Antipituitary Antibodies as Pathogenetic Factors in Patients with Pituitary Disorders

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Abstract. Our previous reports showed detection of antipituitary antibodies (APA) by immunoblot analysis and enzyme linked immunosorbent assay (ELISA) by using rat pituitary tissue as antigen in patients with certain endocrine disorders. In the present report, we evaluated APA by using our immunoblot and ELISA technique in 76 patients with various pituitary disorders. The prevalence of a 22 kDa band of APA detected by immunoblot was found to be significantly higher (P<0.01) in patients with pituitary disorders (20 of 76, 26%) than in the controls (3 of 209, 1%). APA levels detected by ELISA were significantly higher in patients with GH deficiency, isolated ACTH deficiency, acromegaly, and idiopathic panhypopituitarism compared with control (mean ± SD; 2.40 ± 2.66, 2.36 ± 1.87, 2.09 ± 1.87, 3.10 ± 1.96 versus 1.42 ± 0.64 (C.I.) P<0.05, respectively). APA levels detected by ELISA in 7 patients with GH deficiency showed a statistically significant decrease ( p<0.05) after administration of GH replacement therapy. APA detection by immunoblot is useful in examining as pathogenesis, while ELISA may be useful as an objective index of pathological state in patients with autoimmune-related pituitary disorders.

Key words: Antipituitary antibodies, Pituitary disorders, Immunoblot, Enzyme linked immunosorbent assay


IN 1975, Bottazzo et al. [1] developed an assay method for detecting antipituitary antibodies (APA) with an immunofluorescence technique. Sugiuera et al. [2] subsequently developed a sensitive assay for APA based on avidin-biotin detection technique and rat pituitary antigens. These researchers also established a sensitive assay that relied on an immunofluorescence method as well as AtT-20 cells and GH3 cells [3]. Furthermore, APA were detected through immunoblotting by using an antigen derived from a human pituitary cell membrane by Crock et al. [4]. Although the development of these technologies has established the existence of APA on both experimental and clinical grounds [5], the etiological roles of APA that may be responsible for various autoimmune disorders remain unexplained. To make available a more quantitative assay method of detecting APA, we developed an assay technique with ELISA [6] using rat pituitary soluble antigen in place of human pituitary tissues [7]. We demonstrated that the positive findings for APA detected by ELISA correlated with the prevalence of 22 kDa bands of APA by immunoblot analysis [7].

Detection of APA by immunoblot and ELISA is one of the most valuable assays with respect to high sensitivity and specificity to date. In a recent report, using APA detection by immunoblot and ELISA, we have identified patients with various autoimmune endocrine disorders including insulin-dependent diabetes mellitus, Graves' disease, and Hashimoto's thyroiditis [7]. In the present report, we used APA detection using immunoblot and ELISA to examine patients with various pituitary disorders.
Subjects and Methods

Subjects

Serum samples were obtained from 76 previously untreated Japanese patients with pituitary disorders, 41 men 5 to 63 years of age, and 35 women 3 to 64 years of age. Diagnoses included GH deficiency (n=20), pituitary nonfunctioning adenoma (n=16), isolated ACTH deficiency (n=14), acromegaly (n=13), idiopathic panhypopituitarism (n=7), and Sheehan’s syndrome (n=6). Lymphocytic adenohypophysitis was not to be confirmed on the basis of a history, clinical and laboratory findings or magnetic resonance imaging in these patients.

Control sera were collected from 219 healthy volunteers, 108 men and 111 women 21 to 60 years of age. Written informed consent for participation was obtained from each subject.

Preparation of rat pituitary antigens

Preparation of rat pituitary antigens was made according to methods previously described [7].

Rat pituitary glands (RKL, Gilbertsville, PA) were homogenized in 3 ml of buffer (0.25 M sucrose, 0.1 mM ethylenediamine tetraacetic acid, 3 mM Tris-HCl buffer, pH 7.4) with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and centrifuged at 10,000×g at 4°C for 10 min. The resultant supernatant was used as pituitary antigens for subsequent steps. The concentration and protein standard were determined with a protein assay kit (Bio-Rad, Hercules, CA).

