A New Immunoradiometric Assay for Corticotropin Evaluated in Normal Subjects and Patients with Cushing's Syndrome

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We evaluated a new, commercially available two-site immunoradiometric assay (IRMA) for corticotropin (ACTH) in human plasma. The precision and detection limit were an improvement over radioimmunoassay (RIA). Addition of ACTH 1-24 or ACTH 18-39 to plasma containing ACTH 1-39 resulted in a decrease in measured ACTH. Results by both IRMA and RIA are reported and compared for normal subjects, patients with Cushing's disease, and patients with ectopic ACTH. Effects of administering dexamethasone, metyrapone, ACTH 1-24, and corticoliiberin were evaluated. The present assay is more sensitive, specific, and reproducible than RIA. The presence of fragments of ACTH or "big" ACTH that are reactive in RIA may lead to artifically low ACTH concentrations by IRMA. Therefore, RIA may still be necessary for differential diagnosis of ectopic ACTH syndromes. However, the IRMA is an improvement for evaluating pituitary-adrenal function in patients with low concentrations of ACTH in plasma.

Additional Keyphrases: radioimmunoassay compared · pituitary-adrenal function · dexamethasone · metyrapone · corticoliiberin (corticotropic-releasing factor)

The ability to measure corticotropin (ACTH) in plasma not only has improved the differential diagnosis of pituitary-adrenal disorders but has also greatly expanded basic research in the control of hypothalamic-pituitary-adrenal function. Some clinicians have been reluctant to accept RIA measurements of ACTH in plasma because of early problems with sensitivity and specificity (1).

The development of the two-site immunoradiometric assay (IRMA) has greatly improved the evaluation of thyroid and parathyroid disease (2, 3). In this type of assay, two antibodies—nowadays often monoclonal—are directed at two different sites (e.g., the N- and C-terminals) on the hormone to be measured. Because both sites must be bound to generate an assay response, fragments of the hormone may not be detected, which enhances the sensitivity and specificity of the measurement.

Here we report our evaluation of the IRMA for measurement of ACTH in plasma, which has recently been made available commercially. This assay requires relatively small volumes of plasma, is rapid (<22 h incubation), and has very high specificity and sensitivity. We also compared samples from normal subjects and patients with ACTH-dependent Cushing's syndrome, using both the new IRMA and the non-extraction RIA already in use in our laboratory. Responses to ovine corticoliiberin (corticotropic-releasing factor) in normal subjects and in patients with Cushing's syndrome highlighted the advantages of the IRMA.

Materials and Methods

Normal Subjects

Six ostensibly normal healthy male volunteers, 23 to 28 years of age, were studied on four mornings at least three days apart. Blood was sampled between 0745 and 0930 hours. The protocol was approved by the Institutional Review Board of St. Luke's Medical Center, and informed consent was acquired from each subject.

ACTH stimulation test: From each subject, we collected 10 mL of blood before and 30 min after administration of the standard stimulation dose of ACTH 1-24 (250 µg, intravenously, Cortrosyn; Organon, West Orange, NJ). Metyrapone stimulation test: Each subject took 2.5 g of metyrapone (Metopiron; Ciba, Summit, NJ) orally at 2200-2300 hours on the night before sampling, and 15 mL of blood was drawn the next morning.

Dexamethasone suppression test: Each subject took 1 mg of dexamethasone (Roche; Columbus, OH) orally at 2200-2300 hours the night before sampling. Ten milliliters of blood was drawn the next morning.

Corticoliiberin stimulation test: After a control sample of blood was collected, we injected 100 µg of ovine corticoliiberin (a generous gift of Dr. George Chrousos, NIH) into each subject. Blood samples (10 mL each) were drawn at 15, 30, 45, 60, 75, and 90 min after the administration of corticoliiberin.

Patients

Cushing's syndrome: All patients were studied during clinically indicated bilateral simultaneous sampling of blood from the inferior petrosal sinuses with corticoliiberin administration as previously described (4, 5). After catheters were placed in the right and left petrosal sinus and in the inferior vena cava, 5-mL blood samples were drawn (control). Corticoliiberin was then administered as described above and blood samples were drawn 2, 5, and 10 min later.

