

## IDENTIFICATION OF A SHRIMP-DERIVED ALLERGEN AS tRNA

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During an attempt to isolate shrimp allergens, evidence was obtained that shrimp ribonucleic acid was capable of eliciting a specific IgE response in man and an experimental animal model system. The shrimp ribonucleic acid was extracted from boiled whole shrimp (*Peneaus indicus*), and was isolated by salt precipitation and sequential chromatography over DEAE-Sephacel and BioGel P-100. The allergenic material was identified as a ribonucleic acid based on the following criteria: a maximal absorption at 258 nm, failure to stain positively with Coomassie Brilliant Blue on slab gel electrophoresis, positive staining with ethidium bromide, comigration with yeast tRNA on submerged gel electrophoresis in 1.5% Agarose M, and sensitivity to ribonuclease T<sub>2</sub> and 0.3 M NaOH. Treatment with protease did not alter its allergenic activity. The RNA was capable of binding allergen-specific IgE in sera from two shrimp-sensitive patients, as demonstrated by microELISA and solid-phase radioimmunoassay (SPRIA) using antigen-coated nitrocellulose filter paper discs and purified <sup>125</sup>I-labeled goat anti-human IgE. RNA isolated from shrimp by a conventional tRNA isolation procedure also had the ability to specifically bind IgE in the sera of shrimp-sensitive patients. IgE antibodies to shrimp RNA did not recognize yeast tRNA or salmon testes DNA, and were not detected in sera of other subjects. The shrimp-derived RNA was further able to induce a reaginic response in mice. A combination of in vitro aminoacylation of shrimp tRNA and SPRIA resulted in the identification of the allergenic tRNA as tRNA<sup>Tyr</sup> and tRNA<sup>Arg</sup>. Thus, shrimp tRNA is capable of inducing a specific IgE response in man.

The allergens which trigger IgE-mediated hypersensitivity reactions when isolated and characterized have been found to be proteins or low m.w. compounds covalently coupled to carrier molecules (1). Although nucleic acids are recognized to induce antibodies of IgG, IgM, and IgA class, as in the case of autoimmune diseases including systemic lupus erythematosus (2, 3), there is virtually no information in the literature on the ability of a nucleic acid to trigger IgE-mediated allergic reactions. In this

paper, we report the identification of a ribonucleic acid from shrimp (*Peneaus indicus*) which has allergenic activity in man and is capable of provoking an IgE response in cyclophosphamide-treated mice.

### MATERIALS AND METHODS

**Materials.** The following materials were used: Tween 20, BSA (RIA grade), swine skin gelatin, Evans blue dye, acrylamide, Tris, sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide,  $\beta$ -mercaptoethanol, DEAE-cellulose, PPO, POPOP, yeast tRNA, ribonuclease T<sub>2</sub>, proteinase K, salmon testes DNA, and ATP (Sigma Chemical Co., St. Louis, MO); we also used hydrogen peroxide and trichloroacetic acid (TCA; Glaxo Laboratories India Ltd., Bombay, India); o-phenylenediamine (Aldrich Chemical Co., Milwaukee, WI); horseradish peroxidase-avidin conjugate (Vector Laboratories, Burlingame, CA); iodogen (Pierce Chemical Co., Rockford, IL); cyclophosphamide (Khandelwal Laboratories, Bombay, India); Casamino acids (Difco Laboratories, Detroit, MI); DEAE-Sephacel and protein A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden); BioGel P-100 (Bio-Rad Laboratories, Richmond, CA); BA-85 nitrocellulose paper (Schleicher & Schuell, Keene, NH); <sup>125</sup>I-, <sup>14</sup>C-labeled Chlorella hydrolyzate and <sup>14</sup>C- and [<sup>3</sup>H]-labeled amino acids (Bhabha Atomic Research Center, Bombay, India); Agarose M (LKB, Bromma, Sweden); and polyvinyl chloride microtiter plates (96-well, U-shaped) with lids (Dynatech Co., Alexandria, VA). All other reagents were of analytical grade and are available commercially.

**Sera.** Sera were obtained from two patients with documented immediate hypersensitivity reactions following the ingestion of cooked shrimp. Patient 1 was a 36-yr-old white male with an 11-yr history of adverse reactions to shrimp. On multiple occasions and within 1 to 15 min after ingesting cooked shrimp, he experienced generalized pruritus and erythema, and periorbital edema. Symptoms were reproduced after a double-blind challenge with cooked shrimp meat on an approved protocol at the National Institutes of Health. Patient 2 was a 26-yr-old Indian female with a 7-yr history of episodes of cramping, abdominal pain, and diarrhea after ingestion of shrimp. These reactions occurred within 1 hr after eating shrimp and were not accompanied by other allergic manifestations.

**Skin tests.** Skin prick-puncture tests were performed on the volar surface of the forearm of the two patients by using shrimp allergen extract (1:10 w/v) obtained from the Walter Reed Army Medical Center, Allergy Extract Laboratory, Washington, D.C. On evaluation at 15 to 20 min, those skin test sites with a net wheal diameter equal to or greater than 3 mm larger than the negative control site were considered positive.

**Preparation of shrimp extract.** Whole shrimp (*Peneaus indicus*) (250 g) were placed in distilled water at 100°C for 10 min. The resulting supernatant was decanted, was cooled to 4°C, and was clarified by centrifugation at 20,000 × G for 20 min. Ammonium sulfate was added to 50 ml of this crude extract to achieve 90% saturation. The resultant precipitate collected after centrifugation at 20,000 × G for 20 min was dissolved in 5 ml of 0.05 M Tris-HCl buffer, pH 8, and was dialyzed overnight at 4°C against the same buffer before chromatography. Protein was measured by the method of Lowry et al. (4).

