Isolation and identification of hevein as a major IgE-binding polypeptide in *Hevea* latex

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Background: Polypeptides in *Hevea* latex are known as the major cause of latex type I sensitivities. So far, only a few of them have been characterized.
Methods: Proteins with a molecular weight lower than 10 kd in fresh *Hevea* latex were separated by ultrafiltration and further characterized by liquid chromatography on-line–coupled electrospray mass spectrometry. Hevein in this fraction was then purified by preparative reverse-phase high-performance liquid chromatography and characterized by matrix-assisted laser desorption ionization mass spectrometry and protein sequencing. Skin prick tests, enzyme-linked allergosorbent tests, and inhibition immunoblotting were performed to show the allergenicity of the purified hevein.
Results: Hevein, a 4.7 kd polypeptide, is the predominant component in the fraction with latex proteins of smaller than 10 kd. Specific IgE antibodies to hevein were detected by enzyme-linked allergosorbent test in 48 of 64 (75%) sera from health care workers allergic to latex and in three of 11 (27%) sera from patients with spina bifida and hypersensitivity reactions to latex. Inhibition immunoblotting demonstrated that the preincubation of 14 sera and a serum pool from patients allergic to latex with purified hevein completely inhibited IgE binding to the 20 kd protein, which has been recently reported to be a major allergen in latex (prohevein). Skin prick testing showed a positive reaction to hevein in 17 of 21 (81%) patients with latex allergy.
Conclusions: The results clearly demonstrate that hevein is an important latex allergen, and the IgE-binding capacity of prohevein in latex is mostly attributed to hevein, the N-terminal domain of prohevein. (J Allergy Clin Immunol 1997; 99:402-9.)

Key words: *Hevein, latex allergy, IgE antibody, latex proteins*

Natural rubber latex, derived from the rubber tree *Hevea brasiliensis,* is the most important raw material in the production of latex examination and surgical gloves. In recent years, with the enormous increase in the use of latex gloves in hospitals, the IgE-mediated reactivity to natural rubber latex has become a potentially serious problem, especially for health care workers.[1-3] In addition, patients with conditions requiring repeated surgery or long-term hospitalization have been shown to have a higher risk of developing latex allergies.[4, 5] There is a common agreement that the soluble proteins remaining on the surface of latex-made products are responsible for latex immediate hypersensitivity.[6, 7] Recently, many efforts have been made to isolate and identify the IgE-binding proteins from natural latex and latex-made products. By using the immunoblotting method,[8-13] it has been shown that more than 10 protein bands are capable of binding IgE antibodies. The molecular weights (MWs) of these proteins range from 10 to 100 kd. In combination with protein sequencing, several known proteins in natural rubber latex such as rubber elongation factor (Hev b 1),[14] prohevein,[10, 15] and hevamine[10, 15] were found to react with IgE antibodies in sera of individuals allergic to latex.

Abbreviations used

BSA:	Bovine serum albumin
EAST:	Enzyme-linked allergosorbent test
ESI-MS:	Electrospray ionization mass spectrometry
HPLC:	High-performance liquid chromatography
MALDI:	Matrix-assisted laser desorption ionization mass spectrometry
MW:	Molecular weight
RP-HPLC:	Reverse-phase high-performance liquid chromatography
SPT:	Skin prick test
TBS:	Tris-buffered saline
TBST:	0.05% Tween-20 dissolved in TBS
TFA:	Trifluoroacetic acid

Recently, we have focused our work to characterize the low MW allergens (<10 kd) in latex. Using two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients, followed by immunoblotting with sera of patients allergic to latex, we observed that a protein spot (isoelectric point, approximately 4.9; MW < 6 kd) reacted with serum IgE antibodies. (Detailed results of two-dimensional polyacrylamide gel electrophoresis will be published separately.) Further investigations showed that this spot represented a 4.7 kd polypeptide called hevein in *Hevea* latex. The experiments in this study were undertaken to isolate the hevein in latex and to elucidate its immunologic role in latex allergy. The purified hevein was used in enzyme-linked allergosorbent test (EAST), EAST inhibition, and in

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vivo skin prick tests (SPTs). The results revealed that hevein is a very important allergen in latex. Moreover, the results of inhibition immunoblotting of latex proteins demonstrated that the hevein was able to completely inhibit IgE binding to the 20 kd protein, which has been recently identified as prohevein and reported to be a major allergen in latex.

