Analysis of the major epitope of the α2 chain of bovine type I collagen in children with bovine gelatin allergy

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Background: Anaphylaxis to measles, mumps, and rubella vaccines has been reported. It has been found that most of these reactions to live vaccines are caused by type I allergy with the bovine gelatin present in the vaccines as an allergen. Gelatin mainly includes denatured type I collagen, which consists of α1 and α2 chains. We previously reported that allergic reactions to gelatin are caused by the type I collagen α2 (α2[I]) chain.
Objective: To aid in the development of gelatin that has little or no allergenicity in human subjects, we investigated epitopes of bovine α2(I) chain with use of IgE in gelatin-sensitive children.
Methods: Serum samples were collected from 15 patients who had systemic allergic reactions to vaccines and high levels of specific IgE to bovine gelatin. Eleven overlapping recombinant proteins that cover bovine α2(I) were prepared with a bacterial expression vector. We examined IgE reactivity to these recombinant proteins by means of ELISA. Fifteen peptides covering a major reactive recombinant protein were synthesized. The IgE-reacting epitope was identified by means of IgE-ELISA inhibition with these synthetic peptides and pooled serum from the patients.
Results: We found that of the 15 patients, 13 showed IgE reactivity to a recombinant protein (no. 3) spanning the central region of the collagenous domain (^{418}Gly-^{662}Pro). Furthermore, all 13 patients showed IgE reactivity to the 4-kd recombinant protein (no. 3a) spanning the region from ^{461}Pro to ^{500}Glu. In IgE-ELISA inhibition we found that a minimum IgE epitope of gelatin allergen was composed of the 10-amino-acid sequence ^{485}Ile-Pro-Gly-Glu-Phe-Gly-Leu-Pro-Gly-Pro^{494}. This sequence is not observed in the human type I collagen α1 and α2 chains, nor is it found in the bovine type I collagen α1 chain.
Conclusions: We found that Ile-Pro-Gly-Glu-Phe-Gly-Leu-Pro-Gly-Pro is a major IgE epitope of the α2 chain of bovine type I collagen in patients with gelatin allergy. The degree of anaphylaxis to gelatin in vaccines might be reduced by digestion of this IgE-binding site in gelatin. (J Allergy Clin Immunol 2002;110:652-7.)

Key words: *Allergen, anaphylaxis, gelatin, IgE, epitope*

Anaphylaxis to measles, mumps, and rubella vaccines and to the combined measles-mumps-rubella (MMR) vaccine has been reported.[1-4] Keslo et al[5] reported that a child with anaphylaxis to gelatin-containing MMR vaccine had IgE to gelatin. Also, in Japan most children who had systemic immediate-type reactions, including anaphylaxis, to gelatin-containing vaccines had antigelatin IgE.[6-8] Recently, we found that bovine gelatin in erythropoietin products and suppositories caused anaphylaxis.[9,10] Furthermore, it has been reported that gelatin causes food allergy.[11,12] It has been suggested that anaphylaxis is caused by gelatin present in these items.

Gelatin is widely distributed in all multicellular animals and has long been considered to be nonimmunogenic and to cause no immune reactions in human subjects. Therefore gelatin has been generally used as a stabilizer in vaccines. The gelatin obtained from bone and skin consists mainly of denatured type I collagen.[13] We found that all children with anaphylaxis to vaccines who had specific IgE to gelatin also showed specific reactions to purified denatured type I collagen.[14] The collagen molecule is a heterotrimer composed of 2 α1 chains and one α2 chain.[13] In our previous study IgE from children with gelatin allergy strongly reacted with the bovine type I collagen α2 (α2[I]) chain, and thus we found that the α2(I) chain is a major allergen in gelatin allergy.[14] Furthermore, we reported that the amino acid sequence homology (98%) between α1 chains of human and bovine type I collagen is higher than that (93%) between α2 chains.[13,15,16] These homology data support that allergic epitopes exist in the α2 chain.

To aid in the development of gelatin with little or no allergenicity in human subjects, we analyzed the precise IgE-binding sites in the bovine α2(I) chain. The degree of anaphylaxis caused by gelatin in vaccines might be reduced by means of enzymatic digestion of the IgE-binding sites in gelatin.