Surgery: We also assayed plasma samples from a previous study (6), which had been stored at −70°C. These samples were drawn from patients undergoing coronary revascularization, who had been premedicated with diazepam and anesthetized with pentothal and ethane as described previously (7). Samples were taken before (control) and 30 min after the start of thoracotomy.

Pools

Pooled specimens of human plasma were used to determine assay performance: a low-concentration pool (normal), a medium-concentration pool (combination of plasma from 30 clinical patients), and a high-concentration pool (plasma from a patient with ectopic ACTH-dependent Cushing's syndrome). In addition, we used the low- and high-concentration pools supplied by the IRMA manufacturer. Parallelism was determined by serial dilutions of synthetic ACTH 1-39 and of plasma from patients with above-normal ACTH concentrations (pituitary and ectopic ACTH-dependent Cushing's syndrome and primary adrenal insufficiency).
Procedures

**IRMA:** This is a non-extraction assay supplied by the Nichols Institute, San Juan Capistrano, CA. In it are used (a) synthetic human ACTH 1-39 standard (Peninsula Labs; Belmont, CA); (b) 125I-labeled monoclonal antibody [Ab(m)] directed against ACTH 1-17 (the N-terminal region of ACTH 1-39); and (c) polyclonal antibody [Ab(p)], affinity purified against ACTH 34-39 (C-terminal region of ACTH 1-39); and coupled to biotin. Addition of an avidin-coated bead allows a "sandwich" complex to form. Standard or sample (200 μL) is assayed in duplicate. After 20 to 22 h of incubation, the bead is washed and its radioactivity is counted. ACTH 1-39, which has bound both Ab(p) and Ab(m), is measured. The standard curve generated has a positive slope and is linear; therefore, little data reduction is required. Cross-reactivities are minimal for ACTH 11-24, ACTH 1-10, alpha- and beta-melanotropin, beta-lipotropin, and beta-endorphin. Addition of ACTH 1-24 and ACTH 18-39 to zero standard had no effect on zero standard counts (i.e., nonspecific binding). However, addition of 341 pmol (1000 pg) of ACTH 1-24 or 406 pmol (1000 pg) of ACTH 18-39 to 33 pmol (150 ng) of ACTH 1-39 per liter resulted in decreases in assayable ACTH concentrations of 15 pmol/L (70 ng/L) and 4.4 pmol/L (20 ng/L), respectively. This is due to binding of ACTH 1-24 and ACTH 18-39 to Ab(m) and Ab(p), respectively, without forming a "sandwich" complex. We made a more detailed evaluation of this phenomenon, using the biologically active fragment, ACTH 1-24. We used five post-metyrapone samples of plasma with an ACTH concentration of 55.5 (SEM 8.5) pmol/L by IRMA. Addition of 341 and 682 pmol of ACTH 1-24 per liter significantly (P <0.001) decreased ACTH 1-39 concentration, as measured by IRMA, to 42.3 (SEM 9.2) and 36.3 (SEM 9.0) pmol/L, respectively.

We determined sensitivity by assaying serial dilutions of the low-concentration pool. Sensitivity was defined as the lowest duplicate concentration of ACTH 1-39 measuring greater than one standard deviation above the zero standard (nonspecific binding). Sensitivity determined in this way, in one assay, performed in duplicate, was approximately 0.4-0.7 pmol/L (2-3 ng/L) when sample radioactivity was counted for 1 min. Because the low end of the curve has low radioactivity (<600 counts/min in our gamma counter), increasing counting time to 20 min (or <1% counting error) increased the sensitivity to 0.3 pmol/L (1.5 ng/L).

**RIA:** This is a standard non-extraction RIA in which National Pituitary Agency antiserum (West), as supplied by the Nichols Institute, is used (6-8). Intra- and interassay coefficients of variation were 6% (n = 10) and 10% (n = 8), respectively. This antiserum has equimolar cross-reactivity with ACTH 1-39 and ACTH 1-24. The reliable lower limit of detection of the RIA was 4.4 pmol/L (20 ng/L).

