coupled immunosorbent and could be recovered by acid elution (Fig. 7). Considering the mol. wt of the protein, the actual concentration (ng/ml) of the 55 kDa protein in the sera would be 4-fold higher than that estimated by ELISA.

In our previous studies using murine T cell hybridomas and homologous transfectants of TCR α chain cDNA in the hybridoma (4,5), which formed antigen-specific GIF upon stimulation with anti-CD3, we obtained evidence that the murine 55 kDa GIF is a subunit of antigen-specific GIF and represents a post-translationally formed conjugate of a TCR α chain with a 13 kDa GIF peptide (5). The association of both the GIF determinant and TCR α chain determinant with the human 55 kDa GIF indicates that the human GIF protein detected in the present experiments is a human homologue of the murine 55 kDa GIF.

Previous studies by Thomas et al. (6) on human GIF have shown that human T cell hybridomas specific for bee venom phospholipase A2 constitutively secreted the 13 kDa GIF and produced antigen-specific GIF upon stimulation with anti-CD3. The molecular size of the antigen-specific GIF was estimated to be ~80 kDa by gel filtration, but biochemical identification of the protein was unsuccessful (15). Thus, the present findings were the first demonstration of human 55 kDa GIF, which may represent a subunit of antigen-specific GIF.

Another important finding obtained in the present study is that the 55 kDa GIF is formed in vivo. It should be noted that all previous studies on the murine 55 kDa GIF were carried out using T cell hybridomas and their homologous transfectants with the TCR α chain cDNA and the protein was detected in the culture supernatant of anti-CD3-stimulated cells. The presence of the human homologue in the sera of healthy aged individuals suggests that the 55 kDa GIF is formed in vivo under physiological conditions.
The association of the TCR α chain determinant with the human GIF indicates that the cell source of the 55 kDa GIF is probably Tγδ cells, which bear TCR αβ. In the murine system, the 55 kDa GIF was formed only after stimulation of the Tγδ hybridomas with antigen-pulsed antigen-presenting cells or anti-CD3 (4,5), which facilitated translocation of the cytosolic 13 kDa GIF into the endoplasmic reticulum (16). When Tγδ cell clones and Tγδ hybridomas were stimulated with anti-CD3, the translocation of inactive cytosolic GIF to subcellular organelles did not occur and these cells failed to form the 55 kDa GIF. If the same principles apply to the human system, the cell source of the human 55 kDa GIF described here would be Tγδ cells, which constitutively secrete 13 kDa bioactive GIF.

The results of previous experiments on OVA-specific murine Tγδ hybridomas and a homologous transfectant of TCR α chain cDNA in the hybridoma indicated that the 55 kDa GIF is a product of TCR α chain cDNA (5). When the OVA-specific effector-type Tγδ hybridoma cells were stimulated by antigen-pulsed APC, essentially all 55 kDa GIF released from the cells was associated with a peptide having the TCR β antigenic determinant (4). However, the homologous transfectant secreted both the TsF and 55 kDa GIF, the latter of which is not associated with the TCR β chain (5). The results indicated that association of the TCR β chain is not required for the secretion of 55 kDa GIF from Tγδ cells. Studies by Kuchroo et al. (17) on the inducer-type murine Tγδ hybridomas suggested that the inducer-type TsF, i.e. TsF1, is a single chain molecule having the TCR α determinant but lacking the TCR β chain determinant. They also showed that the TCR α chain gene, but not the TCR β chain gene, is required for the formation of TsF1 (18). Further studies are required to determine whether the 55 kDa GIF detected in human sera is associated with the TCR β chain to form an effector-type TsF or whether it represents a single chain molecule. In any event, it seems likely that the 55 kDa GIF detected in human sera in the present study is a product of antigen-specific Tγδ cells and may represent TsF or its subunit. Previous studies have shown that the murine 55 kDa GIF is bioactive (5) and that the bioactive GIF has immunoregulatory effects on Ig synthesis (19). If the same principle applies to the human GIF system, it is reasonable to speculate that the 55 kDa GIF in human sera has immunosuppressive activities.

It is surprising that the 13 kDa GIF peptide was not detected in human sera in which 55 kDa GIF was detected. In our previous experiments on murine Tγδ hybridomas, one half or more of the 13 kDa GIF in culture supernatants of the cells was of an inactive form (20). After stimulation of the cells with anti-CD3 or antigen-pulsed APC, the majority of the bioactive 13 kDa GIF appears to be utilized for the formation of the 55 kDa GIF (5), but culture supernatant of the stimulated cells contained much more inactive 13 kDa GIF than bioactive 55 kDa GIF. Lack of the 13 kDa GIF in human sera suggests that the 13 kDa GIF peptide is easily metabolized in vivo. In vivo effect of human 55 kDa GIF is not known. However, the increase in the 55 kDa GIF in the sera of patients with malignant tumors suggests that antigen-specific GIF is formed in these patients and plays a role in the immunosuppressive state in such patients. Takahashi et al. (21) reported that Tγδ cells are generated in melanomas in mice. The 55 kDa GIF may also be involved in the decrease in immune responsiveness in aged individuals. Further studies are required for elucidation of the mechanisms involved in the formation of 55 kDa GIF in vivo, and for identification of its bioactivity.

**Abbreviations**

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<tr>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>GIF</td>
<td>glycosylation inhibiting factor</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>OVA</td>
<td>ovalbumin</td>
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<tr>
<td>TsF</td>
<td>suppressor T cell factor</td>
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**References**

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