METHODS

Source material

Fresh latex was obtained from the Rubber Research Institute of Malaysia. It was collected from 22 rubber trees *(Hevea brasiliensis)* of the clone RRIM 600 and was immediately ammoniated at 0.7% and shipped without freezing. The isolation of hevein was carried out 1 week after the harvest.

Human sera

Two groups of sera were used in the IgE antibody–binding study. The first group comprised all available serum samples from 45 individuals allergic to latex who had visited our department of medicine between July 1994 and March 1995 and 20 sera from the skin testing group (see Skin testing). All of the patients were diagnosed by clinical history and showed a positive SPT responses to latex extract prepared in our laboratory. Sixty-four showed the presence of specific IgE antibodies to latex in their sera as determined by latex RAST (CAP system, Pharmacia). Among the sensitized persons, 62 were health care workers, and two worked in the textile industry with exposure to latex products. One serum sample was from an atopic child with latex allergy. In the other group, sera from 11 patients with spina bifida were used. They were collected by Dr. R. Cremer from the Children's Hospital, Cologne, Germany, and were all demonstrated to have increased latex-specific IgE antibodies by the CAP method. A serum pool from five individuals with latex allergy was also prepared and studied. Twelve additional sera, 10 from nonallergic adults and two from patients with spina bifida without latex allergy, were used as negative controls.

Purification and identification of hevein from natural rubber latex

The latex C-serum proteins in fresh ammoniated latex sap were separated from rubber particles as previously described.[16] They were further fractionated by ultrafiltration by using Amicon Centriprep 10 (Amicon GmbH, Witten, Germany) with a molecular mass cutoff of 10 kd according to the manufacturer's instructions. The fraction containing the proteins with MW lower than 10 kd was diluted with 2 volumes of distilled water and concentrated to about 20% of the original volume to remove most of the low MW salts by using a Centricon 3 filter (Amicon GmbH, with a molecular mass cutoff of 3 kd). The concentrated sample was separated by analytic reverse-phase high-performance liquid chromatography (RP-HPLC) (column: Nucleosil C-8, 4.6 × 250 mm) with a linear gradient from 15% to 60% solution B (0.06% trifluoroacetic acid [TFA] in acetonitrile; solution A was 0.1% TFA in water) for 25 minutes at a flow rate of 1 ml/min. The identification of hevein was achieved by micro-HPLC on-line–coupled electrospray mass spectrometry (ESI-MS).[17] Twenty microliters of concentrated sample containing about 300 pmol of peptides was subjected to HPLC on a C-18 column (SGE glass inline, Weiterstadt, Germany; 100 mm × 1 mm I.D.; particle size: 5 μ; pore size: 300 Å). The peptide was eluted with a gradient from 10% to 60% solution B for 50 minutes and from 60% to 100% solution B for 5 minutes at a flow rate of 30 μl/min. The eluate was on-line injected into the electrospray ion source of a Finnigan TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT Ltd., Bremen, Germany). The spectra were recorded under unit-mass resolution in the positive ion mode. The total ion current chromatogram was obtained by scanning the mass range corresponding to mass-to-charge ratio values of 100 to 2500 every 4 seconds. The spectra representing the peptide were accumulated and deconvoluted to get the calculated mass.

After the hevein fraction in the HPLC separation had been identified, the hevein in this sample was purified by preparative HPLC with a 20 mm × 250 mm Nucleosil C-18 column (Grom, Herrengerg, Germany) and a 20-minute linear gradient of 25% to 45% solution B at a flow rate of 15 ml/min. The purity of the isolated hevein was carefully controlled by analytic HPLC, and the hevein peptide was further characterized by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)[18] with a linear mode Lasermat MALDI mass spectrometer (Lasermat 2000; Finnigan MAT Ltd., Hemel Hempstead, U.K.) equipped with a nitrogen laser (337 nm; pulse duration, 3 nsec). The spectrum was obtained by summing up 10 scans and was externally calibrated by using a standard peptide mixture.

Protein sequence analysis

N-terminal sequence analysis was carried out by automated Edman degradation on an Applied Biosystems model 476A protein sequencer equipped with an on-line phenylthiohydantoin amino acid analyzer (120A).

