別種の構成成分を含む大分子IgGの存在

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Existence of the Large Molecules of Immunoglobulin G with Another Component

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ABSTRACT. The larger molecules of immunoglobulin G (LIgG) were found in ascites fluid containing monoclonal antibody against *Clostridium botulinum* toxin, and were purified by gel filtration and affinity chromatography. The purified material had the antigenicity and antibody activity similar to those of IgG obtained from the same ascites fluid. The molecular weight (Mr) estimated by gel filtration of the purified material was 250,000, while LIgG molecules dissociated into a kind of molecule with Mr 245,000 and nine kinds of the component molecules with Mr 220,000, 188,000, 152,000, 135,000, 105,000, 75,000, 67,000, 52,000 and 25,000 in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) in the absence of 2-mercaptoethanol (2ME). In SDS-PAGE in the presence of 2ME, the bands of Mr above 135,000 disappeared and three bands of Mr 120,000, 82,000 and 67,000 newly appeared besides the usual heavy and light chains of IgG. These results suggest that hybridoma cells produce not only normal IgG but also LIgG.—KEY WORDS: IgG, LIgG, monoclonal antibody.

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In the process of studying on the molecular properties and biological activities of monoclonal antibodies against *Clostridium botulinum* type C_1 and D toxins [1, 2, 9], antibody activities were often detected on two separated positions, at normal IgG position and between IgM and IgG, in gel filtration of ascites fluid on a column of Sephadex G-200 (superfine).

Generally, the antibody activity would be eluted at only normal IgG position because only one kind of antibody might be secreted in the ascites fluid. Therfore, the elution profile mentioned above suggests the existence of the larger molecule (s) of immunoglobulin. To confirm the existence of the larger molecules of immunoglobulin, purification and characterization of the materials are necessary.

In this paper, a purification procedure of immunoglobulins with larger size in the ascites fluid is described and some properties of the purified materials are compared with immunoglobulins known.

MATERIALS AND METHODS

Buffer A: Borax-sodium phosphate buffer (Buffer A) was prepared by mixing in the portion of 55 m*l* of 0.05M $Na_2B_4O_7 \cdot 10H_2O$ and 45 m*l* of 0.1M $NaH_2PO_4 \cdot 2H_2O$ to give pH 8.0 at 18°C [14].

Antisera: Goat anti-mouse IgA, goat anti-mouse IgG, goat anti-mouse IgM and rabbit anti-mouse whole serum were purchased from Cappel Laboratories Inc. (U. S. A.). Rabbit anti-mouse LIgG was prepared as follows; 300 μg of the purified LIgG in 1 m*l* of Buffer A was mixed with 1 m*l* of Freund's complete adjuvant and subcutaneously injected to a rabbit. Then, 200 μg of the purified LIgG with Freund's complete adjuvant was injected 10 days after the first injection, followed by a booster injection with 100 μg of the purified LIgG without adjuvant 30 days after the first injection.

The rabbit was bled 7 days after the booster injection.

Ascites fluid containing monoclonal antibody: Monoclonal antibodies against *Clostridium botulinum* toxin were prepared by the methods reported previously [9, 10]. A clone CA12, which was prepared by immunization with the toxoid of *Clostridium botulinum* type C_1 toxin [9], was used throughout the experiments.

Toxins: Toxins of *Clostridium botulinum* type C strains Stockholm (CST toxin) and 6813 (C6813 toxin), type D strains South African(DSA toxin) and 1873 (D1873 toxin) were purified by chromatographies described previousuly [15, 6, 11, 17]. Only CST toxin was used for the analyses of the binding and neutralizing activities of LIgG to toxin. All four toxins were used for the analysis of the binding specificity of LIgG.

Affinity column: An affinity column was prepared as follows; 2 mg of CST toxin was coupled to 1 m*l* of CNBr-activated Sepharose 4B gel according to the manual (Pharmacia Fine Chemicals) and the gel was packed into a pasteur pipet.