METHODS

Subjects

Physicians and vaccine manufacturers submitted serum samples from 15 patients (mean ± SD age, 23 ± 10 months) with systemic immediate-type reactions, including anaphylaxis to live vaccines, to the Japan National Institute of Infectious Diseases. As a negative control, serum samples from 26 children (mean ± SD age, 28 ± 12 months) who had no reaction to measles vaccine containing gelatin (1 mg per dose) were also collected.

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Abbreviations used
α2(I) chain: Type I collagen α2 chain
FU: Fluorescence unit
MMR: Combined measles-mumps-rubella

Gelatin and collagen

Gelatin for ELISA was prepared as described previously.[12] Bovine type I collagen was prepared from skin dermis by means of 0.5 mol/L acetic acid extraction and purified by means of differential salt precipitation.[17]

Construction, expression, and purification of recombinant proteins in bovine α2(I) chain

A schematic presentation of the recombinant proteins used in this study is shown in Fig 1.[15,18] Five recombinant proteins that covered the entire α2(I) chain were designed as follows: protein 1, residue number ^{1N}Gln-^{9N}Pro/^{1}Gly-^{224}Leu; protein 2, residue number ^{213}Asn-^{417}Ala; protein 3, residue number ^{418}Gly-^{662}Pro; protein 4, residue number ^{663}Val-^{890}Glu); and protein 5, residue number ^{879}Val-^{1014}Ser/^{1C}Gly-^{15C}Ala (Fig 1, *A*). The numbers of the amino acid sequences represent the position number from the N-terminus of the pepsin-treated type I collagen.[18] In addition, recombinant proteins 3a (residue number ^{418}Gly-^{510}Ala), 3b (residue number ^{491}Leu-^{588}Pro), 3c (residue number ^{572}Leu-^{675}Ser), 3a-1 (residue number ^{408}Lys-^{444}Pro), 3a-2 (residue number ^{431}Ala-^{475}Gly), and 3a-3 (residue number ^{461}Pro-^{500}Glu) were also designed (Fig 1, *B*). The cDNA fragments encoding these recombinant proteins were amplified by means of PCR from full-length bovine pro-α2(I) chain cDNA.[15] Each forward primer contained a *Bam*HI site and methio-nine codon (GGATCCATG) at the 5′ end, and each reverse primer contained a stop codon and *Hin*dIII site (AAGCTTTTA) at the 3′ end.[19] The resulting PCR products were cloned into the *Bam*HI/*Hin*dIII sites of the expression vector pRSET (Invitrogen, San Diego, Calif). The vector contains a polyhistidine tag sequence and 25 amino acids, with an enterokinase cleavage site at the N-terminus to facilitate purification and detection. The plasmid constructs encoding recombinant proteins of bovine α2(I) chain transformed into BL21 for expression (Novagen, Madison, Wis). Positive clones were sequenced on both strands. An overnight bacterial culture was used to inoculate a 500-mL culture in LB medium. This culture was grown for approximately 4 hours until the cells reached an OD_{600} of 0.6. Then protein expression was induced for 4 hours by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mmol/L. A purification procedure for His-tagged recombinant proteins from bacterial cells was performed according to the manufacturer's instructions (Novagen). Briefly, bacterial cells were collected by means of low–speed centrifugation, followed by lysis with 6 mol/L urea. The lysate was then applied to a His-Bind Resin column. After washing with 6 mol/L urea containing 10 mmol/L imid-azole, recombinant protein was eluted with 6 mol/L urea containing 250 mmol/L imidazole. Finally, recombinant proteins were dialyzed against water and lyophilized. Recombinant proteins were digested with cyanogen bromide, blotted onto PVDF membrane after separation with SDS-PAGE,[20] and sequenced with an LF3000 Peptide Sequencer (Beckman Instruments, Inc, Fullerton, Calif) to confirm N-terminal amino acid sequences.

