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PRECIPITIN TO MITE DETECTED IN ASTHMATIC PATIENTS

By

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It was reported by Reisman and associates (11), that following antigen E therapy in ragweed allergy, numerous post-treatment sera contained antibodies in sufficient quantities to be demonstrated by direct precipitation. There is no positive finding about direct precipitation with mite antigen.

The author tried to detect precipitin to mite using mite extract and ^{125}I labelled mite antigen, and examined the characteristics of the antigen with immunoelectrophoresis. It will be shown that approximately 40 per cent of sera from patients allergic to mite showed positive reactions with mite antigen in double gel diffusion, and that the main immunogenic component in mite antigen migrated to the beta region in immunoelectrophoresis.

MATERIALS

Patients' Sera

Blood was collected from 37 patients who showed immediate positive skin reaction to both mite extract and house dust extract. Blood was kept for 2-3 hours at room temperature, and sera were separated. Sodium azide (0.1 mg/ml) was added to the sera, which were frozen at -20°C , usually for 1 to 2 months until they were analyzed. Of 37 patients, seven were untreated, five were treated with house dust of various concentrations up to the highest concentration (1:10), and 24 were treated with extract from *dermatophagoides farinae* of various concentrations. The highest concentration of mite extract used for hypsensitization when the sera were collected was 1:10⁵. By 1:10 extract is meant extract obtained, when 10 ml of phosphate buffered saline of pH 7.2 was added

to 1 g of lyophilized defatted material. By 1:10⁵ extract is meant extract prepared from 1:10 extract by 10⁴ times dilution. One of the 37 patients was treated with house dust up to the highest concentration without effect, and subsequently with mite extract up to 1:10⁵ extract.

Normal Serum

Sera were collected from 20 normal individuals who showed no reaction in skin tests with both mite extract and house dust extract; the sera were stored as above.

Mite Extract and House Dust Extract

Lyophilized extract from the mite *dermatophagoides farinae* and house dust and their solution were supplied from Torii Pharmaceutical Co. Ltd., Tokyo, Japan.

Radioactive Iodine

Carrier-free iodine-¹²⁵I was purchased from New England Nuclear, Boston, Mass., U.S.A.

X-ray Film

Sakura® X-ray film, New Y type was purchased from Konishiroku Photography Engineering Co. Ltd., Tokyo, Japan.

Anti IgE Antiserum and IgE Control Serum

Sheep anti IgE antiserum and IgE control serum containing approximately 10,000 units of IgE/ml were purchased from Pharmacia, Uppsala, Sweden.

Antiserum to Human Serum and its Components

Goat antisera to whole human serum, to IgA and IgM were purchased from Hyland Div., Travenol Laboratories Inc., Costa Mesa, Calif., U.S.A. These antisera were used only in radioimmuno-electrophoresis.

METHODS

Labelling of Mite Extract and House-dust Extract with ¹²⁵I

Mite antigen was labelled with ¹²⁵I by the method of Hunter & Greenwood (4), with some modification. 5 mg of lyophilized mite extract was dissolved in 0.5 ml of borate buffered saline of pH 8.0 (BBS). After dialysis against BBS overnight at 4° C, insoluble substance was removed by centrifugation. The protein concentration of the supernatant was determined by Lowry's method (9).

A total of 100 µg of the protein was mixed with 0.1 ml of potassium iodide (16.6 mg/l in BBS), 0.1 ml of chloramin T (0.7 mg/ml in BBS) and 2 mCi of

¹²⁵I. The mixture was kept at room temperature for 5 min, and added to 0.1 ml of sodium metabisulfite (0.5 mg/ml in des. water) to stop the reaction. The free iodine was removed by gel filtration using sephadex G-25 fine. Approximately 60 per cent of total ¹²⁵I was bound to protein.

20 mg of house-dust extract was dissolved into 0.5 ml of BBS, and labelled with ¹²⁵I in the same way as described above.

Immunoelectrophoresis

Immunoelectrophoresis was done by the method of Scheidegger (12), with some modification. 1.2 g of agarose was dissolved into 100 ml of 0.05 M barbital buffer of pH 8.2. Two ml of agarose solution was put on a 2.5 × 7.6 mm slide glass. 5 µl of solution containing labelled or non-labelled antigen was put into a well of 2.5 mm diameter. Electrophoresis was run at 4° C for 1.5 h using 10 volt/cm. When labelled antigen was electrophoresed, 5 µl of labelled antigen, containing approximately 0.15 µg of protein and 2 µC of radioactivity, was put into the well. When non-labelled antigen was used, 5 µl of solution containing 1.7 µg of protein was put into the well.