Affinity purification of LIgG: Fractions containing LIgG (from fraction 31 to 35 in Fig. 1) were pooled and loaded on the affinity column. The column was washed with 50 m*l* of Buffer A at a flow rate of 10 m*l*/hr and then the bound materials were eluted with 6 m*l* of 3M KSCN at a flow rate of 4 m*l*/hr and at 0°C. The effluent was subsequently dialysed against a 300-fold volume of Buffer A for 24 hours at 4°C. The outside buffer was changed three times. The dialysate was concentrated by ultrafiltration with 8/32 cellulose tubing under suction. About 50 μg of the purified material was obtained from 1 m*l* of the ascites fluid.

Electrophoresis: SDS-PAGE with or without 2ME was carried out by the method of Weber *et al.* [18].

Immunological assays: Immunodiffusion test and immunoelectrophoresis were carried out according to Ouchterlony and Nilsson [12].

Protein determination: Protein concentation of the effluent in gel filtration was monitored at 280 nm by using a Uvicord II (LKB Products). Toxin concentrations were determined by absorbance at 278 nm using the extinction coefficients of 14.18 for CST toxin [15], 10.14 for C6813 toxin [17], and 11.4 for D1873 toxin [6]. Protein concentrations of other samples were determined by the method of Lowry *et al.* [5] using bovine serum albumin as the standard.

Binding activity assay: The binding activities of the samples to toxin were assayed by the controlled electrophoresis [16] with slightly modification. A 100 μl aliquots of the fraction obtained by gel filtration or affinity chromatography was mixed with 10 μl of toxin solution containing 1–20 μg of toxin. The mixture was allowed to stand for 15 min at room temperature, and then electrophoresed at a constant current of 0.55 mA per gel. When marker dye migrated 14 cm from the top of the gel, the current was stopped. The gels were stained with 0.05% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid solution at 50°C for 1 hr, then destained by incubation at the same temperature in the destaining solution containing 10% methanol and 10% acetic acid until the background became clear. Appearance of new band of toxin-antibody complex was observed.

Toxicity assay: Toxicities of the toxins were estimated by time-to death method [4] and represented as the minimum lethal dose (MLD) unit.

Neutralizing activity assay: Neutralizing activity of the fractions obtained by gel filtration was estimated by the decrease of toxicity of the control toxin; a 0.1 m*l* portion of a mixture of 0.5 m*l* of a fraction and 0.1 m*l* of the control toxin (1.3×10^5 MLD/m*l*) was intravenously injected to a female

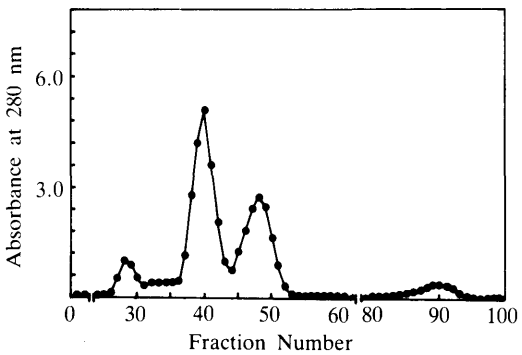

Fig. 1. Gel filtration of ascites fluid on a column of sephadex G-200 superfine. CA12 ascites fluid, 5 m*l*, was applied on a column of sephadex G-200 superfine, 2.55×63.5 cm, equilibrated with Buffer A, and eluted with the same buffer at a flow rate of 5 ml/hr. Protein was monitored by an absorbance at 280 nm. Each fraction contained 4.0 m*l*.

