



Case Report

Antigen-specific free immunoglobulin light-chain antibodies: Could it be a new diagnostic marker for patients with allergy?

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Abstract

Objectives: Human free immunoglobulin light chains (FLCs) have long been considered as nonmeaningful spillover remnants from the process of immunoglobulin production; however, recent findings suggest that the antibody activity of FLCs may be involved in the pathology of allergic responses. We therefore assessed the antigen-binding ability of FLCs to evaluate their usefulness as diagnostic markers for patients with allergy.

Design and methods: FLCs were separated from the serum samples of patients seropositive against cedar pollen and mice immunized with bovine serum albumin and 1-fluoro-2,4-dinitrobenzene by ultrafiltration and protein G absorption. A sensitive immunoassay confirmed the absence of any IgG in the separated FLC fractions from the human serum samples.

Results: Solid-phase immunoassay for cedar pollen showed that none of the human serum samples possessed any antibody activity against the antigen after the removal of whole immunoglobulins. Furthermore, while the immunized mice also showed high antibody titers against the antigens, but the serum specimens showed no residual antibody activity against the antigens after the FLCs were separated from the whole immunoglobulins.

Conclusions: The results of the present study suggested that the FLC fractions may possess little or no antigen-binding activity, and that therefore, they may not serve as useful diagnostic markers in patients with allergy.

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Keywords: Free immunoglobulin light chain; Allergy; Antibody; Immunoassay

Introduction

Human immunoglobulin kappa and lambda light chains, protein components of intact immunoglobulins, are detected in their free form in serum and urine samples because they are produced in excess over heavy chains by plasma cells [1,2]; it is noteworthy that free light chains (FLCs) account for approximately two-fifths of the total light-chain production. However, the biological functions of FLCs remain unclear, and they have long been

considered as nonmeaningful spillover remnants from the process of immunoglobulin production.

IgE mediates the allergic inflammatory responses of mast cells in patients with atopic dermatitis. This is the most prevalent form of allergic diseases, accounting for about 70–85% of patients with allergies [3]. On the other hand, 15–30% of the population show neither elevated levels of total IgE nor the presence of antigen-specific IgE and are referred to as having non-IgE allergy [4]. Recently, Redegeld et al. [5,6] reported that kappa-type free light chains separated from trinitrophenol-specific IgG₁ or oxazolone-specific IgG₁ monoclonal antibodies (mAbs) can elicit immediate hypersensitivity-like responses. They also suggested that antigen-specific FLCs were involved in the pathology of allergic diseases in both patients with and without the presence of antigen-specific IgE. This report prompted us to investigate whether antigen-specific FLCs might serve as new diagnostic markers for patients

Abbreviations: BSA, bovine serum albumin; DNFB, 1-fluoro-2, 4-dinitrobenzene; DNP, 2,4-dinitrophenol; FLC, free immunoglobulin light chain; HRP, horseradish peroxidase; HSA, human serum albumin; mAb, monoclonal antibody.

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with allergy, especially those without elevation of specific IgE titers in serum. In this study, in order to examine the potential of FLCs as diagnostic markers in patients with allergy, we assessed the antigen-binding ability of intact FLCs, i.e., those originally present in their free form, in specimens obtained from immunized mice and patients with allergy.

Subjects and methods

Subjects

Serum samples were collected at the first medical examination, in February 2004, from outpatients of Asahi General Hospital (Chiba, Japan) who were suffering from allergy to cedar pollen. None of the patients were receiving any medication at the time of sampling. Sera were assayed for specific IgEs to identify allergen (s) using UniCAP system (Pharmacia Diagnostics, Woerden, Sweden). The first ten sera were found to be seropositive for cedar pollen (class 2: $n=5$, 0.7 to 3.5 kU/l; class 3: $n=4$, 3.5 to 17.8 kU/l; class 4: $n=1$, 17.5–50 kU/l) were used for this study. The serum samples were numerically coded and tested in a blind manner in accordance with the guidelines of the Japanese Society of Laboratory Medicine (Tokyo, Japan) for the use of residual samples for methodological study, and then stored at -80°C until use.

FLC assays

The concentrations of human FLC kappa and FLC lambda were determined by immunoassays, as described previously [7], with a slight modification [8], using purified intact FLCs from the urine as the standards [9]. We also quantified human serum IgG by an in-house immunoassay [10].