150 µl of patient serum was put into the trough, and the agarose plate was placed for 1 day at room temperature, washed for 2 days with phosphate buffered saline of pH 7.4 (PBS), dried and stained with solution of amido black 10B.

Radioimmunoelectrophoresis

Radioimmunoelectrophoresis was done by the method described by Yagi et al. (13). Conditions in immunoelectrophoresis were the same as above. 5 µl of patient serum was put into the well, and electrophoresis was run for 1.5 h. Goat anti-serum to whole human serum, to IgA or to IgM was put into the trough. 24 h later the agarose plate was washed with PBS for 2 days. 150 µl of radioactive antigen, containing approximately 4.5 µg of protein (ca. 50 µC of radioactivity), was put into the trough, and the plate was kept for 2 days, washed for 2 days, dried and stained as above.

Radioimmunodiffusion

Radioimmunodiffusion using anti IgE was done by the method of Ishizaka et al. (5). 20 µl of anti IgE was put into the center well (5 mm diameter), and 50 µl of patient serum was put into the peripheral well (8 mm diameter). 24 h later the agarose plate was washed for 2 days. Then 20 µl of ¹²⁵I labelled mite antigen (ca. 0.6 µg of protein and 7 µC) was put into the center well. Twenty-four hours later, the agarose plate was washed and stained as above.

Double Gel Diffusion

Precipitin was looked for by a microimmunodiffusion technique (Ouchterony). 20 µl of ¹²⁵I labelled mite antigen (ca. 0.6 µg protein and 7 µC) or nonlabelled

antigen (ca. 7 μ g protein) was put into the central well, and 50 μ l of patient serum was put into the peripheral well. Twenty-four hours later the agarose plate was washed and stained as above.

Autoradiography

X-ray films were put in contact with slide glass of radioimmuno-electrophoresis, radioimmunodiffusion or double gel diffusion, and developed 24 h later.

RAST

Radioallergosorbent test (RAST) was carried out by Ceska's paper disc method (2). Coupling of mite antigen to paper discs was done by the method of Miyamoto et al. (10). 5 mg of mite extract was dissolved into 10 ml phosphate buffered saline of pH 7.4. By preliminary experiments, it was determined that this concentration of antigen was enough to obtain a maximum count of RAST. One hundred CNBr-activated paper discs were put into 10 ml of antigen solution. The procedure thereafter was the same as Ceska's method. After reaction of antigen-coupled paper discs with patient sera (0.1 ml of each), they were washed with incubation buffer and mixed with ^{125} I labelled anti IgE of 10^5 C.P.M. in 0.1 ml.

The relative concentration of IgE antibodies to mite in patients' sera was calculated from a standard curve as described by Johansson et al. (7), and represented as per mille of the concentration of the standard serum (1,000 per mille). The serum which was used as standard serum for RAST was obtained from an untreated patient who was highly allergic to mite and house dust. This serum gave a RAST count of 4×10^4 C.P.M. when undiluted serum was used. At the points where the slope of RAST became parallel to the standard curve by serial dilution of patients' sera, the concentration of IgE antibodies could be determined as described by Johansson et al. (7). To standardize the whole experiment, the same lot of CNBr-activated paper discs, the same lot of antigen and the same lot of ^{125} I-labelled anti IgE were used.

RESULTS

Radioimmunodiffusion

Three sera (I, A and T) were examined by radioimmunodiffusion. Serum I was obtained from a patient who was highly allergic to both mite and house dust and had never received hyposensitization. Serum A and serum T were obtained from patients who had never been treated and were moderately allergic to both allergens. IgE control serum was used as control.