Peptides

Fifteen overlapping peptides spanning the region from ^{461}Pro to ^{500}Glu of the bovine α2(I) chain were synthesized by Sigma Genosis Japan on the basis of the primary sequence (Table I).[15]

Measurement of specific IgE antibody to bovine type I collagen and recombinant proteins

Specific IgE antibody levels to denatured bovine type I collagen and recombinant proteins were determined by using a fluorometric ELISA, as described previously.[12] Microplates were coated with denatured collagen or recombinant protein (5 μg/mL). After incubation overnight at 4°C, 0.1 mL of the serum sample was added to the wells. Each patient's serum was diluted to approximately 1 Ua/mL as the level of specific IgE to gelatin. Negative sera were diluted 1:10. After incubation for 3 hours at room temperature, anti-human IgE antibody conjugated with β-D-galactosidase (Pharmacia, Uppsala, Sweden) was added. The enzyme reaction substrate was 0.1 mmol/L 4-methylumbelliferyl-β-D-galactoside (Sigma Chemicals, St Louis, Mo). The fluorescence unit (FU) of the reaction product, 4-methylumbelliferone, was measured on a fluorometric microplate reader (Fluoroskan; Flow Laboratory, McLean, Va) with 1 FU corresponding to 1 pmol of 4-methylumbelliferone per well.

Identification of the minimum IgE epitope

The minimum epitope in a recombinant protein (no. 3a-3) was identified by using a fluorometric IgE-ELISA inhibition assay.[21] Briefly, the recombinant protein was adsorbed to microplate wells. The pooled serum from 3 patients (nos. 1, 2, and 6) who showed a strong reactivity to 3a-3 protein was used. There was enough volume of these sera to be used in ELISA inhibition. This pooled serum was preincubated with each peptide from nos. 3a-3-P1 to 3a-3-P5-11 (50 μg/mL) as an inhibitor (Table I), respectively, and the mixtures were added to the wells. After 3 hours of incubation at room temperature, anti-human IgE antibody conjugated with β-D-galactosidase was added. The subsequent procedures were the same as those in the fluorometric ELISA described above. The percentage of inhibition was calculated as follows:

$$\left(1 - \frac{(\text{FU in the presence of inhibitor})}{(\text{FU in the absence of inhibitor})}\right) \times 100.$$

RESULTS

IgE reactivity of overlapping recombinant proteins spanning the bovine α2(I) chain

We measured specific IgE to the recombinant proteins in patients with allergic reactions to vaccines. As shown in Table II, 13 of 15 patients showed IgE reactivity to recombinant protein 3, 6 patients to protein 2, 3 patients to protein 4, and 1 patient to protein 5. These data suggested that more than 4 IgE-binding epitopes are present in the bovine α2(I) chain and that the major epitope of the bovine α2(I) chain is localized in recombinant protein 3. None of the sera from these patients reacted to another recombinant protein derived from pRSET vector alone (data not shown). Furthermore, none of the 26 control children had specific IgE to type I collagen or to these recombinant proteins (<40 FU, data not shown).

To further analyze the major epitope identified above, we examined the IgE reactivity of 3 recombinant proteins, 3a, 3b, and 3c, covering protein 3. Of the 13 patients, all had IgE reactivity to recombinant protein 3a, and only one patient had high IgE reactivity to recombinant protein 3b (Fig 2). Five patients had weak IgE activity to recombinant protein 3c.