**Antibodies.** Monospecific goat anti-human IgE ( $\epsilon$ -chain-specific) purchased from Meloy Laboratories, Springfield, VA, was further purified by affinity chromatography using human IgE conjugated to CNBr-activated Sepharose 4B (5). Specific antibodies were eluted by using 3 M sodium thiocyanate. The eluate was dialyzed overnight against BBS, pH 8. The solution was centrifuged to remove denatured protein and was concentrated by ultrafiltration to give an antibody concentration of 1 mg/ml. Biotinylation of the affinity-purified goat anti-human IgE was performed as described (5).

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**Avidin-biotin microELISA (AB-microELISA).**<sup>3</sup> The allergenicity of various shrimp fractions obtained during purification was evaluated by measuring the ability of specific IgE in shrimp-sensitive patient's serum (1/25 dilution) to bind to the immobilized shrimp allergen. PVC microtiter plate wells were coated with each fraction (5 µg/ml). The AB-microELISA (5) was followed after blocking the allergen-coated wells with 3% BSA in serum diluent buffer for 1 hr at 37°C. One ELISA unit is defined as one absorbance unit at 492 nm. The activity (mean of three experiments) is expressed as ELISA U/ml undiluted serum.

**Solid-phase radioimmunoassay (SPRIA).** Affinity-purified goat anti-human IgE was labeled with <sup>125</sup>I by the lodogen method (6). Discs (6.5-mm) punched from nitrocellulose filter paper were incubated for 1 hr at 37°C with 1 ml of each fraction in PBS, pH 7.4, containing one A<sub>260</sub> unit. After washing with rinse buffer (0.05% Tween-20 in PBS), the allergen-coated discs were incubated successively for 1 hr at 37°C with 100 µl of 3% BSA in rinse buffer, shrimp-sensitive patient's serum (diluted 1/5 with 1% BSA in rinse buffer), and <sup>125</sup>I-labeled anti-human IgE (50,000 cpm). The discs were washed with rinse buffer at the end of each incubation. The results from three experiments are expressed as the mean percentage of radiolabeled anti-human IgE bound (B) to the allergen discs over total counts (T) of added radiolabeled anti-human IgE (B/T × 100). In the SPRIA inhibition assay, undiluted serum from shrimp-sensitive patient 1 (100 µl) was preincubated with different concentrations of shrimp allergen (0.001 µg/ml to 1 µg/ml) for 1 hr at 37°C.

**Passive cutaneous anaphylaxis.** Male Balb/c mice, 8 to 10 wk old, were administered cyclophosphamide i.p. (100 mg/kg body weight) 3 days before immunization to inhibit suppressor T cells concerned with IgE antibody synthesis (7). Mice were immunized by i.p. administration of antigen (10 µg of shrimp extract mixed with 4 mg of aluminium hydroxide gel suspended in 300 µl of 0.9% saline) on day 0, day 7, and day 35. The resulting IgE response to shrimp antigens was verified by passive cutaneous anaphylaxis (PCA) reactions (8). For this PCA reaction, male Wistar rats were injected intradermally with 100 µl of pooled serum (1/50 dilution) from mice immunized with the shrimp extract. After a latent period of 4 hr, different antigen preparations were injected i.v. with 0.5% Evans blue dye in 500 µl of 0.9% saline. The animals were sacrificed 20 min after antigen challenge and the diameter of the PCA reaction was measured on the everted skin surface. Three rats were used for each test dose.

**Electrophoresis.** Polyacrylamide slab gel electrophoresis and submerged agarose gel electrophoresis were performed according to the procedures of Davis (9) and Maniatis et al. (10), respectively.

**Isolation of shrimp tRNA.** tRNA from shrimp was isolated by the following procedure, which is a combination of the methods reported previously (11–14). Descaled whole shrimps (50 gm) were ground and were extracted with 0.01 M Tris-HCl buffer (pH 7.5) containing 0.5% SDS, 0.14 M NaCl, and 0.01 M MgCl<sub>2</sub> (25 ml). An equal volume of freshly distilled, water-saturated phenol was added and the mixture was stirred for 30 min. The aqueous fraction containing nucleic acids was recovered after centrifugation at 10,000 × G for 10 min at 4°C. After two more extractions with an equal volume of water-saturated phenol followed by centrifugation, the aqueous layer was diluted twofold with distilled water and was passed through a DEAE-cellulose column (1.2 × 12-cm) previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.5), containing 0.1 M NaCl. The column was washed with 10 vol of 0.3 M NaCl in 0.01 M Tris-HCl buffer (pH 7.5). RNA was eluted with 1 M NaCl. Two volumes of 95% ethanol were added to the eluant fractions to precipitate RNA, and the fractions were kept at –20°C overnight. The fractions containing RNA were pooled and were centrifuged at 10,000 × G for 10 min at 4°C. The precipitated RNA was washed twice with 70% ethanol containing 0.1 M sodium acetate and was dried under vacuum. Next, a 0.15 vol of 1-propanol and a 0.5 vol of 4 M NaCl were added in succession to RNA in a 0.35 vol of 0.01 M Tris-HCl buffer (pH 7.5) containing 0.5% SDS, 0.14 M NaCl, and 0.01 M MgCl<sub>2</sub>. This mixture was vortexed and was kept at –20°C for 16 hr. The precipitated ribosomal RNA was removed by centrifugation at 10,000 × G for 10 min at 4°C, and the supernatant containing tRNA was collected. The precipitate was washed once with 3 M sodium acetate and the washings were added to the supernatant. Two volumes of 95% ethanol were added to the supernatant and were kept at –20°C for 2 hr. The precipitated tRNA was washed three times with 70% ethanol containing 0.1 M sodium acetate and was dried under vacuum. The tRNA (20 A<sub>260</sub> units) was dissolved in 500 µl of 0.1 M sodium acetate. Two volumes of a 1% aqueous solution of cetyltrimethylammonium bromide were added dropwise to the tRNA solution while the sample was vigorously stirred on a vortex mixer. The precipitated cetyltri-