Immunoblot analysis

Immunoblot analysis was performed according to methods previously described [8].

SDS-PAGE was performed according to the method of Laemmli [10]. In brief, 50 μl of rat pituitary antigen was mixed with 50 μl of sample buffer and was assayed by using electrophoresis in gradient 4-20% polyacrylamide gel. Separated proteins were transferred to a polyvinylidene difluoride membrane using a semi-dry blotting apparatus, and then incubated in diluted biotinylated rabbit anti-human IgG polyclonal antibodies. The membrane was then incubated in diluted streptavidin-biotin complex peroxidase. The labeled bands were revealed by chemiluminescence using POD Immunostain Set (Wako Pure Chemicals, Kyoto, Japan).

ELISA

ELISA was performed according to methods previously described [7].

Wells of microtiter were coated with 100 μl of rat pituitary antigen. One hundred μl of sample sera or APA-positive control sera which showed positive for 22 kDa bands of APA by immunoblot were added to each well. A volume of 100 μl of peroxidase-labeled rabbit anti-human IgG polyclonal antibody was added to each well, and 100 μl of substrate solution was added to each well. Absorbance at 450 nm was then measured with ELISA Processor III, and the cut-off index was calculated, where index value = absorbance at 450 nm of test serum/absorbance at 450 nm of the serum of APA-negative normal subject. Assays were typically performed in duplicate.

APA levels before and after treatment

Difference of APA levels determined by ELISA was examined of 7 patients with GH deficiency before and after administration of GH replacement therapy during 2 or 3 month period.

Further, difference of APA levels determined by ELISA was examined of 7 patients with nonfunctioning pituitary adenoma before and after surgical resection during an 1 or 2 week period.

GH and APA after administration of levodihydroxyphenylalanine (l-dopa) in patients with GH deficiency

The patient, a 5-year-old male, 10 mg/kg of l-dopa was orally administered. The patient had fasted for 12 h after dinner the previous day and had a rest after rising that day. Blood was drawn for APA measurement by ELISA and GH at 30 min intervals until 120 min after administration.

Relationship between the degree of lymphocyte accumulation in specimens of pituitary tumors and APA levels

Twenty-five patients with pituitary tumor including
acromegaly (n=11) and nonfunctioning adenoma (n=14) were tested for the relationship between degree of lymphocyte accumulation in specimens of pituitary tumors and APA levels determined by ELISA. The grade of lymphocyte accumulation in the specimens of pituitary tumors was grouped in 10 classes with a microscope and numbered from G1 to G10, and further, divided into 3 subgroups as follows: slight (from G1 to G3), moderate (from G4 to G7) and severe (from G8 to G10) according to the degree of lymphocyte accumulation.

Immunohistochemical study employing APA

Immunohistochemical study was made of 7 patients with pituitary tumor involved 3 patients with acromegaly and 4 patients with a pituitary nonfunctioning adenoma who showed severe lymphocyte accumulation above G8 and 3 healthy controls. Examination for reaction with APA positive sera against these specimens was performed by using immunohistochemical study.

Staining procedure for cryostat sections and paraffin-embedded tissue sections

Indirect staining method with sera positive and negative for APA was examined. Staining procedure is as follows. Paraffin fixed sections of pituitary tissues were cut and placed on slides for a histological examination. They were fixed in acetone for 10 min, air dried, and incubated for 5 min with 0.6% hydrogen peroxide in methanol. The slides were rinsed with distilled water and placed in Tris-buffered saline (TBS) for 5 min and incubated for 20 min with normal rabbit serum diluted at 1:5 in TBS to block any non-specific background. Sera positive and negative for APA were added to the pituitary tissues on slides. Pituitary tissues were incubated for 20-30 min with the sera. The sera were rinsed off, and the slide was placed in a TBS bath for 5 min. This was followed by incubating for 20-30 min with biotinylated rabbit anti-human immunoglobulins (DAKO, Glostrup, Denmark) that were diluted between 1:200 and 1:600 in TBS. The slide was incubated for 5-15 min with an ABC complex/HRP (DAKO, Glostrup, Denmark) that was prepared according to the manufacturer’s instructions. After incubation between 5 and 15 min with peroxidase substrate solution, the slide was rinsed with distilled water, counterstained, and mounted with coverslip.