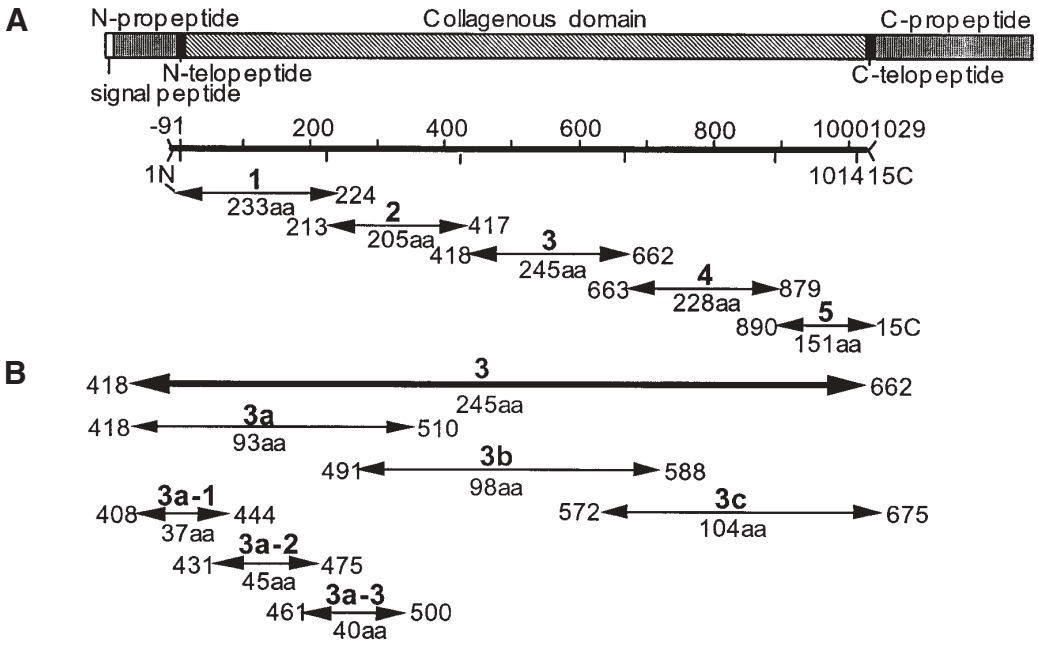

FIG 1. Schematic presentation of recombinant proteins of bovine pro-α2(I) chain: **A,** 5 recombinant proteins covering the collagenous domain and both telopeptides; **B,** 6 recombinant proteins covering the ^{418}Gly–^{675}Ser and ^{408}Lys–^{500}Glu residues of the α2(I) chain. The numbers above the amino acid sequences represent the number from the N-terminus of the pepsin-treated type I collagen.[18] The first amino acid position of the collagenous domain corresponded to the ^{89}Gly residue of the pro-α2 chain.[15] *1N-9N,* N-telopeptide; *1C-15C,* C-telopeptide.

TABLE I. Inhibition of IgE binding to recombinant protein 3a-3

Inhibitor Peptide	Amino acid sequence	Inhibition IgE to 3a-3*
3a-3-P1	461 PPGFQGLPGPAGTAGEAGKP 480	–
3a-3-P2	471 AGTAGEAGKPGERGIPGEFG 490	–
3a-3-P3	481 GERGIPGEFGLPGPAGARGE 500	+
3a-3-P4	RGIPGEFGLPGPAGA	+
3a-3-P5-1	IPGEFGLPGPA	+
3a-3-P5-2	IPGEFGLPGP	+
3a-3-P5-3	IPGEFGLPG	–
3a-3-P5-4	IPGEFGLP	–
3a-3-P5-5	IPGEFGL	–
3a-3-P5-6	IPGEFG	–
3a-3-P5-7	PGEFGLPGPA	–
3a-3-P5-8	GEFGLPGPA	–
3a-3-P5-9	EFGLPGPA	–
3a-3-P5-10	FGLPGPA	–
3a-3-P5-11	GLPGPA	–
Recombinant protein		
3a-3	461 PPGFQGLPGPAGTAGEAGKPGERGIPGEFGLPGPAGARGE 500	

*In pooled serum each child's serum was diluted to the same levels of specific IgE to 3a-3. The FU levels in wells that do not contain each inhibitor ranged from 1035 to 1257.
+, Greater than 80% inhibition; –, less than 10% inhibition.

Next we produced 3 additional recombinant proteins (nos. 3a-1, 3a-2, and 3a-3) covering the 3a protein and examined the IgE reactivity to the 13 patients' sera (Fig 3). All 13 sera had higher IgE reactivity to recombinant protein 3a-3 than to recombinant proteins 3a-1 and 3a-2. These results clearly show that a major IgE-binding epitope for allergic reactions to gelatin is localized in the central region of the bovine α2(I) chain corresponding to a 40-amino-acid sequence (^{461}Pro-^{500}Glu).