**Steroids:** Cortisol was measured by double antibody RIA (Dupont-NEN) as described previously (8). 11-Deoxycorticisol (Compound S) was measured at the Nichols Institute Reference Laboratory.

**Data analysis:** Data were analyzed by paired and unpaired t-test, one- and two-way analysis of variance for repeated measures followed by Duncan’s multiple-range test, and linear regression by least-squares best fit. A P of <0.05 was considered significant.

**Results**

**Assay performance (Table 1):** The intra-assay and interassay coefficients of variation were higher for the low pool and control because the counts per minute were low (300–600).

<table>
<thead>
<tr>
<th>Table 1. ACTH IRMA Performance</th>
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<tbody>
<tr>
<td><strong>Plasma</strong></td>
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<tr>
<td>Mean</td>
</tr>
<tr>
<td>Low pool</td>
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<tr>
<td>Med. pool</td>
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<tr>
<td>High pool</td>
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</table>

As stated in the Methods section, the IRMA distinguished between 0 and 0.6 pmol/L (2.5 ng/L) when the radioactivity was counted for 1 min. Increasing the counting time to 15-20 min improved the sensitivity such that results for 0 and 0.3 pmol/L (1.4 ng/L) were significantly different. Figure 1 shows results for parallel dilutions of synthetic human ACTH 1-39 and plasma from the patients. The standard curve has a positive, steep slope and is linear on a log-log scale.

**Normal subjects:** Data from the normal subjects are shown in Table 2 and Figure 2. The mean baseline concentration of ACTH in plasma from samples drawn at 0730–0830 hours was 7.5 (SEM 0.9) pmol/L [34 (SEM 4) ng/L] by IRMA, and the concentration was below the reliable detection limit of the RIA in three of the six subjects. Dexamethasone suppressed ACTH concentration measured by IRMA (0.86 SEM 0.18 pmol/L; 3.9 (SEM 0.8) ng/L). ACTH concentration was undetectable by RIA in five of the six subjects after dexamethasone administration. Interestingly, ACTH concentration measured by RIA in one subject was actually higher (7.1 pmol/L; 32 ng/L) after dexamethasone as compared with baseline, although suppression was detected by IRMA and assay of plasma cortisol concentration (39 nmol/L; 14 μg/L). Dexamethasone produced a significant decrease in plasma cortisol concentration in all six subjects and averaged 44 (SEM 10) nmol/L [18 (SEM 4) μg/L].

Administration of metyrapone resulted in a significant increase in ACTH, which was similar whether measured by IRMA or RIA. Plasma cortisol concentration was decreased [179 (SEM 36) nmol/L; 65 (SEM 13) μg/L] after metyrapone administration. 11-Deoxycorticisol was significantly increased from a baseline of 2.4 (SEM 0.5) nmol/L [0.83 (SEM 0.3) μg/L].
Table 2. Concentrations of ACTH in Plasma from Normal Subjects (pmol/L)*

<table>
<thead>
<tr>
<th>Subject</th>
<th>Baseline</th>
<th>Dexamethasoneb</th>
<th>Metyraponec</th>
<th>CoisyntrOIfld</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IRMA</td>
<td>RID</td>
<td>IRMA</td>
<td>RID</td>
</tr>
<tr>
<td>1</td>
<td>5.3</td>
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<td>0.64</td>
<td>&lt;4.4</td>
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<td>2</td>
<td>7.1</td>
<td>&lt;4.4</td>
<td>0.86</td>
<td>7.1</td>
</tr>
<tr>
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<td>&lt;4.4</td>
<td>0.40</td>
<td>&lt;4.4</td>
</tr>
<tr>
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<td>8.1</td>
<td>4.6</td>
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</tr>
<tr>
<td>6</td>
<td>11.0</td>
<td>7.1</td>
<td>1.30</td>
<td>&lt;4.4</td>
</tr>
</tbody>
</table>

Mean 7.5 --- * 0.86' --- 62.8' 59.5 3.3 1079'

SEM 0.9 0.18 . . . .