methyammonium salt of tRNA was allowed to stand for 2 min at room temperature, was centrifuged at 10,000 × G for 10 min, and was washed three times with 70% ethanol containing 0.1 M sodium acetate and twice with 95% ethanol. It was finally dried under vacuum.

**Deacylation of shrimp tRNA.** Shrimp tRNA (20 A<sub>260</sub> units) was dissolved in 500 µl of 0.5 M Tris-HCl buffer (pH 8.8) and was incubated at 37°C for 2 hr (11). A 0.1 M solution of MgCl<sub>2</sub> (100 µl) was added to the incubation mixture and the tRNA was precipitated with 95% ethanol (15). The resultant deacylated tRNA was used for in vitro aminoacylation.

**Preparation of aminoacyl tRNA synthetases.** Crude aminoacyl tRNA synthetases were prepared by the method of Muench and Berg (16). In brief, rabbit liver (6 gm) was homogenized in 15 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl<sub>2</sub>, 30 mM NH<sub>4</sub>Cl, 5 mM β-mercaptoethanol, and 5% autoclaved glycerol (extraction buffer). The homogenate was centrifuged at 15,000 × G for 30 min. The supernatant was decanted and was centrifuged at 105,000 × G in an ultracentrifuge for 1 hr. The supernatant was removed, was diluted with an equal volume of 5 mM β-mercaptoethanol, and was applied to a DEAE-cellulose column (1.3 × 23-cm) previously equilibrated with the extraction buffer. The column was washed with 200 ml of the same buffer and aminoacyl tRNA synthetases were eluted with 0.3 M KCl in the extraction buffer. The synthetases containing fractions were pooled, were aliquoted, and were stored at –80°C.

**In vitro aminoacylation of shrimp tRNA.** The in vitro aminoacylation of shrimp tRNA was performed according to the procedure of Ofengand et al. (17). In brief, shrimp tRNA (0.4 A<sub>260</sub> units) was added to a reaction mixture (200 µl) containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 2 mM ATP, a specific <sup>14</sup>C-amino acid (0.1 µCi) or [<sup>3</sup>H]-amino acid (0.5 µCi), and 300 µg aminoacyl tRNA synthetases preparation and was incubated for 20 min at 37°C in a metabolic shaker bath. The reaction mixture (150 µl) was then spotted on a Whatman No. 3 filter paper disc (presoaked in 10% TCA and dried) and was dipped in cold TCA (10%) containing casamino acids (10%) for 30 min. The discs were placed between the folds of two Whatman No. 3 filter papers on a Buchner funnel and were washed in succession with 10% cold TCA, 5% cold TCA, diethyl ether:ethanol (1:3), and diethyl ether. Discs were dried and the radioactivity was determined in a liquid scintillation counter.

**Measurement of specific tRNA-binding activity.** Aliquots of shrimp-sensitive patient's serum (100 µl) were immobilized by spotting on nitrocellulose paper discs (25-mm-diameter). The discs were subsequently blocked with PBS (pH 7.4) containing 1% BSA and 0.05% Tween-20. Shrimp tRNA, charged with a single species of either <sup>14</sup>C- or [<sup>3</sup>H]-labeled amino acid (50 µl, 0.1 A<sub>260</sub> unit) was spotted on each disc. The discs were placed between the folds of two Whatman No. 3 filter papers on a Buchner funnel and were washed with PBS (pH 7.4) containing 0.05% Tween-20, were dried, and the radioactivity was measured in a liquid scintillation counter.