Identification of the minimum IgE epitope

To determine the minimum epitope of the recombinant protein (no. 3a-3) of the bovine α2(I) chain, we analyzed the IgE reactivity by using synthetic peptides covering

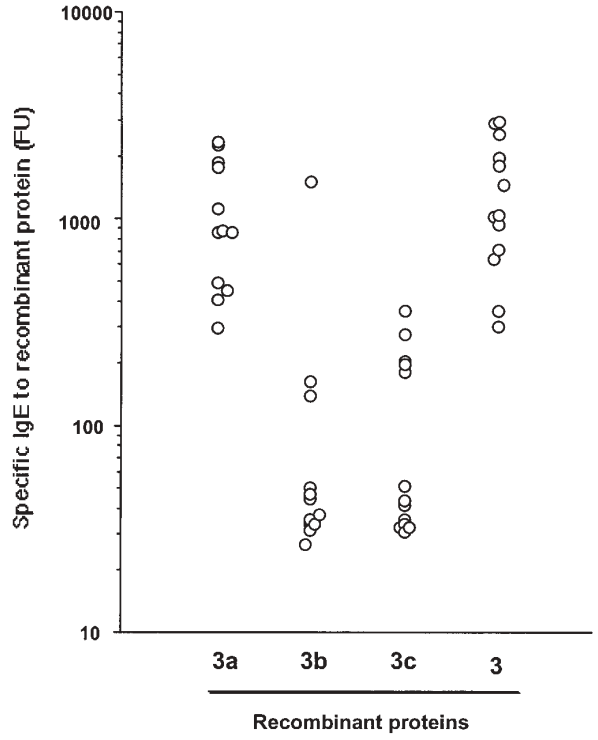

FIG 2. IgE reactivity to 3 overlapping recombinant proteins covering residues ^{418}Gly-^{675}Ser of the bovine α2(I) chain. *3a,* ^{418}Gly-^{510}Ala; *3b,* ^{491}Leu-^{588}Pro; *3c,* ^{572}Leu-^{675}Ser.

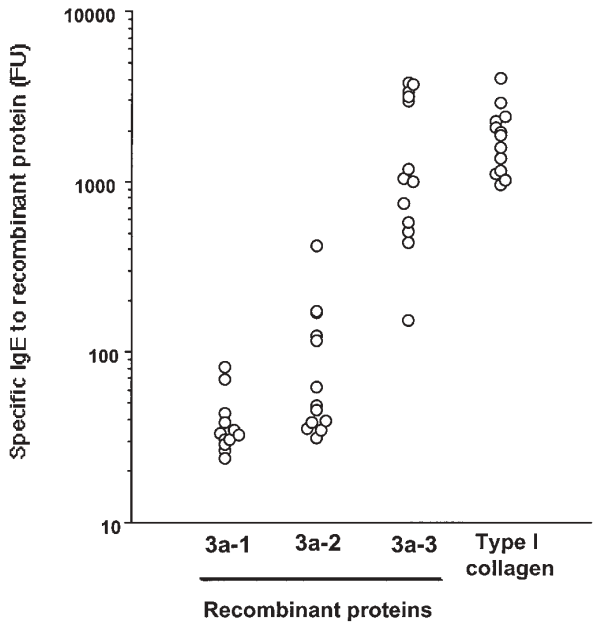

FIG 3. IgE reactivity to 3 overlapping recombinant proteins covering residues ^{408}Lys-^{500}Glu of the bovine α2(I) chain. *3a-1,* ^{408}Lys-^{444}Pro; *3a-2,* ^{431}Ala-^{475}Gly; *3a-3,* ^{461}Pro-^{500}Glu.

the recombinant protein (Table I). It was difficult to immobilize the shorter peptides to the wells of the microplate, and therefore IgE-ELISA inhibition was performed for this assay with pooled serum from 3 patients