Mice and immunization

Eight-week-old female BALB/c mice (Clea Japan Inc., Tokyo, Japan) were divided into three groups of five mice ($n = 5$) each: Group 1, consisting of mice immunized by intraperitoneal administration of human serum albumin (HSA) (Sigma, St. Louis, MO), as described previously [7]; group 2, consisting of mice sensitized by painting their shaved backs with 20 μl of 0.5% 1-fluoro-2,4-dinitrobenzene (DNFB; Sigma) in acetone/olive oil (4:1) and 10 μl of 0.2% DNFB at 2-week intervals for 2 months; group 3, consisting of non-immunized control mice. Urine and serum samples of the mice were then collected, and all the specimens were stored at -80°C until use. The serum antibody titers were determined based on the ED_{50} in sigmoidal dose–response curves. Mouse serum IgE was quantified by a Mouse IgE EIA kit (Yamasa corp.). All the animals were cared for in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, Tokyo, Japan.

Separation of FLCs

FLCs were separated from the whole immunoglobulins present in the serum as follows: Serum samples were diluted 10-fold with phosphate-buffered saline containing 1% bovine serum albumin

(BSA) and filtered in a Microcon[®] YM-100 100-kDa NMWL centrifugal filter unit (Millipore, Billerica, MT). The filtered fractions were mixed with protein G–sepharose (Pharmacia, Uppsala, Sweden) for absorption of any residual IgG [10]. After the separation, all the fractions were diluted or concentrated to obtain a final concentration of the FLCs of 1 $\mu\text{g}/\text{ml}$. The concentrations of the FLCs in the mouse specimens were not adjusted because of the lack of suitable quantitative methods for such adjustment.

ELISAs for cedar pollen-specific human FLCs

Polystyrene 96-well microtiter plate were coated with an extract of Japanese cedar pollen [11] at 0.1 $\mu\text{g}/\text{well}$. One hundred microliters of specimens containing 1 mg/l of the FLCs were applied to the wells and incubated for 24 h. Horseradish peroxidase (HRP)-labeled anti-human kappa and lambda light-chain mAbs (Yamasa, Chiba, Japan) were added as the secondary antibodies at a concentration of 1 $\mu\text{g}/\text{ml}$. The antibody reactivity of the specimens is expressed only in terms of the absorbance; no quantitative evaluation was performed.

Anti-HSA and DNP-BSA assays for mouse FLCs

To examine antigen-specific mouse FLC antibodies, we conducted solid-phase immunoassays as follows: plates were coated with HSA and DNP-BSA at 0.1 $\mu\text{g}/\text{well}$ each. Then the diluted samples were added to the plates, followed by incubation for 24 h. HRP-labeled anti-mouse kappa and lambda light-chain mAbs (Yamasa) were used at a concentration of 1 $\mu\text{g}/\text{ml}$ as the secondary antibodies.

Results

Separation and recovery of the immunoglobulin FLCs

We recovered FLCs kappa and lambda on the order of milligrams from the human serum samples subjected to centrifugal filtration. The human FLCs showed $\sim 0.12\%$ cross-reactivity with light chain structures included in whole immunoglobulins, thus, the serum FLCs could not be measured accurately. After the filtration,

Table 1
Concentrations of serum IgG and FLCs after filtration and IgG adsorption

Analytes		Fractions of the serum specimens		
		Serum	Filtration ^a	Absorption ^b
IgG	g/l	13.5 $\pm$ 3.9	0.12 $\pm$ 0.06	Not detectable
FLC kappa	mg/l	41.8 $\pm$ 12.9 ^c	1.92 $\pm$ 0.71	0.80 $\pm$ 0.31
FLC lambda	mg/l	57.5 $\pm$ 36.7 ^c	3.86 $\pm$ 1.99	1.22 $\pm$ 0.59

^a Ten serum specimens from patients suffering from allergy to Japanese cedar pollen were filtered through a 100-kDa NMWL filter unit.

^b The filtered fractions were then adsorbed onto a protein G sepharose column. The concentrations of IgG and the FLCs were determined by specific monoclonal antibody-based enzyme immunoassays.

^c In FLC kappa and FLC lambda assays, the cross-reactivity of light chains bound to whole immunoglobulins were 0.11% and 0.12%, respectively; thus, these data probably represent considerably overestimated values [7,10]. Data are expressed as means $\pm$ SD.