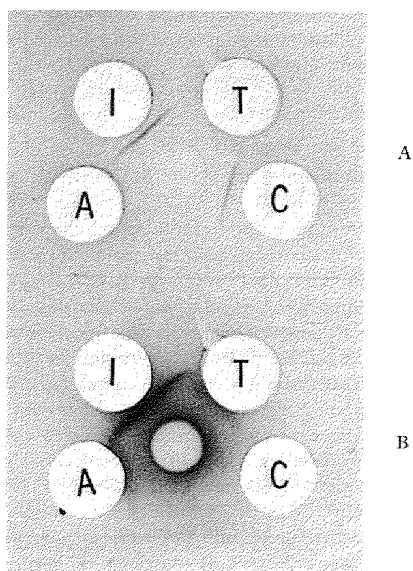


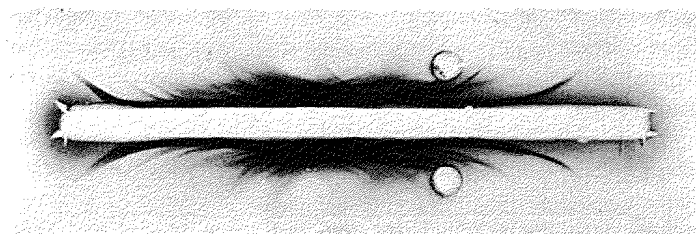
Fig. 1.

Radioimmunodiffusion. 1A: stained plate of radioimmunodiffusion. 1B: radioautogram of 1A.

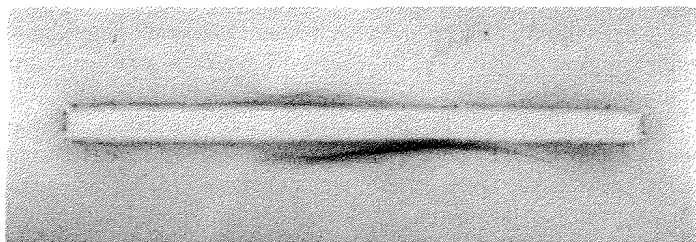
In peripheral wells are 50 μl of sera I, A and T from patients allergic to mite. IgE control serum (C) in peripheral well as control. In center well, 20 μl of anti IgE at first, and after appearance of precipitin lines, 20 μl of ^{125}I labelled mite antigen (ca. 0.6 μg protein, 7 μC).

Sera I and C formed precipitin lines against anti IgE, but sera A and T did not (Fig. 1A). ^{125}I labelled mite antigen reacted strongly with IgE of serum I, slightly with sera A and T (Fig. 1B).

The results are shown in Fig. 1. Serum I and IgE control serum formed precipitin lines in double gel diffusion against anti IgE, but the two other sera (A and T) did not (Fig. 1A). The uptake of ^{125}I labelled mite antigen to IgE of serum I was clearly demonstrated (Fig. 1B). Some uptake of antigen was observed in serum A and serum T (Fig. 1B). These results agree with the results of RAST. Although IgE control serum formed a precipitin line with anti IgE, only a trace amount of



A



B

Fig. 2.

Radioimmuno-electrophoresis (left is positive). 2A: stained plate. 2B: radio-autogram of 2A.

Upper well: normal serum ($5\ \mu\text{l}$). Lower well: patient serum (serum I, $5\ \mu\text{l}$). $150\ \mu\text{l}$ of goat anti-human serum was put in trough, at first. After appearance of precipitin bands (Fig. 2A), the plate was washed and $150\ \mu\text{l}$ of ^{125}I labelled mite antigen (ca. $4.5\ \mu\text{g}$ protein, $50\ \mu\text{C}$) was put into the trough. Uptake of ^{125}I labelled antigen to IgG of patient serum can be seen (Fig. 2B). Trace amount of antigen reacted with IgG of normal serum and several components of both sera (Fig. 2B).

antigen reacted with IgE of the serum. This very weak reaction is probably nonspecific because the RAST count of IgE control serum was in the normal range.

Radioimmuno-electrophoresis

Radioimmuno-electrophoresis of patient serum (serum I) was carried out using normal serum as control. After the appearance of precipitin lines against goat anti-human serum,

as shown in Fig. 2A, the plate was washed and radioactive mite antigen was put into the trough. The plate was washed again 24 h later, dried, stained and put in contact with X-ray film.

It was revealed by autoradiography that IgG of patient serum reacted with this antigen (Fig. 2B). IgG of the normal serum and several components of both sera showed weak reaction with the labelled antigen, but albumin did not react with either serum. The meaning of this weak reaction was not studied further. In two other sera from patients who received long-term hyposensitization with house dust, similar uptake of mite antigen to IgG, as seen in serum I, was observed in radio-immunoelectrophoresis.