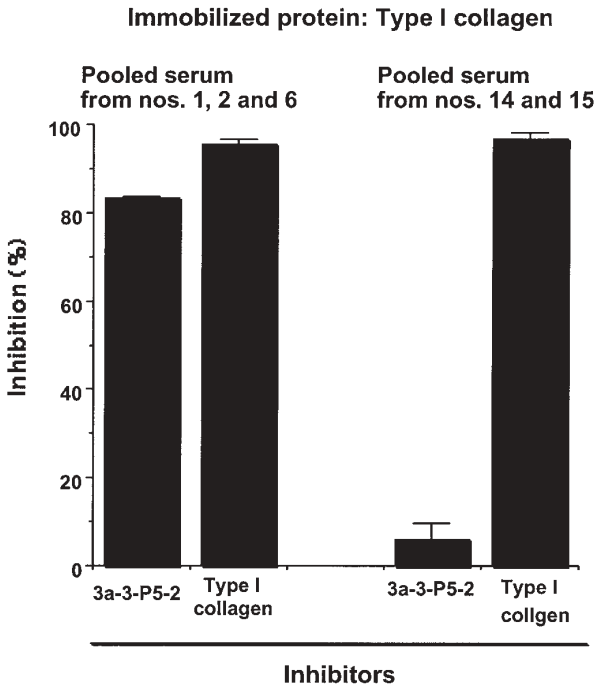

FIG 4. Inhibition of specific IgE to type I collagen by the minimum epitope. In pooled serum each child's serum was diluted to the same levels of specific IgE to type I collagen. The pooled serum from 3 patients (nos. 1, 2, and 6) who showed strong IgE reactivity to protein 3 was used in the inhibition for type I collagen. The FU level in wells that do not contain inhibitor was 1128. The pooled serum from 2 patients (nos. 14 and 15) who weakly reacted to protein 3 was used in the inhibition assay to type I collagen. The FU level in wells that do not contain inhibitor was 1346.

(nos. 1, 2, and 6). First, we prepared 3 synthetic peptides (nos. 3a-3-P1 to 3a-2-P3) overlapping protein 3a-3 for inhibitors (Table I). Only peptide 3a-3-P3 inhibited IgE binding to the immobilized protein 3a-3 (Table I). In contrast, peptides 3a-3-P1 and 3a-2-P2 did not inhibit IgE binding to protein 3a-3. Second, we prepared 2 synthetic peptides (nos. 3a-3-P4 and 3a-3-P5-1) that lacked 2 or 3 amino acid residues from peptide 3a-3-P3 (Table I). These peptides inhibited IgE binding to the immobilized protein 3a-3 (Table I).

Furthermore, to determine the minimum IgE-binding epitope, we prepared shorter peptides (3a-3-P5-2 to 3a-3-p5-11, Table II) with a few amino acid residues deleted from peptide 3a-3-P5-1 and tested them with the inhibition assay (Table I). Peptides 3a-3-P-5-1 and 3a-3-P-5-2 inhibited IgE binding to protein 3a-3, but other peptides did not inhibit IgE binding (Table I). We found that the core of the IgE-binding epitope of the bovine α2(I) chain is ^{485}Ile-Pro-Gly-Glu-Phe-Gly-Leu-Pro-Gly-Pro^{494}.

Specific IgE to type I collagen in pooled serum from those patients (nos. 1, 2, and 6) who strongly reacted to protein 3 was markedly inhibited by the minimum epitope (3a-3-P5-2, Fig 4). In contrast, specific IgE to type I collagen in pooled serum from 2 patients (nos. 14 and 15) who weakly reacted to protein 3 was not inhibited by the epitope (Fig 4).

TABLE II. Reactivity of antigelatin IgE to recombinant proteins for α2(I)

	IgE reactivity to:					
Patient no.	**Type I collagen***	**No. 1, 1N-224**	**No. 2, 213-417**	**No. 3, 418-662**	**No. 4, 663-890**	**No. 5, 879-15C**
1	2800*	–†	–	+++	–	±
2	2116	–	–	+++	±	±
3	1494	–	–	+++	–	–
4	834	–	–	+++	+	–
5	1833	–	–	+++	–	–
6	2455	–	±	+++	–	–
7	1773	–	±	+++	–	±
8	868	–	±	+++	–	±
9	1019	–	+++	++	–	–
10	2600	–	–	+	++	–
11	1152	–	+	+	±	±
12	1820	–	+	+	+	–
13	814	–	+	+	–	–
14	772	–	+++	±	+	+++
15	2179	–	++	–	–	–

*Levels of IgE to type I collagen are expressed as FU.
†Results are expressed as a percentage of the binding of IgE to each recombinant protein compared with that to bovine type I collagen: +++ ≥75%, 75% > ++ ≥50%, 50% > + ≥25%, 25% > ± ≥5%, – <5%.