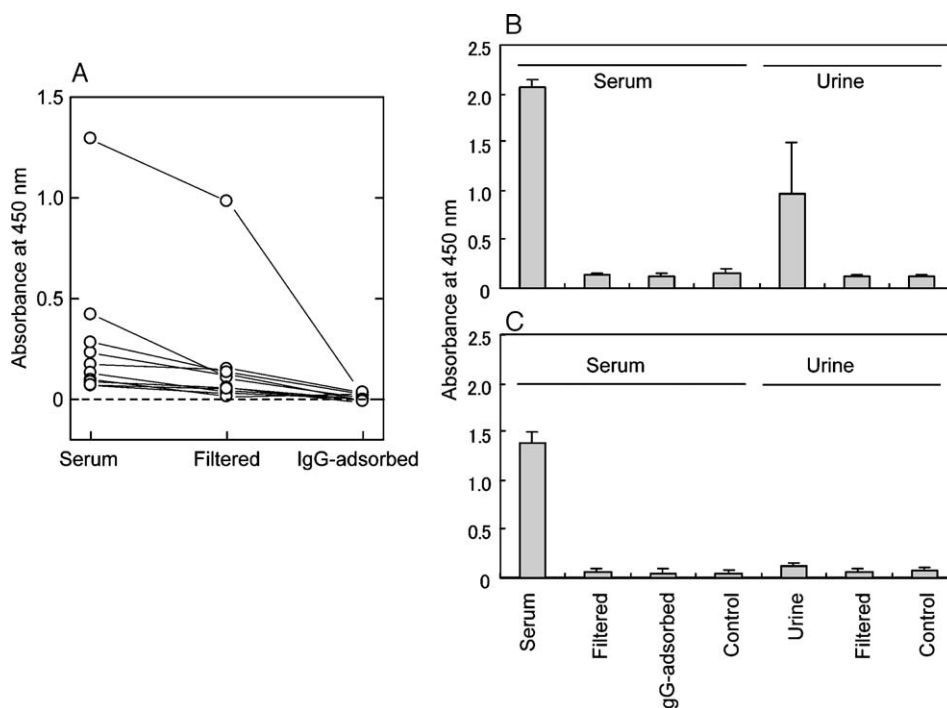


Fig. 1. Antigen-binding activity of free immunoglobulin light chains (FLCs). (A) FLCs in ten serum specimens obtained from patients, who were seropositive for cedar pollen-specific IgE, were successively separated in a 100-kDa NMWL ultracentrifuge filter unit and by protein G sepharose gel adsorption. The sera and the fractions were applied to ELISA plates coated with cedar pollen extract at a concentration of 0.1 $\mu\text{g}/\text{well}$ each. The antigen-binding activity was evaluated based on the absorbance values. Serum and urine specimens from HSA-immunized mice (B) and DNFB-sensitized mice (C) were assayed at 200-fold and 4-fold dilutions, respectively.

the contamination rate by intact IgG was 0.009% (Table 1). The residual whole immunoglobulins were completely adsorbed onto a protein G–sepharose gel, and no IgG was detected in the specimens by a sensitive IgG immunoassay after the adsorption (<0.61 mg/l). We did not estimate the recovery or contamination rates in the separation steps of the mouse FLCs, because no suitable assay methods are available for the quantification.

Anti-allergen immunoreactivity of the human FLCs

The mean absorbance of the human serum samples was 0.29 (± 0.37 SD), but it decreased by approximately half [0.17 (± 0.14 SD)] after the separation through the filter unit (Fig. 1A). After the removal of IgG using protein G, there were no differences in the absorbance between separated human FLCs and the assay background, even though the FLCs (1 $\mu\text{g}/\text{ml}$) were applied in substantial concentrations for the ELISA. These results showed that, in patients seropositive against cedar pollen, intact FLCs in the sera possessed no reactivity with the antigen.

Anti-immunogen immunoreactivity of mouse FLCs

Serum samples from the immunized mice showed high antibody titers against the antigens, ranging from 1:125,000 to 1:625,000 for HSA, and 1:2500 to 1:12,500 for DNP-BSA. The serum IgE concentrations in group 1 and group 2 were about 13-fold and 5-fold greater than that in group 3: group 1 (HSA-immunized), 56.9 (± 49.8) mg/l; group 2 (DNFB-immunized), 20.6 (± 8.9) mg/l; group 3 (non-immunized control), 4.3 (± 2.4)

mg/l. The absorbances in the respective immunoassays at 200-fold dilution were 2.08 (± 0.06) for HSA and 1.39 (± 0.10) for DNP-BSA (Figs. 1B and C). However, after centrifugation in the filter unit, mouse serum samples showed no antigen-binding activity, indicating that there were no antigen-specific FLCs in the samples. We also tested the urine specimens of the immunized mice. The antibody activity of the FLCs in the urine were easier to detect than those in the serum, because FLCs have low molecular masses, readily pass through the glomerular filter, and are excreted into the urine. Urine specimens from mice immunized with HSA also contained substantial antibody-activity while those immunized DNFB had only a little activity (Figs. 1B and C). Similar to the serum samples, the urine samples from the mice in group 1 and group 2 showed loss of their antigen-binding ability after the filtration.