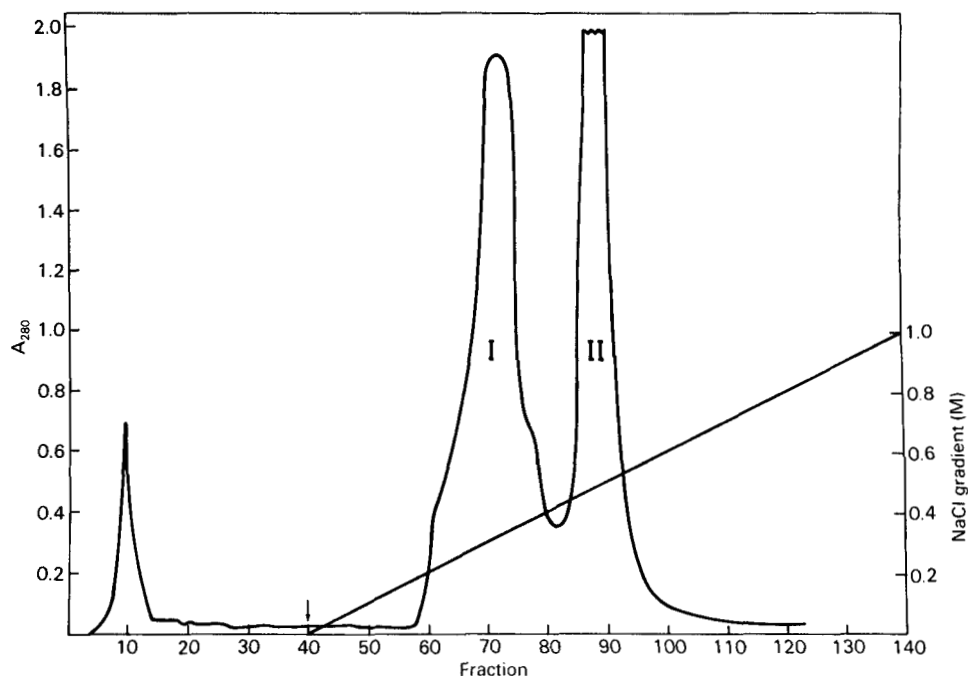
**Quantitation of IgE antibodies specific for shrimp tRNA species.** Goat anti-human IgE (1 µg in 50 µl PBS, pH 7.4, containing 1% BSA and 0.05% Tween-20) was immobilized by spotting on a nitrocellulose filter paper disc (25-mm-diameter). Each disc was blocked by incubation with 1% BSA in PBS (pH 7.4) for 30 min at 37°C. To eliminate the interference of shrimp tRNA-specific IgG antibodies present in the serum of shrimp-sensitive patient (Pt 1) in the quantitation of tRNA-specific IgE antibodies, the patient's serum (5 ml) was passed five times through a protein A-Sepharose 4B column (10 ml) previously equilibrated with BBS (pH 8). The IgG-depleted serum (50 µl) was incubated with shrimp tRNA (50 µl, 0.1 A<sub>260</sub> unit) charged with each labeled amino acid for 30 min at 37°C in a metabolic shaker bath. The mixture was spotted on a nitrocellulose paper disc immobilized with anti-human IgE. The discs were washed on a Buchner funnel between folds of two Whatman No. 3 filter papers with PBS (pH 7.4) containing 0.05% Tween-20. Discs were dried and the radioactivity was measured in a liquid scintillation counter.

## RESULTS

Both the patients demonstrated positive skin test reactions to shrimp. Initial screening studies confirmed that IgE antibodies specific for shrimp were demonstrable in the serum of both the patients by AB-microELISA. To characterize the allergens involved, the dialyzed shrimp extract was first chromatographed on DEAE-Sepharcel (1.7 × 52-cm column), using a gradient of 0 to 1 M NaCl on 0.05 M Tris-HCl buffer, pH 8 (400 ml), for elution. As shown in Figure 1, the IgE binding activity was associated with two peaks (I and II) which resolved at a concentration of 0.35 M and 0.6 M NaCl, respectively.

<sup>3</sup> Abbreviations used in this paper: AB-microELISA, avidin-biotin micro-enzyme-linked immunosorbent assay; SPRIA, solid-phase radioimmunoassay; PCA, passive cutaneous anaphylaxis.

Figure 1. DEAE-Sephacel chromatography of dialyzed shrimp extract. Fifty milligrams of dialyzed shrimp extract in starting buffer were applied to the column, and 4-ml fractions were collected at a flow rate of 20 ml/hr. A 5  $\mu$ g/ml protein solution of peak I used for coating the ELISA plate resulted in the measurement of 29 ELISA units (vs 35.8 ELISA units in the starting material). A 5  $\mu$ g/ml protein solution of peak II contained 10.3 ELISA units of activity. Serum of patient 1 was used in AB-microELISA.



Peak I was found to be protein-rich (24 mg), whereas the second peak contained little measurable protein (800  $\mu$ g). The peak II fractions were pooled (15 ml), were dialyzed against 100 vol of distilled water for 18 hr at 4°C, and were lyophilized. The residue was dissolved in 1 ml of 0.05 M Tris-HCl buffer, pH 8, and was passed through a BioGel P-100 column (1.6 x 40-cm) equilibrated with the same buffer. Greater than 90% of the material showing absorbance at 280 nm appeared in a single peak in the void volume (fractions 10 to 25) (Fig. 2) which contained materials binding to IgE in the shrimp-sensitive individual's (Pt 1) serum.

Initial studies revealed that the allergen, i.e., material in the excluded volume of the BioGel P-100 column, had an absorption maximum at 258 nm, suggesting the pos-

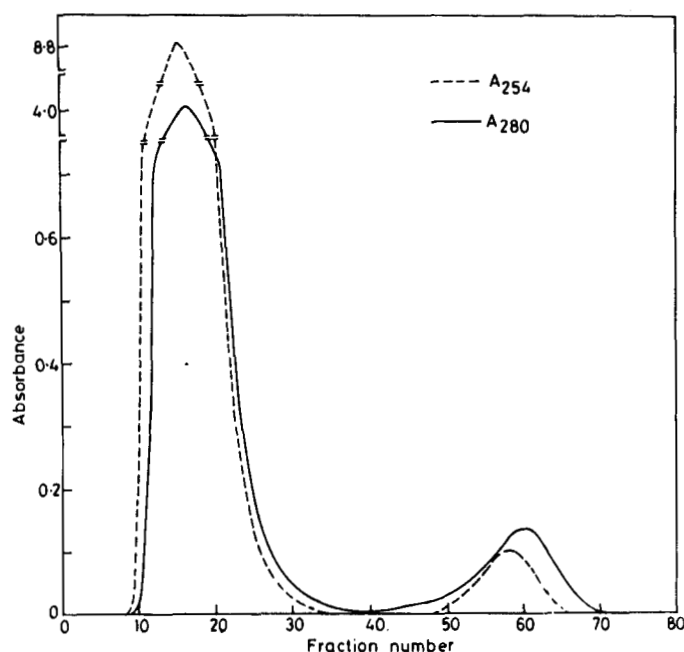


Figure 2. BioGel P-100 chromatography of peak II from DEAE-Sephacel. Fractions of 2 ml were collected at a flow rate of 15 ml/hr. A 5  $\mu$ g/ml protein solution of peak I used for coating the ELISA plate resulted in the measurement of 13.3 ELISA units of activity.