* All sampling performed between 0745 and 0845 hours. b After 1 mg of dexamethasone taken the night before. c After 2.5 g of metyrapone taken the night before. d After 250 μg of ACTH 1-24 given intravenously 30 min earlier. * Not calculated, because some samples were below sensitivity of the assay (4.4 pmol/L). ' Significantly different from baseline (P < 0.05). ** RIA > IRMA (P < 0.05).

Fig. 2. ACTH (by IRMA) and cortisol concentrations in plasma after bolus injection of 100 μg of ovine corticoliberin (CRH) at 0 min (CTL)

* significantly (P < 0.05) different from control (CTL). Data are displayed as mean ± SEM in this and all other figures.

0.16 μg/L) to 254 (SEM 29) nmol/L [88 (SEM 10) μg/L] after metyrapone. Administration of ACTH 1-24 produced a significant decrease in ACTH concentration as measured by IRMA (intact ACTH) and a significant increase in ACTH concentration as measured by RIA (ACTH 1-24 and ACTH 1-39). Plasma cortisol concentration increased significantly from 505 (SEM 72) nmol/L [183 (SEM 26) μg/L] to 676 (SEM 25) nmol/L [245 (SEM 9) μg/L] after administration of ACTH 1-24.

Figure 2 shows the ACTH concentration by IRMA and the cortisol concentration before and after administration of corticoliberin to the normal subjects. Administration of corticoliberin resulted in an increase in ACTH concentration from 5.1 (SEM 0.7) pmol/L [23 (SEM 3) ng/L] to a peak of 9.5 (SEM 0.7) pmol/L [43 (SEM 5) ng/L] 30 min later. The increase in plasma cortisol concentration lagged 15 min behind the ACTH response; cortisol concentration increased from 469 (SEM 39) nmol/L [170 (SEM 14) μg/L], reaching its peak value of 646 (SEM 44) nmol/L [232 (SEM 16) μg/L] at 45 min. ACTH concentration in many of the samples was below the reliable detection limit of the RIA. However, plasma ACTH concentration (by RIA) 45 min after corticoliberin administration [8.1 (SEM 0.7) pmol/L; 37 (SEM 3) ng/L] and 60 min after corticoliberin administration [7.5 (SEM 0.4) pmol/L; 34 (SEM 2) ng/L] was similar to the ACTH concentrations measured by IRMA.

Cushing's syndrome: Figures 3–5 show the concentration of ACTH in plasma sampled from the inferior vena cava and petrosal sinuses in patients with ACTH-dependent Cushing's syndrome of either pituitary or ectopic etiology. Figure 3 shows the concentration of ACTH in plasma from blood sampled from the inferior vena cava after administration of corticoliberin.

Concentrations of ACTH in control samples were significantly lower in patients with pituitary (Cushing's) disease, and they increased significantly after administration of corticoliberin. Results by IRMA and RIA were similar. Patients with ectopic ACTH syndrome had above-normal peripheral concentrations of ACTH by IRMA (42 (SEM 14) pmol/L; 193 (SEM 63) ng/L) and RIA (56 (SEM 17) pmol/L; 255 (SEM 79) ng/L). The ACTH concentration measured by RIA was greater than by IRMA in all six patients (P < 0.05). Concentrations of ACTH in plasma from patients with ectopic ACTH syndrome were unchanged after administration of corticoliberin.

Figure 4 shows the ACTH concentration in plasma sampled from the dominant inferior petrosal sinus in patients with pituitary ACTH-dependent Cushing's syndrome (Cushing's disease). Individual data are plotted on a logarithmic scale because of the extreme heterocedastic scatter inherent in petrosal sinus sampling after administration of corticoliberin (ranging from 7 pmol/L (32 ng/L) to 8103 pmol/L (36 800 ng/L) by IRMA). All patients demonstrated significantly increased ACTH concentrations after corticoliberin administration. The data were very similar, whether measured by IRMA or RIA. Unlike samples from patients with

Fig. 3. ACTH concentrations in peripheral (inferior vena cava) blood plasma before (CTL) and after administration of 100 μg of ovine corticoliberin (CRH) to patients with ACTH-dependent Cushing's syndrome of pituitary or ectopic etiology

Control ACTH concentrations were significantly (P < 0.05) lower in patients with pituitary disease as compared with those with ectopic ACTH. * significantly (P < 0.05) increased from control values. Also, RIA ACTH values significantly (P < 0.05) exceeded IRMA ACTH values in ectopic patients.
ectopic ACTH syndrome, ACTH concentrations were not consistently higher by RIA in samples from patients with Cushing's disease.