DISCUSSION

We previously reported that the repeated helical epitopes of a defined 10-amino-acid sequence in human type III collagen were identified by means of mouse mAb.[17] In this study, however, we analyzed the precise IgE-binding sites in the bovine α2(I) chain by using recombinant proteins and synthetic peptides. Because commercially available gelatin consists of denatured type I collagen in a linear construction, it is possible that the IgE-binding epitope of the bovine α2(I) chain might be a sequential epitope.

In this study we found that many patients had IgE reactivity to recombinant protein 3. We also found that a major IgE-binding epitope might be located in the central region of the bovine α2(I) chain. Finally, we determined a 10-amino-acid peptide, ^{485}Ile-Pro-Gly-Glu-Phe-Gly-Leu-Pro-Gly-Pro^{494}, which corresponds to a major IgE-binding epitope that is responsible for allergic reactions to gelatin. When we compared the bovine 10-amino-acid sequence with the corresponding sequence in human subjects, we found that ^{485}Ile was replaced by Leu, and ^{486}Pro was replaced by His. A homology search of BLAST found no sequence matching to that of collagens in other species.

The results of the present study suggest that some minor IgE epitopes, but not the major epitope, might exist in the bovine α2(I) chain. We observed that of the 15 patients in the study, 6 had IgE reactivity to protein 2, and a minor population of the patients in this study had IgE reactivity to proteins 4 and 5. These results suggested a polymorphism of the IgE reactivity to gelatin in human patients. The reactivity to collagen was probably influenced by unknown genetic factors. It might be worthwhile to analyze the IgE-binding epitopes to understand the cause of gelatin allergy.

Collagen is biosynthesized as procollagen, secreted from cells with triple helical structure, and processed into collagen molecules after cleavage of the N- and C-propeptides. Some specifically modified amino acids, such as hydroxyproline, hydroxylysine, and glycosylated hydroxylysine, are also contained in collagen. Amino acids of the recombinant proteins prepared by the bacterial expression system were not modified. In this study we found that all patients had IgE reactivity to recombinant proteins 2 to 5 covering the bovine α2(I) chain. We also found that specific IgE to type I collagen in pooled serum from patients who showed strong reactivity to recombinant protein 3 was greatly inhibited by the synthetic peptide that consists of a nonmodified amino acid, such as proline. It is suggested that this IgE-binding site is not dependent on these modified amino acids.

Anaphylaxis to gelatin-containing vaccines, drugs, and food has been reported, and it has been suggested that the anaphylaxis is caused by allergic reactions to the gelatin present in these items.[5-12] In the United States between 1990 and 1995, 33 cases of anaphylactic reactions occurred after MMR vaccination. Similarly, in Japan between 1994 and 1996, 37 cases of life-threatening anaphylaxis to gelatin-containing measles, mumps, and rubella vaccines were reported.[22] Gelatin was removed from these live vaccines used in Japan. Human serum albumin has been substituted for gelatin in some vaccines. However, it has been reported that human parvovirus B19 DNA was detected in human serum albumin as a blood product.[23] Thus we need to develop gelatin with little or no allergenicity. In this study we found that there is a major IgE-binding epitope in the bovine α2(I) chain. We found that the digestion of bovine type I collagen with some proteases reduced IgE reactivity to the bovine type I collagen (data not shown). In the future our

data on analysis of an IgE-binding epitope in the bovine α2(I) chain might help in the development of gelatin with little or no allergenicity in human patients. The degree of anaphylaxis to gelatin in vaccines might be reduced by means of digestion of the IgE-binding sites in gelatin.

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