sibility that it was rich in nucleic acid, which could still account for the high absorbance at 280 nm. Further studies were consistent with this hypothesis. Thus, application of 50  $\mu$ l of this solution containing 40  $\mu$ g of protein, as measured by Lowry's method, to slab gel electrophoresis in 7.5% polyacrylamide did not reveal detectable bands when stained with Coomassie Brilliant Blue. On submerged gel electrophoresis in 1.5% Agarose M, material excluded on BioGel P-100 gave a major band co-migrating with that of yeast tRNA and stained positively with ethidium bromide, again consistent with the presence of nucleic acid. The ultraviolet absorption spectrum of the material excluded on BioGel P-100 was identical with that of other nucleic acids including yeast tRNA (data not shown). Treatment of the material excluded on BioGel P-100 (10  $A_{260}$  units) with 0.3 M NaOH for 1 hr at 65°C or with ribonuclease T<sub>2</sub> (20 U) for 18 hr at 37°C resulted in its breakdown with the release of dialyzable 260 nm absorbing material and loss of allergenic activity, as evaluated by AB-microELISA. Chromatography of the alkaline and enzymic hydrolyzates on BioGel P-100 revealed the disappearance of absorbance associated with fractions 10 to 25.

These experiments were consistent with the identity of IgE-binding material excluded on BioGel P-100 as ribonucleic acid. To determine the amino acid content of the material excluded on BioGel P-100, fractions 10 to 25 were pooled, were dialyzed against distilled water, and were lyophilized to dryness. One milligram of this material, when hydrolyzed with 6 N HCl (1 ml) at 110°C for 24 hr and subsequently analyzed in an automated amino acid analyzer (Model LKB 4400), revealed the presence of all the detectable common amino acids, which accounted for only 11% of the total dry weight of the starting material. To determine whether the protein component of the material excluded on BioGel P-100 was essential for allergenic activity, 5  $A_{260}$  units of BioGel P-100-purified material were treated with proteinase K (200  $\mu$ g) at 37°C for 18 hr. This reaction mixture was re-chromatographed on BioGel P-100 without showing a decrease in absorption at 260 nm. Amino acid analysis revealed that after

proteinase K treatment, 84% of the associated amino acids were lost (Table I).

Evaluation of the allergenicity of proteinase K-digested material by AB-microELISA was not feasible because the BioGel P-100-excluded material no longer bound to the surface of PVC microtiter plate wells. This was consistent with controls demonstrating that no radioactivity could be detected on incubating microtiter wells (PVC) overnight with  $^{32}\text{P}$ -labeled yeast tRNA,  $^3\text{H}$ uridine, or  $^3\text{H}$ adenine in ELISA coating buffer (20,000 cpm). Hence, the IgE binding activity was determined by measuring specific IgE antibodies in the sera of shrimp-sensitive patients by radioimmunoassay using antigen-coated nitrocellulose filter paper discs and  $^{125}\text{I}$ -labeled goat anti-human IgE as detailed in *Materials and Methods*. The SPRIA indicated that the proteinase K-treated material was indeed active. There was no significant difference in the binding of specific IgE antibodies to native and proteinase K-treated material, demonstrating that the associated protein was not required for recognition by specific IgE, but rather the associated protein facilitated the binding of antigen material excluded on BioGel P-100 to PVC.

RNA isolated from shrimp by a conventional tRNA isolation procedure, as detailed in *Materials and Methods* (11–14), also had the ability to specifically bind to IgE in the sera of both the shrimp-sensitive patients (Table II). The allergenicity of the material excluded on BioGel P-100, as measured by SPRIA, did not differ significantly from that of shrimp tRNA isolated by this procedure, either from the cooked shrimp extract or an extract from minced raw whole shrimps. A sample of the material excluded on BioGel P-100 (800  $\mu\text{g}$  in 1 ml), when subjected to the same extraction procedure, was recovered with no appreciable loss in the binding capacity. On agarose gel electrophoresis, the isolated shrimp tRNA moved as a single band, with mobility corresponding to that of the standard yeast tRNA (data not shown). The specificity of the IgE antibodies in the serum of the shrimp-sensitive patient (Pt 1) to shrimp RNA was further established by SPRIA inhibition. Preincubation of undiluted serum of the patient (100  $\mu\text{l}$ ) with shrimp RNA

TABLE II  
Specificity of IgE antibodies to BioGel P-100-excluded material, shrimp tRNA, yeast tRNA, and salmon testes DNA<sup>a</sup>

| Material                                                | Allergen-specific IgE Antibodies (B/T $\times$ 100) in the Serum of |                   |
|---------------------------------------------------------|---------------------------------------------------------------------|-------------------|
|                                                         | Patient 1                                                           | Patient 2         |
| BioGel P-100-excluded material                          | 10.2                                                                | 9.8               |
| Proteinase K-digested allergen excluded on BioGel P-100 | 10.7                                                                | N.D. <sup>b</sup> |
| tRNA isolated from boiled shrimp extract                | 11.6                                                                | 10.3              |
| tRNA isolated from raw minced shrimps                   | 10.8                                                                | N.D. <sup>b</sup> |
| Yeast tRNA                                              | 0.8                                                                 | 0.6               |
| Salmon testes DNA                                       | 0.3                                                                 | 0.3               |

<sup>a</sup> For this SPRIA experiment, 0.1  $A_{260}$  unit of each nucleic acid was immobilized per disc. SPRIA binding produced by sera from normal individuals and patients suffering from house dust or pollen allergy, as well as by cord sera, ranged from 0.9 to 1.9.