Discussion

Recent evidence has suggested that antigen-specific FLCs are involved in the pathogenesis of allergic responses and may potentially serve as novel diagnostic markers in patients with allergic diseases [5,6]. We therefore examined the antigen-binding activity of FLC fractions separated from the serum samples of patients with cedar pollen allergy and mice immunized with HSA and DNFB, but the results of the solid-phase immunoassays revealed that the FLC fractions from the samples had no detectable antigen-binding activity. Our findings suggest that the detection of antigen-binding ability of intact FLCs may not serve as diagnostic markers for patients with allergy.

Earlier studies have shown that the antigen-binding ability of immunoglobulins is mainly dependent on their heavy chain variable region [12,13]. Several investigators have reported that some light chains separated from intact immunoglobulins had binding activity, but that was less common and their affinities were several orders smaller than those of the native immunoglobulins [14]. Indeed, the diversity in the variable region of the light chain is less than that of the heavy chain, suggesting a weaker contribution of the light chains to antibody affinity maturation. The results of the present study are consistent with these observations.

The antibody-activity of light-chain fragments has been investigated using light chains from intact immunoglobulin molecules, by separating the light-chain portion under reducing or acetylating conditions [12–14]. Such light chains were denatured and different from the intact FLCs in terms of glycosylation and polymerization patterns [1]. Thus, little is understood on the true antigen-binding ability of intact FLCs, although approximately two-fifths of light chains are produced in the free form and secreted into the circulation [15]. The only study on the antigen-binding ability of intact FLCs was carried out by Contini et al. [16] who showed that patients with *Toxoplasma gondii* encephalitis contained antigen-specific FLCs in their cerebrospinal fluid. However, the antigen-binding ability of FLCs may be overestimated by examination with non-specific antisera for FLCs [2,10].

The “free” and “bound” forms of light chains include the same antigenic expression, and the immunological methods for FLC detection can usually not distinguish between the two forms. The concentration of whole immunoglobulins is several thousand-fold more than that of FLCs in the serum [2,10]. Thus, the measured values of FLCs in the sera (Table 1) is likely to be overestimated in the presence of abundant amounts of whole immunoglobulins. In the present study, we separated intact FLCs in a centrifuge filter unit and by IgG-specific gel adsorption. Since this is known to be a reliable method for the removal of whole immunoglobulins, we could examine the antibody activity of intact FLCs without the confounding influence of whole immunoglobulins. However, since the recovery of the FLCs was quite low (approximately 2% without compensation for the overestimation by the FLC assay), only the effects of small amounts were investigated. Thus, the possibility that antigen-specific FLCs were lost during the purification process cannot be ruled out.

The specific detection of FLCs by immunological methods is difficult [2]. In addition, taking together our present findings with earlier reports [12,13], it would appear that the antibody activity of FLCs, if any, is very low. Even if antigen-specific FLCs were involved in allergic responses as reported by Redegled et al. [5,6] and potentially useful as diagnostic markers, improvement of the assay sensitivity is necessary to obtain reliable data. Moreover, as the removal of whole immunoglobulins prior to the assay is impractical during routine assay, a method to specifically detect FLCs must be developed.

We believe that our present study has two limitations. The first is that the antigen-binding activity of FLCs may be

involved in the pathogenesis of patients without elevation of specific IgE, however, such patients were not enrolled. This is because the involved antigens in these patients are still unknown. The identification of the antigens for the FLCs may facilitate the study of the antibody activity of FLCs in patients with non-IgE responses. Thus, we examined in immunized mice, which could be strongly immunized against certain immunogens. The second limitation of our study relates to the assay sensitivity for antigen-specific FLCs. Light-chain fragments have usually been reported to have no or very low antigen-binding ability. Intact FLCs might possess low antigen-binding activity that would be difficult to detect by the conventional colorimetric assay method. Therefore, we should first elucidate the antigens that may be recognized by the FLCs in patients with allergy and how FLCs may induce hypersensitivity-like responses [17].

In conclusion, we found that intact FLCs detected in blood and urine specimens showed little or no antigen-binding activity as determined by colorimetric enzyme-immunoassays, suggesting that intact FLCs may have little or no antigen-binding activity and thus have little value as diagnostic markers for patients with allergy.

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