Epitope Heterogeneity of Thyrotropin Receptor-Blocking Antibodies in Graves’ Patients as Detected with Wild-Type versus Chimeric Thyrotropin Receptors

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ABSTRACT

The stable transfectants of wild-type (W25) and mutant thyrotropin-receptor (TSH-R) allow detection of the bioactivities of TSH-R antibodies in Graves’ patients. A mutant Chinese hamster ovary (CHO) cell line (Mc1+2) transfected with a chimeric construct, where residues 8 to 165 of the TSH-R are replaced with residues 10 to 166 of the lutropin/choriogonadotropin (LH/CGR) receptor, lacks the cyclic adenosine monophosphate (cAMP) response to most thyrotropin stimulating antibodies (TSAb), yet retains the response to TSH and acquires the response to LH/CG. We compared Mc1+2 cells with wild-type W25 cells for their ability to detect TSAb as well as thyrotropin-blocking antibodies (TBAb) in Graves’ sera. Eighteen normal and 39 Graves’ sera were tested for TSAb and TBAb levels by in vitro bioassays using W25 and Mc1+2 cells. In addition, these sera were also tested for thyrotropin-binding inhibitory activity (TBII) by a radioreceptor assay. Eighteen (47%) Graves’ sera had TBAb activity measured with W25 cells but not with Mc1+2 cells. These TBAbs were, therefore, a population of antibodies with functional epitopes on the N-terminus of the extracellular domain. This TBAb activity by W25 cells exhibited a high degree of correlation with TBII levels by a radioreceptor assay ($r = 0.70, p = 0.001$). Ten (25.6%) Graves’ sera had positive TBAb activity in both W25 and Mc1+2 cells; moreover, their activity in both assays was similar ($r = 0.83, p < 0.001$). The TBAb activity in these sera, however, did not correlate with TBII activity. Eleven (28%) Graves’ sera had no TBAb activity. Overall, thyroid-stimulating antibodies were detected in 87% and 28% of the 39 Graves’ sera by W25 and Mc1+2 cells, respectively. Thus, using the 2 cell lines, at least 2 distinct populations of TBAbs were detected. One is detected in a similar fashion by both W25 and Mc1+2 cell lines and likely interacts with the epitopes residing in the unaltered C-terminus of the TSH-R. The other is reactive in W25 cells only, indicating the loss of TBAb epitope in the chimeric receptor located in the N-terminus of the TSH-R. Furthermore, our results indicate that the TBAb binding epitope in 8–165 residues of the native TSH-R is highly associated with TBII activity in Graves’ disease. These results indicate that patients with Graves’ disease harbor TBAbs with epitope heterogeneity and favor the notion that there are different sites and mechanisms by which TBAbs act in Graves’ patients. It remains to be determined whether or not TBAb subtyping will have a useful predictive role in the management of patients with Graves’ disease.

INTRODUCTION

The clinical picture in autoimmune thyroid disease is dependent on the functional status of the thyroid gland and the heterogeneous population of autoantibodies directed against the thyrotropin receptor (TSH-R). The autoantibodies include both thyrotropin receptor stimulating (TSAb) as well as blocking (TBAb) antibodies. In clinical laboratories, TSAb are measured by a bioassay, which determines the ability of patients’ immunoglobulin to stimulate cyclic adenosine monophosphate (cAMP) production in cultured rat thyroid cells (FRTL-5) (1). The recent availability of TSH-R transfected Chinese hamster ovary (CHO) cell lines has provided us with a new generation of func-

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tional assays for TSABs. These assays have markedly improved the sensitivity, precision, and reliability, as well as the cost effectiveness of TSAb measurement (2,3). The TSAb assay can be modified to measure TBAb by its ability to block cAMP production in cells responding to TSH treatment (4). However, due to the heterogeneity of autoantibodies present in patients’ sera, it is difficult to measure TBAb precisely in the presence of TSAb. TSH-R autoantibodies can also be measured by the thyrotropin-binding inhibitory immunoglobulin (TBII) assay, which detects the ability of patients’ immunoglobulin to compete for TSH binding to the solubilized porcine TSHR (5). However, the TBII assay does not discriminate between TSAb and TBAb.