<sup>b</sup> N.D., not determined.

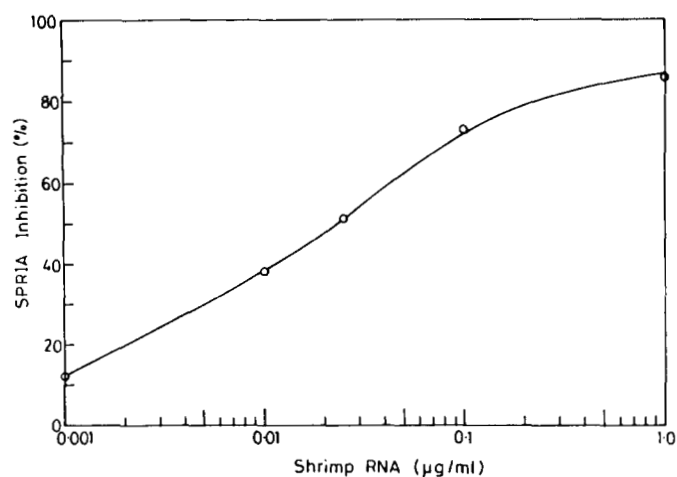


Figure 3. SPRIA inhibition curve for shrimp RNA.

(1  $\mu\text{g}$ ) for 1 hr at 37°C before incubating with shrimp RNA-adsorbed nitrocellulose filter paper discs resulted in 89% inhibition of binding of  $^{125}\text{I}$ -labeled anti-human IgE in SPRIA (Fig. 3).

The IgE antibodies to shrimp RNA measurable in the sera of the shrimp-sensitive patients by SPRIA failed to recognize yeast tRNA and salmon testes DNA (Table II). IgE antibodies specific for shrimp RNA could not be detected in the sera of normal subjects ( $n = 4$ ), patients with established allergy to house dust ( $n = 2$ ) and *Parthenium* (American feverfew) pollen ( $n = 2$ ), as well as in cord sera ( $n = 4$ ). The sera of house dust- and *Parthenium*-sensitive patients contained specific IgE antibodies to the respective allergens when measured by SPRIA using nitrocellulose as the solid support.

The shrimp RNA was also able to induce a homocytotropic antibody response in mice. Thus, BioGel P-100-excluded material, RNA extracted from boiled shrimp extract by the conventional tRNA isolation procedure, and proteinase K-digested material excluded on BioGel P-100 each elicited a PCA reaction in rats sensitized with mouse serum containing reaginic antibodies to the constituents of crude shrimp extract (Table III). The PCA reaction was abrogated when the serum was incubated at 56°C for 2 hr before sensitization of the rats, thus confirming that the antibodies specific for shrimp RNA were of the IgE class.

After establishing the ability of ribonucleic acid from shrimp to induce an IgE antibody response in shrimp-

TABLE I

Amino acid composition of BioGel P-100-excluded material<sup>a</sup>

| Amino Acid | Amount Present (nmol)         |                                           |
|------------|-------------------------------|-------------------------------------------|
|            | Before proteinase K digestion | After proteinase K digestion <sup>b</sup> |
| Asp        | 60.1                          | 8.4                                       |
| Thr        | 40.3                          | 9.4                                       |
| Ser        | 50.0                          | 7.1                                       |
| Glu        | 67.6                          | 7.3                                       |
| Pro        | 37.3                          | 15.4                                      |
| Gly        | 96.0                          | 24.6                                      |
| Ala        | 78.3                          | 5.6                                       |
| Cys        | 4.1                           | 0.8                                       |
| Val        | 39.6                          | 4.7                                       |
| Met        | 4.1                           | 1.1                                       |
| Ile        | 24.2                          | 2.3                                       |
| Leu        | 45.9                          | 3.8                                       |
| Tyr        | 8.1                           | N.D. <sup>c</sup>                         |
| Phe        | 20.6                          | 1.1                                       |
| His        | 12.2                          | 3.6                                       |
| Lys        | 50.0                          | 3.1                                       |
| Arg        | 42.1                          | 12.3                                      |
| Total      | 680.5                         | 110.6                                     |

<sup>a</sup> BioGel P-100-excluded material (10  $A_{260}$  units) was subjected to 6 N HCl hydrolysis.

<sup>b</sup> Percent degradation after proteinase K digestion: 83.7.

<sup>c</sup> N.D., not determined.

TABLE III  
Quantitation of shrimp RNA-specific IgE in mice<sup>a</sup>

| Fraction                                                | Challenge Dose (μg) | PCA Reaction (Diameter in mm) |
|---------------------------------------------------------|---------------------|-------------------------------|
| Crude boiled shrimp extract                             | 300                 | 8.0                           |
| BioGel P-100-excluded material                          | 32                  | 7.5                           |
| Proteinase K-digested allergen excluded on BioGel P-100 | 32                  | 8.0                           |
| Shrimp RNA                                              | 32                  | 8.0                           |

<sup>a</sup> Male Wistar rats were sensitized intradermally with pooled serum from mice immunized with crude boiled shrimp extract and were subsequently injected i.v. with different concentrations of each antigen preparation along with Evan's blue dye. The amount of antigen required to elicit a PCA diameter of 8 mm is expressed as the challenge dose. The diameter of PCA reaction produced by heated mouse sera was less than 2 mm. For experimental details, see *Materials and Methods*.