Electrophoresis and blot analysis

For sodium dodecylsulfate–polyacrylamide gel electrophoresis, 2 mg lyophilized latex C-serum proteins were dissolved in 1 ml of sample buffer containing 50 mmol/L Tris-HCl, 2% (wt/vol) sodium dodecylsulfate, 10% (vol/vol) glycerol, and 30 mmol/L dithiothreitol, pH 6.8. The protein solution was then heated at 90° C for 3 minutes. Three hundred micrograms of proteins was applied to the gels, which consisted of a 1 cm stacking gel (4% T) and a 6 cm separating gel (12.5% T). Latex proteins were resolved by using the buffer system developed by Schägger and von Jagow[19] in a Mini Protean II electrophoresis cell (Bio-Rad). After electrophoresis, the gels were stained with Coomassie Brilliant Blue (Sigma) or transferred to polyvinylidene difluoride membranes (0.2 μm; Pall Filtrationstechnik, Dreieich, Germany) by semi-dry blotting with the buffer system of Kyhse-Andersen.[20] Blotting was performed at 0.8 mA/cm^2 for 1 hour at room temperature. Subsequently, the polyvinylidene difluoride membranes were washed three times with Tris-buffered saline (TBS: 50 mmol/L Tris/HCl, 150 mmol/L NaCl, pH 7.4, containing 5 mmol/L NaN_3 and 1 mmol/L ethylenediaminetetraacetic acid) and were blocked overnight at 20° C with a solution consisting of 1% bovine serum albumin (BSA) and 1% polyvinylpyrrolidone (PVP-40, Sigma) in TBS. After washing with TBS, the membranes were cut into individual 4 mm wide strips. The strips were incubated individually with 800 μl serum samples diluted 1:2 to 1:10 according to the latex-specific IgE values in TBST-BSA (2% BSA dissolved in TBST) for 18 hours at 4° C. After the blots were rinsed three times for 10 minutes with TBST (0.05% Tween-20 dissolved in TBS), they were incubated with alkaline phosphatase–conjugated anti-human IgE antibodies (Sigma) diluted 1:2000 in TBST-BSA for 2 hours at room temperature. IgE binding was demonstrated by adding a BCIP/NBT substrate solution (0.4 mmol/L 5-bromo-4-chloro-3-indolyl phosphate disodium salt, 0.37 mmol/L p-nitro blue tetrazolium chloride in 0.1 mol/L $NaHCO_3$ buffer containing 10 mmol/L $MgCl_2$, pH 9.6). Color