Figure 5 shows the concentration of ACTH in plasma from the left petrosal sinus of patients with ectopic ACTH. The ratio of the left petrosal sinus to peripheral ACTH concentration was 1.2 (SEM 0.1) by IRMA or RIA. The concentrations of plasma ACTH in samples from the petrosal sinus were unchanged after administration of corticotermin.

The concentrations of ACTH in plasma samples from patients during surgery were similar, whether measured by RIA or IRMA. Baseline (i.e., pre-surgery) concentrations of ACTH were 8.8 (SEM 2.0) pmol/L [31 (SEM 9) ng/L] by IRMA and 6.6 (SEM 2.2) pmol/L [30 (SEM 10) ng/L] by RIA. Thirty minutes after the start of surgery, the concentration of ACTH by IRMA had increased significantly to 35.8 (SEM 7.1) pmol/L [162 (SEM 32) ng/L] and by RIA to 31.5 (SEM 5.3) pmol/L [143 (SEM 24) ng/L].

There was a highly significant linear correlation between results by IRMA and RIA (n = 165). The data used in this correlation include those reported in this study except for those drawn after administration of ACTH 1-24. The correlation encompassed ACTH concentrations ranging from <10 to >10 000 pmol/L. The slope of the regression of the logarithm of ACTH concentration determined by RIA (x-axis) vs by IRMA (y-axis) was 0.987 (SD 0.041)—not significantly different from the line of identity. The y-intercept was −0.0186 (SD 0.0013).

Discussion

The two-site IRMA developed by the Nichols Institute for measurement of ACTH concentration in plasma performed well in this comparison with the non-extraction RIA used extensively in our laboratory (6-9). Many of the findings were similar to a clinical evaluation of an IRMA for ACTH previously reported (9). However, we extended these findings over a very wide range of ACTH concentrations. Furthermore, we have identified clinical situations when the use of IRMA alone may be insufficient or misleading.

The coefficients of variation of the assay were quite low, indicating increased precision as compared with the standard direct RIA for ACTH concentration in plasma. The precision of the assay was lower at low ACTH concentrations, owing to the low radioactivity counts per minute. Increasing the counting time to 20 min (or <1% counting error) improved the reliability of measurements made at low ACTH concentration and also increased the sensitivity.

The expression of cross-reactivity is different for IRMA as compared with RIA. If a ligand binds to only one of the antibodies, a "sandwich" complex will not form. This will lead to falsely low ACTH concentration. This is important when ACTH 1-24 (cosyntropin) is administered.

Evaluation of the dynamics of ACTH secretion in normal subjects was vastly improved when the IRMA was used, because of the greater sensitivity and the linearity of the standard curve down to the zero standard (equivalent to nonspecific binding). Several of the normal subjects in our study had baseline ACTH concentrations at or below the reliable sensitivity of the RIA, although well within the limits of the IRMA. The improved sensitivity is necessary for the evaluation of small increases in ACTH or the suppression of ACTH concentration in patients with secondary hypopituitarism. Interestingly, one subject (no. 2) did not suppress ACTH concentration as measured by RIA after dexamethasone administration, although the ACTH concentration as measured by IRMA and the plasma cortisol concentration were clearly suppressed. RIA and IRMA compared favorably when endogenous ACTH concentration was increased after metyrapone administration in normal subjects. However, the IRMA measurements had a smaller standard error.