Mutant TSH-Rs have been used to facilitate the search for TSH binding sites and epitopes for the autoantibodies. Studies with these mutants have shown that the N-terminal portion of the extracellular domain of the TSHR is important for TSAb activity, whereas the C-terminal region is important for TBAb activity in patients with hypothroidism and idiopathic myxedema (6–8). Several laboratories (9–14) also used TSH-R and lutropin/chori‐
ogonadotropin receptor (LH-CGR) chimeras (TSHR/LH-CGR) to evaluate the locus of these epitopes, with similar findings. For example, in several series, 95% of Graves’ patients were found to have their major TSAb epitopes on the N-terminal portion of the extracellular domain of the TSHR, whereas the major TBAb epitope in more than 90% of patients with hypothroidism and idiopathic myxedema was located on the C-terminus (11,12,14). A recent case report, evaluating the monoclonal antibodies generated from lymphocytes of a female patient (with Hashimoto’s thyroiditis and children with transplacental neonatal hyperthyroidism) confirmed the functional heterogeneity of TSHR antibodies and suggested that TBAb epitopes in some hyperthyroid patients might exist on the N-terminus of the TSHR extracellular domain (15). Nonetheless, TBAb epitopes in Graves’ patients were not characterized fully in these studies. This has been the focus of our present investigation.

A CHO cell line stably transfected with a TSHR/LH-CGR chimera termed McI+2, where residues 8 to 163 of the human TSHR extracellular region are replaced with residues 10 to 166 of the LH-CGR, has been shown previously to lack the cAMP response to TSAb but retain the response to authentic TSH as well as to LH/CG (11,12). It seemed possible that this cell line, with minimal or no response to the TSAb in Graves’ immunoglobulin G (IgG) preparations, might enable us to measure accurately the locus and the prevalence of TBAb in patients with Graves’ disease. In the present study, we determined the utility of the McI+2 cell line to detect TBAb in Graves’ sera and compared it with another CHO cell line, W25, stably transfected with the wild-type human TSHR.

**MATERIALS AND METHODS**

**Study subjects**

Sera from 18 normal individuals (median age = 37, range 25–52 years, female: male = 3.5:1) with no history of thyroid disease and from 39 patients with the diagnosis of Graves’ disease (median age = 44, range 13–80 years, female: male = 3.87:1) were analyzed for both TSAb, TBAb, and TBII. In addition, 15 randomly selected sera from patients with Graves’ disease were used to measure their blocking activities against either bovine TSH (bTSH) or human CG (hCG) (see below). The diagnosis of Graves’ disease was based on conventional clinical and laboratory evaluations. All 39 patients were hyperthyroid at the time of diagnosis and all but 5 were hyperthyroid at the time of testing as evidenced by thyroid function tests (suppressed TSH and increased free thyroid index or free thyroxine levels). Retrospective review of the 39 patients’ records revealed that 27 were untreated, 8 had previous antithyroid drug treatment, 2 had previous radioactive iodine treatment, and 2 had previous history of partial thyroidectomy. Twenty-one of 39 had no clinical evidence, 11 had clinical evidence, and 6 had an unknown status for Graves’ ophthalmopathy.

**Cell lines**

Two CHO cell lines were used. W25 was stably transfected with the wild-type human TSHR (11,12,14). McI+2 was transfected with a chimeric receptor construct, where residents 8 to 165 of the human TSHR extracellular region were replaced with residues 10 to 166 of the LH-CGR (11,12,14).

**In vitro bioassay**

Both W25 and McI+2 cells were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum. Cells were seeded in 96-well plates (1 x 10⁴ cells/well) 48 hours prior to assay. Patients’ immunoglobulins (IgGs: crude polyethylene glycol extracts) in NaCl-free hypotonic buffer (100 µL) were incubated with the cells, in the presence or absence of 10 µU/mL bTSH (or 2 nM hCG when performing the hCG blocking assay), for 2 hours at 37°C. Extracellular cAMP levels were determined by means of a radioimmunoassay kit (Instar Inc., Stillwater, MN). All assays were performed in duplicate. TSAb activity was expressed as a percent increase in cAMP compared to the response of a normal IgG (pooled extract). The interassay coefficients of variation (CVs) for a TSAb-positive control sample (n = 5) were 18 and 19% for W25 and McI+2 cells, respectively. TBAb activities were determined by measuring the ability of patients’ IgG to inhibit TSH-stimulated cAMP release in comparison to normal pool IgG response and is calculated as follows (16):