TABLE IV

*In vitro* amino acylation of shrimp tRNA isolated from descaled whole shrimps<sup>a</sup>

| Labeled Amino Acid Used for Charging  | cpm Bound  |
|---------------------------------------|------------|
| <sup>14</sup> C-Chlorella hydrolyzate | 1828 ± 124 |
| <sup>14</sup> C-Methionine            | 0          |
| <sup>14</sup> C-Asparagine            | 0          |
| <sup>14</sup> C-Lysine                | 183 ± 73   |
| <sup>14</sup> C-Glutamine             | 0          |
| <sup>14</sup> C-Threonine             | 246 ± 28   |
| <sup>14</sup> C-Proline               | 0          |
| <sup>14</sup> C-Serine                | 0          |
| <sup>14</sup> C-Isoleucine            | 0          |
| <sup>14</sup> C-Aspartic acid         | 0          |
| <sup>14</sup> C-Phenylalanine         | 1455 ± 58  |
| <sup>14</sup> C-Histidine             | 0          |
| <sup>14</sup> C-Alanine               | 676 ± 31   |
| <sup>14</sup> C-Valine                | 125 ± 21   |
| <sup>3</sup> H-Leucine                | 167 ± 39   |
| <sup>14</sup> C-Arginine              | 651 ± 3    |
| <sup>14</sup> C-Glycine               | 0          |
| <sup>14</sup> C-Glutamic acid         | 0          |
| <sup>3</sup> H-Tyrosine               | 1131 ± 39  |

<sup>a</sup> Shrimp tRNA was aminoacylated by using labeled amino acid in the reaction mixture. "0" indicates the cpm bound equal to or less than that associated with the labeled amino acid blank (range 65 to 95 cpm). For experimental details, see *Materials and Methods*.

sensitive patients, studies were conducted to identify the type of ribonucleic acid. tRNA from shrimp was suspected as the probable species of ribonucleic acid capable of inducing IgE response because of its complex secondary structure and resistant nature. To verify this possibility, experiments were designed to test the ability of the conventionally purified shrimp tRNA preparation to accept labeled amino acids *in vitro*. tRNA isolated from whole raw shrimp had the ability to accept the labeled amino acids, whereas that isolated from boiled shrimp extract lost this ability (data not shown). The allergenic activity of the RNA, however, was unaltered by heat treatment. The *in vitro* aminoacylation of shrimp tRNA isolated from uncooked whole shrimp is shown in Table IV. From these data, it is apparent that tRNA specific for lysine, threonine, phenylalanine, alanine, valine, leucine, arginine, and tyrosine were present in the tRNA preparation.

Antigenicity of the individual tRNA present in the tRNA fraction isolated from uncooked whole shrimp was evaluated by a combination of *in vitro* aminoacylation and SPRIA. As shown in Table V, antibodies against tRNA<sup>Lys</sup>, tRNA<sup>Thr</sup>, tRNA<sup>Ala</sup>, tRNA<sup>Leu</sup>, tRNA<sup>Arg</sup>, and tRNA<sup>Tyr</sup> were detectable in the serum of a shrimp-sensitive patient (Pt 1). Pooled serum from three control subjects or from patients allergic to allergens other than shrimp did not show binding.

Table VI shows the evaluation of IgE antibodies to specific shrimp tRNA in the serum of a shrimp-sensitive

TABLE V  
Evaluation of the antigenicity of shrimp tRNA by a combination of *in vitro* aminoacylation and SPRIA

| Amino Acid Used for Charging tRNA     | cpm Bound <sup>a</sup> |
|---------------------------------------|------------------------|
| <sup>14</sup> C-Chlorella hydrolyzate | 960 ± 24               |
| <sup>14</sup> C-Lysine                | 325 ± 24               |
| <sup>14</sup> C-Threonine             | 1,835 ± 244            |
| <sup>14</sup> C-Phenylalanine         | 0                      |
| <sup>14</sup> C-Alanine               | 3,736 ± 149            |
| <sup>14</sup> C-Valine                | 0                      |
| <sup>3</sup> H-Leucine                | 2,219 ± 302            |
| <sup>14</sup> C-Arginine              | 2,119 ± 140            |
| [ <sup>3</sup> H]-Tyrosine            | 13,446 ± 168           |

<sup>a</sup> Counts bound to shrimp-sensitive patient's serum immobilized on nitrocellulose paper disc as determined by SPRIA. Pooled serum from three control subjects with no history of atopy or from patients sensitive to house dust or *Parthenium* pollen did not show significant binding (range 267 to 295 cpm). "0" indicates the cpm bound equal to or less than that obtained with the control sera. For experimental details, see *Materials and Methods*.