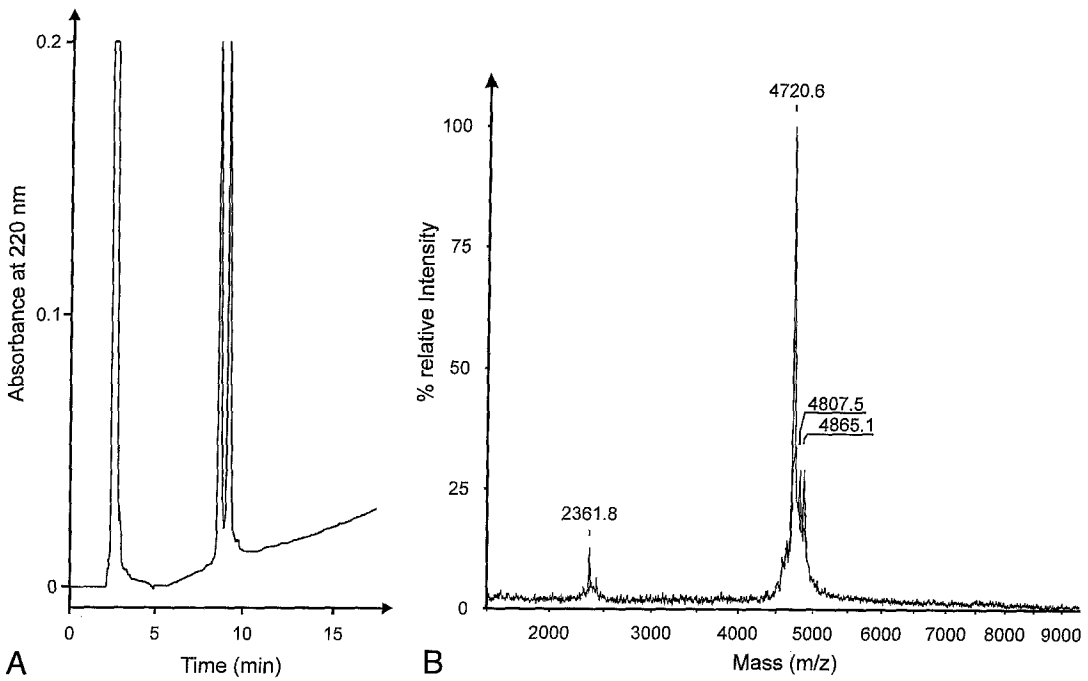

FIG. 1. A, RP-HPLC chromatogram of purified hevein. Analysis was performed with a Nucleosil C-8 column. Gradient condition: 15% to 60% of acetonitrile in 0.1% TFA for 25 minutes at a flow rate of 1 ml/min. Eluate was recorded at 220 nm. **B,** MALDI-MS spectrum of purified hevein. Calculated mass of hevein: M = 4719 d; measured: 4720.6 = M + H^+; 4807.5 = MH^+ plus a Ser (MW 87); 4865.1 = MH^+ plus a Ser and a Gly (MW 57); 2361.8, double protonated hevein molecule (M + $2H^+$).

development was stopped with water, and the membranes were air-dried.

The procedure of immunoblot inhibition assay was the same as described above, except that each diluted serum sample was incubated with 20 μl of hevein solution (0.1 mg/ml) for 16 hours at 4° C before exposure with the blotted latex proteins. Serum samples obtained from 10 adults not allergic to latex were used as controls; five of the adults were health care workers and were occupationally exposed to latex products.

Skin prick testing with latex extract and purified hevein

SPTs were performed with purified hevein at concentrations of 1, 10, and 100 μg/ml in saline solution containing 0.4% phenol (diluent) and with protein extract from latex sap as mentioned above (total proteins in C-serum, concentrations: 1, 10, and 100 μg/ml in diluent). For comparison, a commercially available latex testing solution from Bencard (1%; Smithkline Beecham, Munich, Germany) was also used in this study. Histamine hydrochloride (1 mg/ml) and diluent were used as positive and negative controls, respectively. A total of 31 subjects (health care workers) who were occupationally exposed to natural rubber products and visited our department of medicine between September and December 1995 were tested for their skin reactions to hevein. Cutaneous reactions were read after 15 minutes and were considered positive when a wheal reaction was at least 3 mm surrounded by an erythema as compared with negative control values (wheal ≤ 2 mm, no erythema). To avoid an unexpected overreaction, the subjects first underwent SPTs with the test solution that had the lowest hevein concentration. If no distinct reaction was observed, the SPT was reapplied with more concentrated hevein solutions (up to 100 μg/ml).

Assay for IgE antibodies to latex and hevein

Latex RAST was performed by the CAP method (CAP system, Pharmacia). Latex-specific IgE values greater than 0.35 kU/L were regarded as positive. The presence of specific IgE antibodies to hevein in sera was determined by EAST as previously described.[14] Briefly, the purified hevein was coupled onto the CNBr-activated cellulose disks in 0.1 mol/L sodium bicarbonate buffer, pH 8.4, at a concentration of 0.5 mg of hevein per milliliter. After washing and treatment with diethylamine, the hevein disks were incubated with 50 μl of each patient's serum for 180 minutes at room temperature. A Phadezym RAST test kit (Pharmacia) was used to detect specific IgE antibodies. Values above 0.35 kU/L were regarded as positive, as recommended by Pharmacia. A competitive EAST inhibition was also performed to confirm the test specificity, in which the patient's serum was incubated with 10 μl of hevein solution in phosphate-buffered saline at concentrations ranging from 0.1 to 100 μg/ml before the EAST assay.

RESULTS

Purification and characterization of hevein in latex

The size-fractionated latex C-serum extract containing the proteins with MWs lower than 10 kd was analyzed by RP-HPLC with a C-8 column. The elution profile showed a number of peaks. A major protein species eluted as a double peak at about 10 minutes (peak A).