\[
\text{TBAb activity (X)} = \left[1 - \frac{(\text{Patient} + \text{bTSH}) - \text{Patient}}{(\text{Normal} + \text{bTSH}) - \text{Normal}}\right] \times 100
\]

The interassay CVs for TBAb were 11.5% and 17% for W25 and McI+2 cells (n = 5). Samples were analyzed in duplicate and within run CVs ranged between 3%–13% for both W25 and McI+2 cells.

**TBII assay**

We used a commercially available TSH receptor assay for TBII, which utilizes a solubilized porcine TSH-R (KRONUS, San Clemente, CA) with minor modifications.
A serum pool calibrated against the Medical Research Council reference serum (long-acting thyroid stimulator B) was used as a standard. Results were expressed as units per liter (U/L) (16).

Statistical analysis

Comparison for each variable between different groups was performed by Student's *t* test or by the Mann-Whitney rank test if the normality test failed. The correlations among variables in the whole group as well as in each subgroup were determined by the Spearman rank correlation test. Chi-square analysis (Fisher exact test) was used to compare the diagnostic sensitivities of the 2 cell lines. Statistical analyses were performed using the statistical software package, Sigma Stat (Jandel Scientific, San Rafael, CA).

RESULTS

bTSH and hCG responses in W25 and Mc1+2 cells

We first determined the responses to bTSH and hCG in the wild-type TSH-R-transfected W25 and the chimeric TSH-R-transfected Mc1+2 cells. Both W25 and Mc1+2 cells showed a dose-dependent cAMP response to bTSH (Fig. 1A), confirming previous results (11,14). Treatment with hCG at concentrations up to 100 nM did not elicit a cAMP response in W25 cells. Mc1+2 cells, however, responded to hCG treatment in a dose-dependent manner.

Activities of Graves' sera in blocking bTSH-vs. hCG-stimulated cAMP production in Mc1+2 cells

We compared blocking activities of IgGs from 15 Graves' patients' sera challenged with either bTSH or hCG in Mc1+2 cells. Figure 1B shows that these Graves' sera could block both bTSH- and hCG-stimulated cAMP production in Mc1+2 cells. There was a strong statistical correlation (*r* = 0.883, *p* < 0.001) between the blocking activities against bTSH and hCG.

TSAb and TBAb in normal control sera

We determined the range of TSAb and TBAb activity detected by W25 and Mc1+2 cell lines in IgG extracts from normal individuals. Expressed as percentage increase in cAMP, the TSAb activities in normal sera ranged between 74%-122% (98% ± 12% [mean ± SD]) by W25 cells (n = 18), and ranged between 64%-132% (98% ± 17%) by Mc1+2 cells (n = 17). The actual basal cAMP levels (mean ± SD) in these normal sera were 1.96 ± 0.23 and 2.9 ± 0.75 pmol/well and in the presence of TSH (10 μU) were 6.0 ± 0.58 and 12.8 ± 1.6 pmol/well by W25 and Mc1+2 cells, respectively. The mean percentage cAMP increases by TSH in W25 and Mc1+2 cells were 315% and 440%, respectively. The TBAb activities in normal sera ranged between -30% to +38% (4.2% ± 17%) in W25 cells (n = 18), and from -28% to +44% (8.0% ± 18%) in Mc1+2 cells (n = 17). A value above the 2 SD of these normal sera was used to define the positive responses for both TSAb and TBAb activities. Although, the 2 cell lines had comparable normal ranges, there was no statistically significant correlation found between the TBAb levels by the 2 cell lines in normal sera.