The level of IgG in these sera was in the normal range when determined by single radial immunodiffusion. Sera from 34 other patients showed only weak reaction in radioimmunoelectrophoresis, as normal serum did. Therefore, the strongly positive reaction observed in the three sera was thought to be specific.

Double Gel Diffusion and Immunoelectrophoresis

In double gel diffusion only one (serum I) of 37 sera formed a precipitin band against non-labelled mite antigen (350 μ g protein/ml and its dilutions). The antigen was electrophoresed in agarose, and the same serum was put into a trough. Normal serum was put into another trough as control.

As shown in Fig. 3, a precipitin line can be seen at the beta region between the well and the trough of patient serum, as indicated with an arrow. All sera were examined by double gel diffusion using labelled mite antigen. Sixteen sera out of 37 (43 per cent) showed positive reactions. The positive and negative results are shown in the Figs. 4A and 4B.

Labelled mite antigen was electrophoresed, and the sera which showed positive reactions in double gel diffusion with labelled antigen were put into troughs. After being washed, dried and stained, the agarose plates were put in contact with

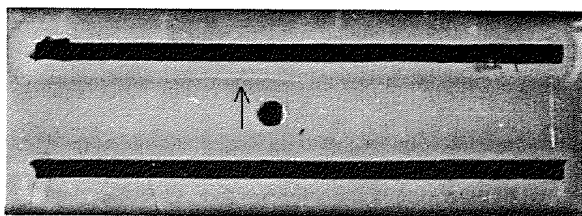


Fig. 3.

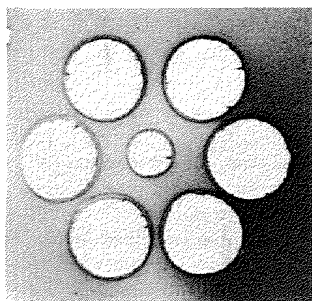
Immunoelectrophoresis (left: positive).

Well: 5 μ l of mite extract (1.7 μ g protein).

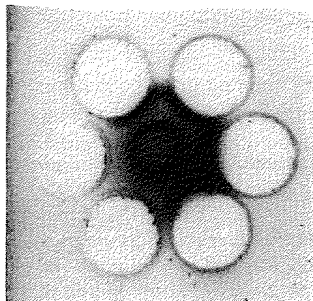
Upper trough: patient serum (serum I, 150 μ l).

Lower trough: normal serum (150 μ l).

A precipitin line can be seen at the beta region in the side of patient serum (as indicated with an arrow), but not in the side of normal serum.



A



B

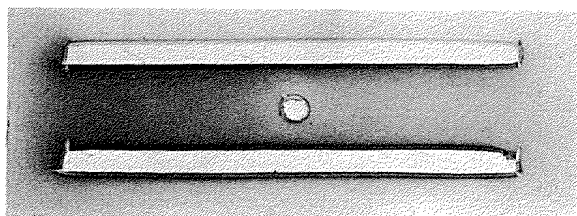
Fig. 4.

Double gel diffusion. 4A: stained plate. 4B: radioautogram of 4A.

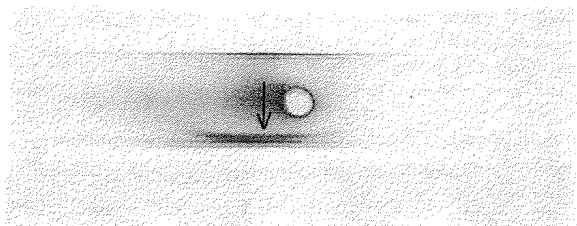
Center well: 20 μ l of ^{125} I labelled mite antigen (ca. 0.6 μ g protein, 7 μ C).

Peripheral wells: sera from patients allergic to mite and house dust (50 μ l of each). Four of six sera reacted with labelled mite antigen (serum I in Fig. 3 is not included).

X-ray film. It was revealed by autoradiography B that all sera examined formed a radioactive line at the beta region. An example is shown in Fig. 5. Twenty normal sera were also examined by double gel diffusion and immunoelectrophoresis



A



B

Fig. 5.