TABLE I. Results of skin prick tests and clinical data of 21 subjects allergic to latex

Subject No.	Sex	Age (yr)	Serum total IgE (kU/L)	IgE antibodies to Latex* (kU/L)	IgE antibodies to Hevein† (kU/L)	Skin reaction Latex	Skin reaction Hevein
1	F	33	36	11.8	7.15	+++	+++
2	F	22	641	29.6	<0.35	+++	−
3	F	22	141	21.3	6.72	+++	+++
4	F	30	54	8.28	3.46	++	+++
5	F	28	94	0.85	nd	+	++
6	F	29	388	47.6	12.20	++	+++
7	M	28	642	8.65	<0.35	++	−
8	F	25	150	1.14	<0.35	++	−
9	F	29	147	6.39	<0.35	++	+++
10	F	31	46	2.99	2.30	+	++
11	F	38	103	1.22	1.26	+++	+++
12	F	23	25	2.84	1.39	+++	+++
13	M	35	496	0.56	0.36	++	+++
14	F	36	168	15.6	13.9	++	+++
15	M	35	72	1.93	1.23	++	++
16	F	22	64	5.23	<0.35	++	++
17	F	27	53	<0.35	<0.35	+	++
18	F	33	188	12.3	9.88	+	+++
19	F	33	72	0.78	0.71	+	+++
20	F	20	235	5.35	4.28	+	+++
21	F	21	514	>100	7.94	+++	−

The grading of skin reactions is defined as follows: −, negative; +, wheal reaction ≥3 mm; ++, wheal reaction ≥5 mm; +++, wheal reaction ≥7 mm.
*Results obtained by CAP method with commercial latex allergen (Pharmacia).
†Results determined by EAST with hevein-coupled disks.

Analyses by liquid chromatography–coupled ESI-MS to determine the masses of polypeptides in the filtered extract revealed that the molecular mass of the peptide in peak A was 4719 d, with two minor peaks of 4863.0 and 4806.0 d, respectively (data not shown). This is in exact agreement with the sequence of hevein.[21] In addition, the mass scanning across the whole eluted double peak (five scans) indicated the presence of a single peptide species, because both peaks revealed identical mass. This suggests that a conformational isoform of the hevein molecule may exist. This polypeptide was then purified by preparative HPLC, and the isolated hevein peptide showed a purity of more than 98% by analytic HPLC (Fig. 1, *A*). Mass analysis of the purified hevein with MALDI-MS (Fig. 1, *B*) confirmed the result from ESI-MS. As demonstrated by the ESI-MS, the MALDI-MS spectrum showed that the isolated hevein sample also contained the sequences with the posttranslational modifications, in which residues of Gly or Gly-Ser were still attached to the C-terminus of the hevein peptide.

The purified hevein was submitted to automatic Edman degradation and was found to have the following N-terminal sequence: Glu-Gln-X-Gly-Arg-Gln-Ala-Gly-Gly-Lys. It is identical to the peptide sequence of hevein (the Cys in position 3 cannot be identified by amino acid sequencing under normal conditions). Two hundred milliliters of latex sap yielded about 7.4 mg of hevein.

Skin testing

Thirty-one individuals with a history of exposure to latex materials underwent skin prick testing with purified hevein. Twenty-one subjects showed positive skin reactions to latex extracts. Among them 17 (81%) also exhibited positive responses to purified hevein (Table I). All 11 subjects who had negative skin test responses to latex extracts also had negative responses to hevein. In general, the hevein SPT solution showed a stronger skin reaction than the latex extract with the same protein concentration.

Measurement of specific IgE antibody to purified hevein

Serum samples of patients allergic to latex were used to evaluate the allergenicity of hevein isolated from *Hevea* latex. Of the 64 sera from group 1 patients (health care workers) that were demonstrated to have latex-specific IgE by the CAP method, 48 (75%) showed the IgE-binding capacity to purified hevein in EAST (Fig. 2). The second group of patients in this study comprised 11 children with spina bifida. All of them were tested and were found to have different levels of IgE antibodies directed against latex. Sera from only three of the 11 children (27%, Table II) had specific IgE directed against hevein. None of the six negative control sera showed detectable IgE antibodies to hevein.