TSAb and TBAb activities in patients' sera detected by W25 and Mc1+2 cells

We examined the ability of both cell lines to detect TSAb and TBAb in sera from 39 patients with Graves' disease. The results are shown in Table 1. As expected, Mc1+2
cells lacked the stimulating response to most TSAb (87.2% by W25 vs. 28.2% by Mc1+2 cells), and when detected (n = 11) the TSAb levels were significantly lower than seen in W25 cells (median 160 [range 150–300] vs. 333 [range 200–1050]% vs. p < 0.001). In addition, Mc1+2 cells also detected TSAb in fewer patients than did W25 cells (71.8% by W25 vs. 25.6% by Mc1+2).

We also tested the ability of W25 and Mc1+2 cells to detect TBAb in a serum obtained from a patient with levothyroxine-treated primary myxedema who had a child born with transient neonatal hypothyroidism. This patient had high TBII activity (468 U/L) and high TBAb activity as detected by FRTL-5 cells (17). The TBAb activity detected by W25 cells was highly positive with a value of 104% ± 12% (mean ± SD, n = 4). On the other hand, TBAb activity in this serum by Mc1+2 cells was consistently within the normal range (19% ± 4%).

**TBAb epitope heterogeneity**

Based on TBAb positivity, patients were divided into 3 groups. Group 1 comprised 10 patients (25.6%) who tested positive for TBAb by both Mc1+2 and W25 cells. Group 2 had 18 patients (46.2%) who tested positive for TBAb by W25 cells only. Group 3 had 11 patients (28.2%) who tested negative by both cell lines. No patient was positive by Mc1+2 and negative by W25. Retrospective review of all study patients’ charts did not reveal any distinctive clinical features in any of the 3 groups. The groups were not different in terms of thyroid function tests, past treatment for Graves’ disease, and Graves’ ophthalmopathy. Table 2 summarizes the results obtained for TSAb and TBAb levels by W25 and Mc1+2 cells as well as TBII levels. Group 3 patients had the lowest TSAb and TBII values among all three groups (p < 0.06). TSAb and TBII values in groups 1 and 2 patients were not statistically different.

We compared TBAb values by W25 and Mc1+2 cells in group 1 patients, who were positive by both cell lines. There was no statistically significant difference among the values obtained by the 2 cell lines and the mean ± SD being 86 ± 23 vs. 77 ± 14% for W25 and Mc1+2 cells, respectively. Also the correlation between the TBAb values by W25 and Mc1+2 cells was strongly positive; (r = 0.88, p < 0.001) in group 1 patients (Fig. 2). There was only a marginally significant correlation (r = 0.59, p = 0.045) between the TBAb levels in group 3 patients, and no significant correlation was observed in group 2 patients (r = 0.26, p = 0.41).

**Correlation between TBII and TBAb**

There was a significantly positive overall correlation between TBII values and TBAb activities detected by W25 cells in all Graves’ patients (n = 39, r = 0.69, p < 0.001). A relatively weaker overall correlation was observed between TBII and TBAb activities measured by Mc1+2 cells (r = 0.48, p < 0.01). However, among the 2 TBAb positive groups, a statistically significant correlation between TBII and TBAb was seen only in group 2 patients (TBAb positive by W25 cells only; r = 0.70, p = 0.001) (Fig. 3A) but not in group 1 patients either by W25 cells (r = 0.47, p = 0.16) or by Mc1+2 cells (r = 0.50, p = 0.14) (Fig. 3B).

There was no significant correlation between TBII and TSAb activities measured by either W25 or Mc1+2 cells.

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**Table 1.** TSAb and TBAb Levels and Positivity in Graves’ Patients (n = 39) as Detected by W25 and Mc1+2 Cell Lines

<table>
<thead>
<tr>
<th></th>
<th>W25</th>
<th>Mc1+2</th>
<th>p value*</th>
<th>W25</th>
<th>Mc1+2</th>
<th>p value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSAb†</td>
<td>317 ± 262</td>
<td>128 ± 57</td>
<td>&lt;0.001</td>
<td>34 (87.2)</td>
<td>11 (28.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TBAb††</td>
<td>64 ± 36</td>
<td>28 ± 35</td>
<td>&lt;0.001</td>
<td>28 (71.8)</td>
<td>10 (25.6)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

†Expressed as % cyclic adenosine monophosphate (cAMP) increase over normal control.
††Expressed as % inhibition of thyrotropin (TSH)-stimulated cAMP compared to normal control.
* t test and **chi-square test for W25 vs. Mc1+2.