Immunoelectrophoresis (left: positive). 5A: stained plate. 5B: radioautogram of 5A.

Well: 5 μ l of ^{125} I labelled mite antigen (ca. 0.15 μ g, 2 μ C).

Upper trough: normal serum (150 μ l).

Lower trough: patient serum (150 μ l, different from serum I used in Fig. 3).

No line was visible in 5A. A single precipitin line can be seen after radioautography (5B) in the side of the patient serum, as indicated with an arrow.

using labelled and non-labelled mite antigen. No positive reaction was observed.

In electrophoresis using labelled antigen, serum I, which formed a precipitin band in double gel diffusion against non-labelled antigen, also formed three or four very weak radioactive bands from the alpha to gamma regions, besides one strong radioactive band at the beta region. These results suggest there is one component which is immunogenic to many patients, whereas in many patients there are only small amounts of antibodies to minor determinants of the mite extract.

Comparison between the Titer of IgE Antibodies to Mite and the Intensity of the Precipitin Bands

The intensity of precipitin lines which were formed between patients' sera and the mite antigen, non-labelled or labelled with ^{125}I , was graded as below.

Grade 0: no reaction to labelled antigen (approximately 30 μg protein/ml).

Grade 1: weak reaction to the same antigen.

Grade 2: reaction of middle grade to the same antigen.

Grade 3: strong reaction to the labelled antigen but no visible precipitation with non-labelled antigen (350 μg protein/ml and its dilutions).

Grade 4: visible precipitation with non-labelled antigen.

In Fig. 6, the relation of the intensity of the precipitin line to the relative concentration of IgE antibodies to mite is shown. Two sera from patients who received long-term hypo-sensitization with house dust showed grade 3 precipitation and low concentration of IgE antibodies to mite. One serum

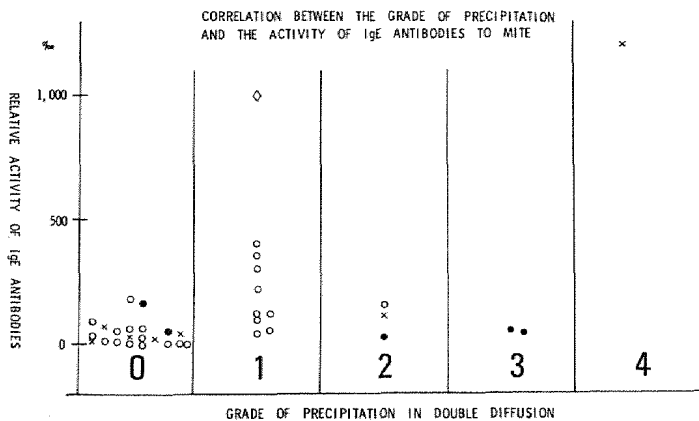


Fig. 6.

from a patient who received hyposensitization with mite extract, following long-term hyposensitization with house dust of the highest concentration, showed only grade 1 precipitation, but it still contained IgE antibodies of high concentration; whereas one serum from a patient who had never received hyposensitization showed grade 4 precipitation and high level of IgE antibodies to mite. From these results it was concluded that there was no significant relation between the intensity of the precipitin line and the relative concentration of IgE antibodies.

Examination of Sera with House Dust

All sera were examined by double gel diffusion, electrophoresis and radioimmuno-electrophoresis using labelled and non-labelled house dust extract. No positive reaction was obtained from these experiments.

DISCUSSION

As shown in the results, 16 out of 37 sera from patients allergic to mite formed a radioactive band against labelled mite antigen. One of the 16 sera also formed a visible precipitin band against non-labelled antigen. Assem & McAllen (1) reported that unconcentrated and concentrated sera did not form precipitin bands against mite antigen from *dermatophagoides pteronyssinus*. The discrepancy of the results from the two laboratories might be due to the difference of the kind of mite used in the experiments. But the most probable reason is the use of a more sensitive technique of labelling mite antigen in the present study.

The main immunogenic component which formed a single precipitin line against patients' sera migrated to the beta region in electrophoresis. It is not known whether this main component is allergenic or not. It is also not known whether the minor components which formed precipitin bands with one serum are allergenic or not. To clarify these points, purification of these components is necessary, or an examination of

whether the precipitin bands are formed by IgE and/or IgG purified from patient serum should be done.