Chromatofocusing

We used a non-metallic high performance liquid chromatography solvent delivery system for liquid chromatography applications (Waters 625LC system, Waters, Milford, MA). The protein was dissolved in 25 mM ethanolamine hydrochloride, pH 9.4 (column buffer), at a protein concentration of 4 mg/ml and applied to a column polybuffer exchanger 94 (Pharmacia, Uppsala, Sweden) equilibrated with a column buffer. The column was eluted with 200 ml Pharmalyte, pH range from 8 to 10.5 (Pharmacia, Uppsala, Sweden), diluted of 1:45 in water and adjusted to pH 8.0 with hydrochloric acid. The flow rate was 50 ml/h. Fractions of 1.7 ml were collected at a temperature of 4°C. Column fractions (20 micro l/ml aliquots) were monitored by using analytical electrofocusing as described below. The Pharmalyte was removed from the pooled fractions by adding sulfate 77% saturation. The pituitary antigens as precipitated protein was collected by centrifugation and resuspended with 1% acetic acid containing 5 M urea. The clear solution was dialyzed against several changes with 1% acetic acid at 4°C.

Amino acid sequence

SDS-PAGE in 8-25% gradient polyacrylamide gel (TEFCO, Tokyo, Japan) was performed for each protein fractions by chromatofocusing. The protein on the gel identified by silver impregnation was measured by molecular weight, and the 22 kDa protein band was transferred to a polyvinylidene difluoride membrane using a semi-dry blotting apparatus. The methods of electrophoresis and transfer to membrane conform to the immunoblot analysis. The first domain from N terminal of a 22 kDa protein derived from rat pituitary was then analyzed for the amino acid sequence and homology. The protein amino acid sequencing was performed with a liquid-phase sequencer (modified Beckmann 890C).

Statistical analysis

Data are expressed as mean ± S.D. unless otherwise
indicated. The differences between groups in titer of ELISA (C.I.) was assayed post hoc by Fisher’s PSLD analysis after analysis of variance. The chi-square test with Yates correction was used to determine the significance of differences between groups for the incidence of positivity for APA. Wilcoxon signed-rank test was used to determine the significance of difference of the titer of APA between before and after treatment. A level of $P < 0.05$ was considered statistically significant.

**Validation of reagents**

Validation of reagents was performed according to methods previously described [7].

**Coating antigen**

For each ELISA, the minimum levels of detection was estimated to be 25 μg/ml of the serum by using the APA positive serum concentration-response curve.

**Reactivity of APA containing serum**

The difference between the absorbance of APA positive serum and that of APA-negative serum was clear at dilutions from 1:100 to 1:1,400.

**Reactivity of peroxidase-conjugated antihuman IgG rabbit polyclonal antibody**

The difference between the absorbance of peroxidase-conjugated anti-human IgG rabbit polyclonal antibody (Sigma)-positive and that of negative samples was clear at dilutions from 1:2,500 to 10,000.

**Assessment of efficiency**

Assessment of efficiency was made according to methods previously described [7].

**Linearity of diluted samples**

Two APA-positive samples and one APA negative sample were tested. At dilutions from 1:50 to 1:6400, the absorbance was linear between 1.0 and 2.0 at 450 nm.

**Interfering experiment**

Billirubin C, bilirubin F, chyle, hemoglobin and ascorbic acid and rheumatoid factor did not affect the results of APA analysis.

**Absorption of nonspecific antibody**

Various tissues including rat stomach, liver, thyroid gland and pancreas did not cross-react with APA. In contrast, APA apparently cross-reacted with human GH.

**Recovery experiment of APA-positive sera on human GH**

The presence of APA-positive sera reduced the amount of GH that was detected.

**Cut off value in healthy volunteers**

Cut off value in healthy volunteers was evaluated according to methods previously described [7]. Among 219 healthy volunteers, APA values ranged from 0.33 to 6.54 (mean $\pm$SD; 1.57 $\pm$0.99). Values did not differ by sex. Immunoblot analysis of 21 healthy volunteers with values $>2.56$ revealed bands that were positive for APA in 10 subjects (47.6%). Excluding those 10 APA-positive subjects, the distribution of values was from 0.33 to 4.05 (1.42 $\pm$0.64). Cut off values $>2.70$ (mean $+2\text{SD}$) were considered as positive for APA.