TSAb, thyrotropin receptor-stimulating antibodies; TBAb, thyrotropin receptor-blocking antibodies.

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**Table 2.** Comparisons of TSAb, TBAb and TBII Values (Mean ± SE) in Subsets of Graves’ Patients Based on TBAb Positivity

<table>
<thead>
<tr>
<th></th>
<th>W25</th>
<th>Mc1+2</th>
<th>W25</th>
<th>Mc1+2</th>
<th>TBII (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TSAb (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (n = 10)*</td>
<td>281 ± 55*</td>
<td>128 ± 21</td>
<td>86.0 ± 7*</td>
<td>77 ± 4.5*</td>
<td>54 ± 14*</td>
</tr>
<tr>
<td>Group 2 (n = 18)b</td>
<td>386 ± 76*</td>
<td>145 ± 14</td>
<td>78.6 ± 6*</td>
<td>14 ± 4.7</td>
<td>36 ± 6.8*</td>
</tr>
<tr>
<td>Group 3 (n = 11)c</td>
<td>238 ± 59</td>
<td>100 ± 22</td>
<td>19.7 ± 4.6</td>
<td>5.4 ± 6.3</td>
<td>13.3 ± 2.5</td>
</tr>
</tbody>
</table>

*TBAb positive by both cell lines.
bTBAb positive by W25, negative by Mc1+2.
cTBAb negative by both cell lines.
*p < 0.05 when compared to Group 3.

TBAb, thyrotropin receptor-blocking antibodies; TSAb, thyrotropin receptor-stimulating antibodies; TBII, thyrotropin-binding inhibitory immunoglobins.
EPITOPE HETEROGENEITY OF THS-BLOCKING ANTIBODIES

FIG. 2. Correlation between thyrotropin receptor-blocking antibodies (TBAb) activities measured by W25 and Mc1+2 cells in Group 1 (O) patients detected positive for TBAb in both W25 and Mc1+2 cells (r = 0.88, p < 0.001). Dashed lines indicate upper limit of normal range. Actual levels of TBAb as obtained by the 2 cell lines, in group 2 (●) and group 3 (+) patients are also plotted for comparison (r = 0.21, p = 0.41 and r = 0.59, p = 0.045, respectively).

DISCUSSION

Previous studies using TSHR/LH-CGR chimeras have identified the importance of the N-terminus of TSHR (residues 30-165) in the detection of TSAb activities in most patients with Graves' disease (11,12,14). In the present study, we used the Mc1+2 cell line, which was engineered to remove the major epitopes for TSAbs, to detect TBAb activities in Graves' sera. As expected most Graves' sera lost their ability to stimulate cAMP in these cells. In addition to their acquired responsiveness to hCG, Mc1+2 cells retained the ability to respond to bTSH, suggesting that the receptor function is not disrupted. Both bTSH- and hCG-stimulated cAMP production could be inhibited by Graves' sera containing blocking activity. Thus, it seemed that Mc1+2 cells would provide a means to measure TBAb in Graves' sera without the interference of TSAb. However, the Mc1+2 cell line was able to detect TBAb in fewer patients than did the wild-type TSHR-transfected W25 line. Of the 28 Graves' disease patients who were TBAb-positive in the W25 assays, most (64%) were negative in the Mc1+2 assays, suggesting that this population (Mc1+2 negative) of TBAbs recognizes the N-terminal portion of the extracellular domain of the TSHR, which is replaced in Mc1+2. This TBAb is different from the TBAb population, which is prevalent in many patients with hypothyroidism and idiopathic myxedema or Hashimoto's thyroiditis and which recognizes the C-terminus of the TSHR extracellular domain (10-15) and is detected by both W25 and Mc1+2 cells. Here we report that only a small subset (approximately 25%) of Graves' patients harbor this C-terminus reactive TBAb. These results establish that there is heterogeneity of TBAb in patients with Graves' disease and that a substantial proportion of Graves' TBAb have recognition sites that involve N-terminal determinants of the TSHR.