The intensity of the precipitin line did not correlate with the relative concentration of IgE antibodies. Since IgG antibodies were detected by radioimmunoelectrophoresis in some patients whose sera formed a strongly positive radioactive line against labelled mite antigen in double gel diffusion, IgG antibodies may have relation to the precipitin line. Ishizaka & Ishizaka (6) reported that IgE antibodies were polyvalent when examined with tanned cell hemagglutination using ragweed antigen. Therefore, the possibility that IgE antibodies might also have some relation to the precipitation could not be excluded.

Although IgA and IgM antibodies to mite were not detected in the experiments, the presence of these antibodies could not be denied because the sensitivity of the method might still be too low for detection. As preliminary experiments, radioimmunodiffusion was done using specific antisera to IgG, IgA and IgM, but did not give such clear results as radioimmunoelectrophoresis. In radioimmunodiffusion using labelled mite antigen and anti IgG, IgA or IgM, the non-specific reaction was sometimes much larger than in radioimmunoelectrophoresis. Improvements of techniques are being investigated. To estimate qualitatively the activity of antibodies which belong to each immunoglobulin class, the double antibody assay using labelled antigens should also be done. In immunoelectrophoresis using labelled mite extract as antigen, IgG of patient serum reacted strongly with this antigen. However, IgG of normal serum and several other components of both normal and patient serum reacted with labelled antigen, although the reaction was very weak. No normal sera reacted with labelled mite antigen in direct precipitation, and this weak reaction was only observed in radioimmunoelectrophoresis. The reaction of IgG of normal serum might mean that normal individuals have a small quantity of IgG antibodies to mite.

In comparison of radioimmunoelectrophoresis (Fig. 2B)

with immunoelectrophoresis (Fig. 2A), it is apparent that the reaction of several components other than IgG is not a reaction of IgA or IgM with mite antigen. It is also probably not due to reaction of IgE with mite antigen, because the normal sera did not react with mite in RAST which is much more sensitive than radioimmuno-electrophoresis. This weak reaction is most probably due to nonspecific binding of labelled mite antigen to some serum components which could be detected only by radioimmuno-electrophoresis. However, the possibility could not be completely excluded that one of these weak reactions in patient serum could be due to a reaction of IgE with mite antigen, because it was not certain whether goat antiserum to whole human serum contained anti IgE or not.

It was reported by Kawai et al. (8) that house dust extract reacted with allergic and non-allergic individuals in counter-electrophoresis. Several sera were examined by the same method using Japanese house dust, and no positive reaction was observed. But further experiments with many sera are necessary before a conclusion can be drawn.

D'Souza et al. (3) reported that uptake of mite antigen to IgG of patients' sera increased after hyposensitization when examined by radioimmunodiffusion. No such results were obtained in my experiments. The reason is probably the low concentration of IgG antibodies to mite, because the concentration of mite extract used for hyposensitization when the sera were collected was very low.

On the other hand, a strongly positive reaction of IgG with mite antigen was observed in two sera from patients who received long-term hyposensitization with house dust. The mite antigen, which is thought to be one of the main antigens in house dust, might have increased these IgG antibodies. The radioimmunoassay of IgG antibodies before and after hyposensitization using the labelled mite antigen is in progress and will be reported in another paper.

No positive result was obtained by radioimmunodiffusion, radioimmuno-electrophoresis, double gel diffusion and immuno-

electrophoresis, when house dust extract labelled with ^{125}I was used. The reason is probably too low a concentration of allergenic protein in house dust extract when compared with mite extract.

SUMMARY

Mite antigen from *dermatophagoides farinae* was labelled with ^{125}I , and used for detection of precipitin to mite antigen in sera from asthmatic patients allergic to mite and house dust.

By double gel diffusion using this labelled antigen, it was found that 16 out of 37 sera formed precipitin bands which were detected by radioautography. Only one serum formed a precipitin band when non-labelled mite antigen was used in double gel diffusion. The main immunogenic component in mite extract migrated to the beta region in immunoelectrophoresis.

The intensity of the precipitin line did not correlate with the relative concentration of IgE antibodies as determined by RAST. IgG antibodies to mite were detected by radioimmuno-electrophoresis in three out of 37 sera.

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