TABLE VI

Quantitation of IgE antibodies specific for antigenic shrimp tRNA by a combination of *in vitro* aminoacylation and SPRIA

| Labeled Amino Acid Used for Charging Shrimp tRNA | cpm Bound <sup>a</sup> |
|--------------------------------------------------|------------------------|
| <sup>14</sup> C-Lysine                           | 0                      |
| <sup>14</sup> C-Threonine                        | 72 ± 20                |
| <sup>14</sup> C-Alanine                          | 222 ± 74               |
| <sup>3</sup> H-Leucine                           | 0                      |
| <sup>14</sup> C-Arginine                         | 1656 ± 57              |
| [ <sup>3</sup> H]-Tyrosine                       | 6936 ± 495             |

<sup>a</sup> IgG-depleted serum of shrimp-sensitive patient (Pt 1) was incubated with shrimp tRNA charged with each labeled amino acid. The mixture was subsequently spotted on nitrocellulose discs immobilized with goat anti-human IgE, and the ability of the labeled immune complexes (IgE-tRNA) to bind to the discs was determined by SPRIA. For details, see *Materials and Methods*. Pooled serum from three control subjects or from patients sensitive to house dust or *Parthenium* pollen did not show significant binding (range 99 to 123 cpm). "0" indicates the cpm bound equal to or less than that associated with the control sera. For experimental details, see *Materials and Methods*.

patient (Pt 1). As shown in the table, IgE antibodies specific for shrimp tRNA present in the serum of Pt 1 were quantitated by a combination of *in vitro* aminoacylation of shrimp tRNA and SPRIA. From the data it can be seen that of the six antigenic shrimp tRNA species, tRNA<sup>Tyr</sup> and tRNA<sup>Arg</sup> had the ability to bind to IgE antibodies in the serum of Pt 1.

#### DISCUSSION

The information presented in this report demonstrates for the first time that a tRNA, in this case derived from food, is capable of eliciting an IgE-mediated reaction in both humans and in an experimental animal model. During conventional isolation procedures to identify proteins from shrimp with allergenic activity, one peak of allergenic activity resolved during DEAE-Sephacel chromatography contained insufficient protein, as assayed by the Lowry method (4), to account for its marked absorption at 280 nm. This suggested the presence of a non-protein allergen (Fig. 1). This material was further purified by molecular exclusion chromatography on a BioGel P-100 column, where the allergenic activity appeared in the excluded volume (Fig. 2). This excluded material exhibited an absorption maximum at 258 nm, suggesting that this peak of activity was nucleic acid-rich. The predominance of nucleic acids in this peak was confirmed by a series of observations: the ultraviolet absorption maximum was identical with that of yeast tRNA, the 260:280 nm ratio was 2, and aliquots of this material

failed to stain positively with Coomassie Brilliant Blue when subjected to slab gel electrophoresis in 7.5% polyacrylamide. Furthermore, a sample of this material on submerged gel electrophoresis in 1.5% Agarose M gave a major band co-migrating with that of yeast tRNA, and both stained positively with ethidium bromide. The major band when electroeluted was found to be allergenic by AB-microELISA.

Together, these observations suggested that the allergenic material was a nucleic acid. To further confirm this hypothesis, the material excluded on BioGel P-100 was treated either with 0.3 M NaOH or ribonuclease T<sub>2</sub> and was dialyzed. Both the procedures resulted in the loss of allergenic activity as well as the 260 nm absorbing material. Treatment of the BioGel P-100-excluded material with proteinase K resulted in the loss of greater than 80% of the associated amino acids without loss of the allergenicity, as measured by SPRIA (Table I). In this instance, however, it was noted that the removal of the protein prevented the binding of the allergenic material to the PVC microtiter plate.

Evidence was thus consistent with the identity of the allergenic material as an RNA. Because tRNA alone is resistant to heating at 100°C for 10 min, it was the most likely candidate among the RNA as the substance with allergenic activity. This hypothesis was confirmed by the demonstration that shrimp tRNA isolated by a conventional tRNA isolation procedure also exhibited allergenic activity (Table II). The specificity of isolated shrimp tRNA was also established by SPRIA inhibition (Fig. 3). To further demonstrate the allergenic potential of tRNA, experimental animals immunized with crude boiled shrimp extract developed IgE antibodies against the shrimp tRNA (Table III).

To identify the specific tRNA with allergenicity, a series of experiments were undertaken to specifically label the shrimp tRNA and to evaluate their binding to IgE antibodies in the serum of the shrimp-sensitive patient by SPRIA. Initial experiments showed that ability of tRNA to accept the corresponding amino acid was lost during boiling, although this did not affect the allergenicity. Subsequent experiments were done by using tRNA extracted from uncooked shrimps. In vitro aminoacylation of isolated shrimp tRNA using radiolabeled amino acids, resulted in the identification of eight tRNA species (Table IV). Among these, six were found to have antibody-binding activity when tested with a shrimp-sensitive patient's serum (Table V). After the removal of IgG antibodies from shrimp-sensitive patient's serum and by using specific anti-human IgE, two of these tRNA, namely tRNA<sup>Tyr</sup> and tRNA<sup>Arg</sup>, were found to have the ability to bind to specific human IgE (Table VI).

This represents the first documentation of the ability of tRNA to induce specific IgE response in man as well as in experimental mice. It may be that nucleic acids from other sources, including pollen, may also be allergenic but have not been identified due to the requirement to associate allergenicity with some measurement of nucleic acids, rather than relying exclusively on protein assays. The ability of food-derived nucleic acids to induce

IgE-mediated hypersensitivity responses demonstrates that nucleic acids from food sources can indeed provoke antibody responses in man.

Although nucleic acid-specific IgE have not previously been implicated in allergenic responses, they may not be unique. The presence of viral-specific IgE has been shown in nasopharyngeal secretions from infants with various forms of respiratory illness due to respiratory syncytial virus (18). Furthermore, a large number of persons suffering from chronic Epstein-Barr virus infection were reported to have IgE-mediated allergic diseases (19). These observations further suggest that viral nucleic acids may also play a role in the manifestation of IgE-mediated allergic reactions with unknown etiology among atopics.

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