TBAbs are also detected in the TBII assay. In a recent study, Kohn et al. (15) have also reported immunoheterogeneity in TBII. Monoclonal antibodies generated using lymphocytes from a hypothyroid woman (who had 3 children with neonatal hyperthyroidism) included TSAb-

FIG. 3. Correlation between thyrotropin receptor-blocking antibodies (TBAb and thyrotropin-binding inhibitory immunoglobulins (TBII) (A) in Group-2 patients detected positive only by W25 cells (r = 0.70, P = 0.001) and (B) in Group-1 patients detected positive by both Mc1+2 cells (open circles and dashed line) and by W25 cells (closed circles and solid line) (r = 0.50, p = 0.14 and r = 0.47, p = 0.16, respectively). Formula \( Y = a_0 + a_1 \ln X \), was used for curve fit. Dotted lines indicate the upper limit of the normal range. Blocking activities were determined by measuring the ability of patients' immunoglobulin extracts to inhibit bovine thyrotropin (bTSH)-stimulated cyclic adenosine monophosphate (cAMP) release as described in Methods.
producing (weak TBII activity) and a number of TBII positive clones with TSH blocking activity. Some TBII positive clones had C-terminus specificity whereas others reacted with the N-terminus of TSHR. Our results, establishing the presence of N-terminus and C-terminus reactive TBAbs in Graves' patients validate their observations and have a number of additional implications.

First, our results may explain the variable correlations and disparity observed between TBAb and TBI activity in different studies using site-directed mutagenesis (7, 10 and 13) and antipeptide antibodies (18). Thus, there is a poor correlation of TBI activity with TBAb activity in Mc1+2 cells because the Graves' TBI epitope on the N-terminus is lost. Second, it is likely that TBAbs reactive with the C-terminus of the TSHR may play an important role in the course of the disease, because these TBAbs appear to be able to block TSAb as well as TSH activity, whereas TBAbs associated with the N-terminal epitopes appear to be able to block only TSH activity (15,19–24). Consequently, these antibodies may affect disease expression as the levels or populations of TSAbs and TBAbs shift in time. Third, the serum from a hypothyroid patient described herein seems to contain the subtype of TBAb dependent on the N-terminus of the extracellular domain of the TSHR, which is prevalent in Graves' patients, despite the fact that its origin was from a patient with idiopathic myxedema. Whereas most patients with idiopathic myxedema and hypothyroidism appear to have TBAbs directed at the C-terminus of the TSHR (6–8,10–15), some may possess a heterogeneous population of TBAbs directed at both the C- and N-terminus just as in patients with Graves' disease.

Increasing evidence indicates that the stimulating TSHRAb is heterogeneous (25,26). Moreover, measuring this heterogeneity of TSAbs has potentially identified a subtype of TSAb with clinical implications, i.e., responsiveness to medical (antithyroid drugs) therapy (13). Less is known about the heterogeneity of TBAbs particularly because their measurement as TBI does not discriminate among them functionally or by epitope distribution. This issue has clouded the relationship of TBI or TBAb activity to clinical relevance, perhaps because a heterogeneous population is being measured. The present report, for the first time, distinguishes 2 populations with different epitopes in Graves' patients. This will now enable us to elucidate the clinical significance in patients, i.e., their response to therapy, and the likelihood of development of hypothyroidism. Based on our results and on previously reported observations, one can speculate that C-terminal specific TBAbs may play a role in causing and maintaining the remission of Graves' disease posttreatment by their ability to block both TSAb and TSH activity. Recently the controversy concerning the clinical use of TBI was debated (27). This report emphasized the need for simple and sensitive assays to discriminate among receptor subtypes in order to better assess the clinical relevance. Our data open the possibility that this subtype analysis can now be accomplished for TBIs and TBAbs as has been the case for TSAbs.

In summary, our results indicate that patients with Graves' disease harbor TBAbs with epitope heterogeneity. Two distinct TBAb subtypes can be measured by combined use of wild type and chimeric TSH receptors. Our results support the notion that there are different sites and mechanisms by which TBAbs act in Graves' patients.

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