A dose-related autoinhibition assay was also performed to assess the binding specificity of IgE antibodies

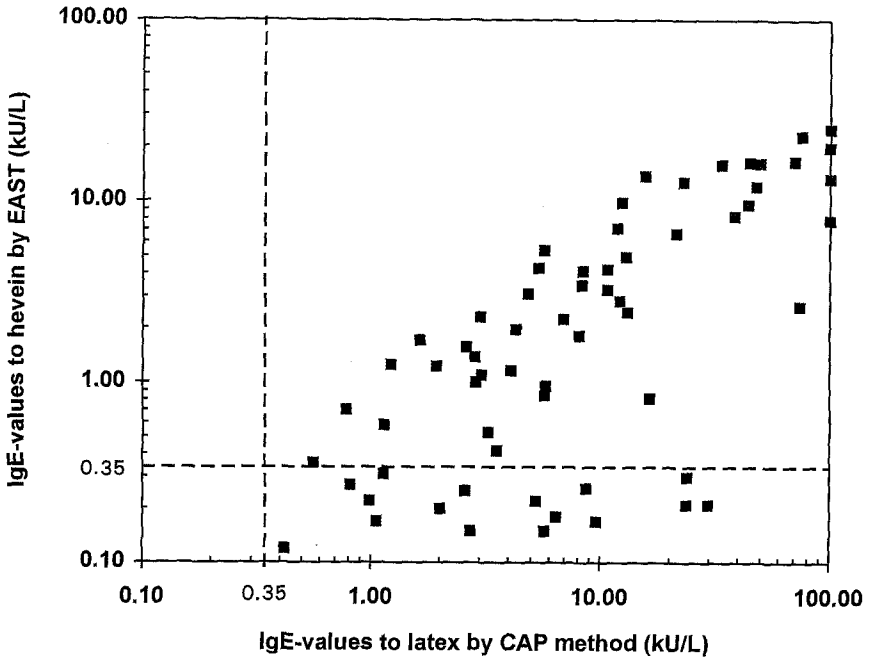

FIG. 2. Comparison of IgE antibodies to latex and hevein in sera of patients allergic to latex (health care workers, $n = 64$). IgE values to latex were determined by the CAP system with commercial latex allergen (Pharmacia), and IgE values to hevein were measured by EAST with hevein-coupled disks. Values below 0.35 kU/L are considered negative.

TABLE II. IgE antibodies to latex and hevein in sera of patients with spina bifida

Subject No.	Sex	Age (yr)	Serum total IgE (kU/L)	IgE antibodies to	
				Latex* (kU/L)	Heveint (kU/L)
SB-1	M	5	784	>100	12.9
SB-2	M	13	347	41.9	<0.35
SB-3	M	5	105	12.3	<0.35
SB-4	F	16	546	>100	<0.35
SB-5	M	12	1256	49.6	0.77
SB-6	F	16	219	1.77	0.53
SB-7	F	12	130	31.6	<0.35
SB-8	F	17	35	1.02	<0.35
SB-9	M	11	274	>100	<0.35
SB-10	F	13	31	15.8	<0.35
SB-11	F	18	272	18.6	<0.35

*Results obtained by CAP method with commercial latex allergen (Pharmacia).
†Results determined by EAST with hevein-coupled disks.

to hevein. One serum sample and one serum pool from five patients allergic to latex were used in this test. Inhibition was shown to be dose-dependent, and the results are summarized in Fig. 3. The IgE binding in both sera was completely inhibited by 5 μg of hevein. Fifty percent inhibition was achieved by less than 0.05 μg of hevein peptide.

Because the determined concentrations of IgE antibodies directed against latex and hevein in our studies were achieved by different methods (CAP and EAST), we could not make a direct comparison of these IgE values to show the binding capability of hevein to the serum IgE antibodies. However, we did find that in about 20 sera from patients in group 1, the values of the IgE antibody directed against hevein corresponded to their IgE values to latex allergens (Fig. 2). To determine to what extent the IgE antibodies to latex measured by the CAP system can be inhibited by preincubation of serum samples with hevein, 11 of these sera were randomly selected for a RAST inhibition assay in the CAP system. Soluble hevein (0.5 mg/ml) was used as an inhibitor, and the patient's serum was incubated with