**Results**

**Detection of APA in patients with pituitary disorders**

The presence of APA in patients with pituitary disorders was evaluated by ELISA and in some cases confirmed by immunoblot analysis. The positive bands of 22 kDa APA detected by immunoblot analysis in patients with pituitary disorders are shown in Fig. 1. The concordance between positive APA findings with ELISA and immunoblot were investigated. In normal subjects (n=39), there was 100% concordance. All of the ELISA-negative subjects (n=36) tested negative by immunoblot, and another 3 subjects were both positive [7]. Twenty-two kDa APA determined by immunoblot was significant in patients with GH deficiency, pituitary adenoma, ACTH deficiency, acromegaly, idiopathic panhypopituitarism and Sheehan’s syndrome compared with healthy controls (20% of 20, 19% of 16, 21% of 14, 46% of 13, 29% of 7, and 33% of 6 patients versus 1% of 209 healthy controls, $P < 0.01$, respectively) (Fig. 2).

The APA titer determined by ELISA was significantly higher in patients with GH deficiency, isolated ACTH deficiency, acromegaly, idiopathic panhypopituitarism compared with controls (mean $\pm$SD, C.I.; 2.40$\pm$2.66, 2.36$\pm$1.87, 2.09$\pm$1.87, 3.10$\pm$1.96.
versus 1.42 ± 0.64, P < 0.05, respectively), but was not significantly higher in patients with nonfunctioning adenoma and Sheehan’s syndrome (mean ± SD, C.I. 1.49 ± 0.62, 1.03 ± 0.69, respectively) (Fig. 3). The incidence of positive cases for APA as determined by ELISA was significant in patients with GH deficiency, isolated ACTH deficiency, acromegaly, and idiopathic panhypopituitarism compared with that in controls (20% of 20, 36% of 14, 23% of 13 and 29% of 7 patients versus 5% of 209 healthy controls, P < 0.01 respectively) and was not significantly higher in patients with nonfunctioning

Fig. 1. Results of immunoblot analysis in patients with various pituitary disorders. Lane 1, negative control; lane 2, positive control; lanes 3, 4, 8, 14 and 15, pituitary nonfunctioning adenoma; lanes 5, 6, 7 acromegaly; lanes 9, 10, 11 and 12, GH deficiency; lane 13, isolated ACTH deficiency.

Fig. 2. Comparative incidence of positive for APA determined by immunoblot in patients with various pituitary disorders versus that in healthy controls. The incidence of positive APA in patients with pituitary disorders was significantly higher (*p < 0.01) than that in healthy controls.

Fig. 3. Distribution of APA determined by ELISA in patients with various pituitary disorders. The statistical significance in GH deficiency, isolated ACTH deficiency, acromegaly and idiopathic panhypopituitarism was p < 0.05 against healthy controls. The upper and lower limits of the dotted range indicate 2SD, Bar indicates mean value.
adenoma and Sheehan's syndrome compared with controls (6% of 16 and 0% of 6 patients, respectively) (Fig. 4).

**APA levels before and after treatment**

APA levels determined by ELISA in patients with a GH deficiency showed a significant decrease after GH hormone replacement therapy (Fig. 5, A; p < 0.05). However, APA levels determined by ELISA of pituitary nonfunctioning adenoma showed no significant change after surgical resection (Fig. 5, B).

**GH and APA levels after l-dopa in administration of a GH deficiency**

Although no correlation was found between the levels of GH and APA determined by ELISA, APA levels showed a downward trend with elevation of GH levels. (Fig. 6)

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**Fig. 4.** Comparative incidence of positive APA determined by ELISA in patients with various pituitary disorders versus that in healthy controls. The statistical significance in GH deficiency, isolated ACTH deficiency, acromegaly and idiopathic panhypopituitarism was *p < 0.01 against healthy controls, respectively.