Administration of ACTH 1-24 resulted in a significant decrease in ACTH concentration as measured by IRMA in all six normal subjects, whereas ACTH concentration as measured by RIA significantly increased to the predicted concentrations. This is consistent with a previous study in which it was reported that infusion of ACTH 1-24 decreased ACTH concentration as measured by IRMA (10). The dynamics of IRMA methodology would suggest that this observation may represent an artifact, rather than "ultrashort" loop feedback in vivo as was suggested (10). Addition of ACTH 1-24 decreased the concentration of ACTH 1-39 measured by IRMA because ACTH 1-24 bound to the N-terminal antibody [radiolabeled Ab(m)] without forming a "sandwich" complex. Therefore, a decrease in ACTH concentration as measured by two-site IRMA during the administration of ACTH 1-24 may be an artifact of the assay rather than true feedback.

Administration of corticotermin to normal subjects (Figure 2) clearly demonstrated the virtues of the IRMA. ACTH concentration before corticotermin administration was at or
below the sensitivity of the RIA. IRMA clearly and reproducibly detected baseline ACHT concentrations and the small increases provoked by administration of corticoliberin. This will be of great benefit in the differential diagnosis of Cushing’s syndrome by measurement of ACHT in the peripheral blood after corticoliberin administration (II).

The effect of the administration of corticoliberin on ACHT concentrations in the peripheral and petrosal sinuses of patients with Cushing’s syndrome were consistent with previous findings using RIA (4, 5). Baseline values for peripheral-blood ACHT concentration were lower in patients with Cushing’s disease than in patients with ectopic ACHT syndrome. Corticoliberin administration resulted in significant increases in ACHT concentration in samples from the petrosal sinus of patients with Cushing’s disease. Results by IRMA and RIA were virtually identical for samples from the inferior vena cava of patients with Cushing’s disease, whereas patients with ectopic ACHT syndrome had higher peripheral ACHT concentrations by RIA than by IRMA. This illustrates another caveat when the two-site IRMA is used for measurement of ACHT. Ectopic tumors may secrete fragments of ACHT that are biologically active and detected by N-terminal RIA (12) but not detectable by IRMA. Furthermore, ectopic ACHT-secreting tumors can also secrete “big” ACHT, which is detected by RIA (13, 14) but not necessarily by IRMA (see below). This would account for higher concentrations of ACHT by RIA in samples from patients with ectopic ACHT syndrome. If a patient secreted only a short, fully active form of ACHT (e.g., 1-24) or only “big” ACHT, the patient would have an increased concentration of ACHT in the peripheral blood as measured by RIA, but very low concentrations of endogenous, intact ACHT as measured by IRMA, owing to corticosteroid negative feedback. This may lead the clinician to an incorrect diagnosis of ACHT-independent Cushing’s syndrome. We have preliminary results from one patient with a supranormal cortisol concentration in the plasma and free cortisol excretion in the urine, a very high ACHT concentration by RIA (67.2 pmol/L; 306 ng/L), but a very low ACHT concentration by IRMA (0.33 pmol/L; 1.5 ng/L). Preliminary chromatographic data indicated a 12-29 kDa peptide without detectable ACHT 1-39. If small-cell carcinoma of the lung had not been diagnosed in this patient, the low ACHT concentration by IRMA would have suggested primary adrenal disease. Therefore, both RIA and IRMA may be required in the differential diagnosis of Cushing’s syndrome in some patients.

Plasma samples from the petrosal sinus of patients with Cushing’s disease were very helpful in evaluating the IRMA because of the very high ACHT concentrations achieved after administration of corticoliberin. IRMA and RIA were in agreement at these very high concentrations of ACHT. IRMA is clearly better at low concentrations; otherwise, the assays are indistinguishable except after injection of ACHT 1-24 and in patients with ectopic ACHT secretion (discussed above).

The advantages of the IRMA for ACHT are obvious. It is rapid (<22 h incubation), highly reproducible, specific, and sensitive, and results parallel sample dilutions. It will be particularly useful for studying the suppression of ACHT secretion from baseline as well as for the dynamics of ACHT secretion in normal subjects with otherwise low baseline values. A disadvantage is, ironically, that it may be too specific. In some instances one may wish to routinely measure biologically active fragments of ACHT or “big” ACHT, which are detected by N-terminal RIA but not necessarily by IRMA.

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References