**Fig. 5.** A. Changes in APA levels determined by ELISA in patients with GH deficiency before and after GH replacement therapy. APA levels showed a significant decrease after replacement therapy (P < 0.05). B. Changes in APA levels detected by ELISA in patients with a nonfunctioning pituitary adenoma before and after surgical resections.

**Fig. 6.** Correlation between GH and APA levels determined by ELISA in patient with GH deficiency after l-dopa administration.
Relationship between the degree of lymphocyte accumulation in specimens of pituitary tumors and APA levels

Negative correlation was found between the degree of lymphocyte accumulation and APA levels detected by ELISA ($r = -0.436$, $p < 0.05$) (Fig. 7).

**Immunohistochemical study employing APA**

All 7 specimens of pituitary tumor and 3 healthy pituitary tissues were reacted with sera positive for 22 kDa APA. The cytoplasm of GH cell of pituitary adenoma grew N/C ratio may have been stained with hematoxylin and eosin. However, no reaction occurred with sera negative for 22 kDa APA (Fig. 8).

**Amino acid sequence of antigen to APA**

The amino acid sequence of P-A-M-P-L-S-S-L-F-A-N-A-V-L-R of the first domain from the N terminal was recognized. Results of the analysis showed that the first domain from the N terminal of a 22 kDa protein derived from rat pituitary had a 67% homology to human and 100% to rat and pig GH (Table 1).

![Fig. 8. Immunohistochemical study of pituitary tumors using APA positive sera. Arrows indicate positivity for APA. HE: hematoxylin eosin stain in APA positive tumors; APA: specimens from APA positive patients; NO: specimens from APA negative patients.](image)

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<tr>
<th>Table 1. Amino acid sequence in the first domain of the 22 kDa protein</th>
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<td>Protein</td>
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<td>22 kDa protein</td>
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<td>Rat growth hormone</td>
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<td>Porcine growth hormone</td>
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Discussion

The relationship between APA and various autoimmune disease has been shown in previous reports to date. APA have been identified in patients with isolated ACTH deficiency [11], panhypopituitarism [12], empty sella syndrome [13, 12], Hashimoto thyroiditis [14], Graves' disease [15]. APA also seem to be expressed in the pituitary and pancreas, and are very frequently detected in patients with insulin dependent diabetes mellitus [9, 16, 17].

In the present report, the relationship between APA and pituitary disorders was examined by using immunoblot and ELISA. The incidence of positive cases for 22 kDa APA determined by immunoblot in all patients with pituitary disorders was significantly higher than that of controls (P < 0.01). On the other hand, the titer of APA determined by ELISA was significantly higher in patients with GH deficiency, ACTH deficiency, acromegaly, idiopathic panhypopituitarism (P < 0.05), and was not significantly higher in patients with nonfunctioning adenoma and Sheehan's syndrome than that of controls. It is generally accepted that at least one of the pathogenesis of GH deficiency, ACTH deficiency and idiopathic panhypopituitarism is ascribed to an autoimmune mechanism, which is also thought to provoke lymphocytic adenohypophysitis. The incidence of positive for APA determined by ELISA in patients with pituitary disorders was significantly higher compared with controls (Fig. 4, P < 0.01), which was almost the same as that of titers of APA determined by ELISA (Fig. 3, P < 0.05). The reason for the difference of detection of APA between by using immunoblot and ELISA in these disorders may be explained by the following consideration. The detection of APA by immunoblot used only the 22 kDa rat pituitary protein for subjects positive for APA, whereas APA determination by ELISA, used the overall soluble fractions of rat pituitary protein for the antigen of the assay. If sera which is weakly positive for the 22 kDa band APA is examined by immunoblot, the remarkably low titer of APA can be detected by ELISA. It is suggested that the titer of APA determined by ELISA can increase in patients with hormone deficit state caused by autoimmune mechanism.

In our previous report, APA was detected by ELISA in a low percentage (3/28, or 8.0%) of patients with GH deficiency and in none of 12 patients with pituitary adenoma [7]. In the present report, positivity for APA by both immunoblot and ELISA was detected frequently in 20% of patients with GH deficiency. The reasons for the difference between these results are not well understood.

Next, we must examine the pathogenetic role for APA not only in patients with pituitary disorders but also autoimmune disease such as lymphocytic adenohypophysitis. In previous reports, APA have been shown to be a possible cause of pituitary damage in patients with empty sella [13]. One case of empty sella has been reported to reflect the late stage of a lymphocytic adenohypophysitis [18]. A recent report showed APA positivity in 7% of patients with lymphocytic adenohypophysitis and idiopathic panhypopituitarism [19]. It should be taken into consideration that low incidence of positive APA may result from low sensitivity of the assay system.

Clinical implications of APA in patients with lymphocytic adenohypophysitis associated with painless thyroiditis have been described in case reports [18, 22]. Further, autoantibodies to vasopressin producing cells in approximately 30% in patients with idiopathic diabetes insipidus have been identified [20]. In order to examine the existence of antigenicity in the pituitary tumor itself, we determined the changes in APA by ELISA in patients with pituitary nonfunctioning adenoma before and after surgical resection. Nonfunctioning adenoma was chosen for examination, since it did not release hormones such as GH, which might react with APA. The examination showed no significant difference in APA as determined by ELISA. It could be that the interval before and after operation may have been too short to evaluate the APA detection in patients.

On the other hand, a significant decrease of APA levels detected by ELISA was shown in patients with GH deficiency after administration of GH hormone replacement therapy. The results indicate a possible competition effect against APA by GH. However, by using immunoblot, we were unable to detect any difference of 22 kDa bands of APA between samples before and after GH administration. These results indicate the superiority of ELISA system that is able to detect APA quantitatively, besides its usefulness as a screening test for APA on mass scale.

In regard to the relationship between APA and
GH, our previous report has described the detection of a 22 kDa band of APA as a soluble cytoplasm in GH-related protein [8]. In another report, detection of anti-GH, anti-TSH and anti-ACTH antibodies have been described by Majorie et al. in 45 percent of patients with pituitary adenoma and empty sella syndrome [21].

Because of the limited availability of pituitary tissues in patients with lymphocytic adenohypophysitis, we examined relationship between the degree of lymphocyte accumulation in specimens of pituitary tumors and levels of APA determined by ELISA in order to elucidate the pathogenetic role of APA. Unexpectedly, we found a negative correlation was found between the degree of lymphocyte accumulation and the levels of APA. Lymphocyte accumulation in the tissues of pituitary tumor might have been caused by the antigenicity of the pituitary tumor itself, or from a down regulation against increasing productivity of APA. Data are as of yet in sufficient to conclude whether there is relationship between APA and lymphocytic adenohypophysitis, because the diagnoses in patients with pituitary tumors are made by exclusion of lymphocytic adenohypophysitis on the basis of the history, physical and laboratory findings. Next, we examined the reaction to the pituitary tumors with sera positive for 22 kDa APA. The cytoplasm of a GH cell of pituitary adenoma may have been stained with hematoxylin and eosin.

Furthermore, in an attempt to identify a binding protein against a 22 kDa band of APA, we examined the first domain from the N terminal of a 22 kDa protein derived from rat pituitary gland by using a liquid chromatography and the amino acid sequence. Results of the analysis showed that the first domain from the N terminal of a 22 kDa protein had a 67% homology to human GH. These results may simply prove the existence of GH in the region of a molecular weight of 22 kDa. For the purpose of distinct analysis of the 22 kDa protein against APA, a protein purified by using an affinity chromatography with high titer antibody must be analyzed with the amino acid sequence.

In the recent report by Aso, the flexibility in detecting various classes or subclasses of immunoglobulins by simply changing second anti-human antibodies has been recommended for the purpose of the advantage of our ELISA system [23]. On the other hand, APA detected by an immunoblot and ELISA are not necessarily caused during autoimmune process in all patients with autoimmune disorders. In order to examine the role of APA, we need to develop an assay system for detecting antibodies to various human tissues.

In conclusion, we were unable to clarify a role of APA in patients with lymphocytic adenohypophysitis by using immunoblot and ELISA. However, the present study suggests that the detection of APA by immunoblot and ELISA developed by us is clinically useful in patients with certain pituitary disorders.

Acknowledgment

The present study was supported in part by a grant-in-aid (804454545 to I.K.) for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

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