Table 1. Neutralization activities of fractions

Mixture	Toxicity (MLD/m*l*)
Toxin 100μl+Buffer A 500μl	22000
Toxin 100μl+Fraction 28 500μl	27000
Toxin 100μl+Fraction 33 500μl	1500
Toxin 100μl+Fraction 36 500μl	13800
Toxin 100μl+Fraction 41 500μl	700
Toxin 100μl+Fraction 47 500μl	16000
Toxin 100μl+Fraction 90 500μl	21000

mouse (dd strain) weighing 18–20 g, and its survival time was recorded. The effect of anti-mouse IgA, anti-mouse IgG or anti-mouse IgM on the neutralizing activity of the fraction was examined by the recover of the toxicity; a 0.1 m*l* of the control toxin (1.3×10^5 MLD/m*l*) and 0.05 m*l* of antiserum was intravenously injected to a female mouse, and its survival time was recorded. Changes of the toxicities were calculated by means of the standard curve [10]. Five mice were used as a group.

RESULTS

Fractionation of CA12 ascites fluid by gel filtration: Four peaks were eluted in the Sephadex G-200 gel filtration (Fig. 1). The first peak contained IgM and β-lipoprotein, the second peak IgG, the third peak transferrin and albumin, and the fourth peak small peptides and amino acids.

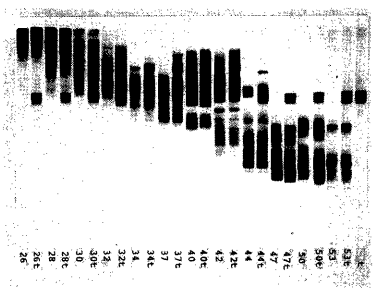

Fig. 2. Electrophoretic analysis of toxin binding activity of the fraction. Number only, fraction without toxin. Number with t, fraction mixed with toxin.

The antibody activities (binding and neutralizing activities) to the toxin were detected on two separate positions, between IgM and IgG peaks (fraction 31–36), and IgG peak (fraction 37–44) as shown in Fig. 2 and Table 1. When a fraction was mixed with toxin and electrophoresed, each fraction from 31 to 36 and from 37 to 44 formed new four and two bands, respectively. All of those bands appeared in different positions; the mixture of the fraction 33, 41 and the toxin formed six bands. When these fractions were mixed with anti-mouse IgG, anti-mouse IgA or anti-mouse IgM in the various ratios, the neutralizing activities to the toxin were inhibited by only anti-mouse IgG. Neither anti-mouse IgA nor anti-mouse IgM inhibited the activity.

Characterization of the purified CA12-LIgG: Purity of the purified material was assayed by electrophoretical and immunological methods. The purified LIgG showed three bands in controlled electrophoresis (Fig. 3A), and formed one precipitin line with anti-mouse IgG and two lines with anti-CA12-LIgG or anti-mouse whole serum in agar gel (Fig. 3B, C, Fig. 4).

The binding activity of the purified LIgG

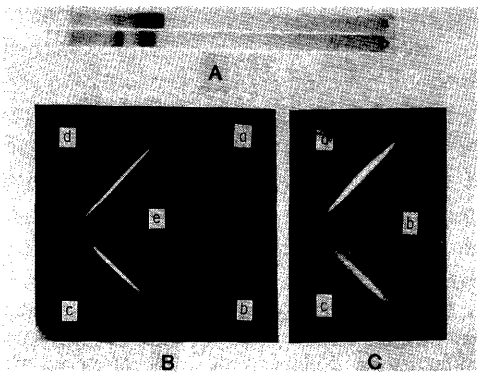

Fig. 3. Purity and antigenicity assays of the purified LIgG. Panel A, controlled electrophoresis analysis. a, purified IgG from CA12 ascites fluid. b, purified LIgG from CA12 ascites fluid. Panel B, agar gel double diffusion test of the purified LIgG. a, anti-mouse IgA. b, anti-mouse IgM. c, anti-mouse IgG. d, anti-mouse whole serum. e, purified LIgG. Panel C, agar gel double diffusion test of the purified LIgG and IgG with anti-LIgG. a, purified IgG. b, anti-LIgG. c, purified LIgG. Added volume of the sample was 25 μl in a well. LIgG concentration was 400 μg per ml.

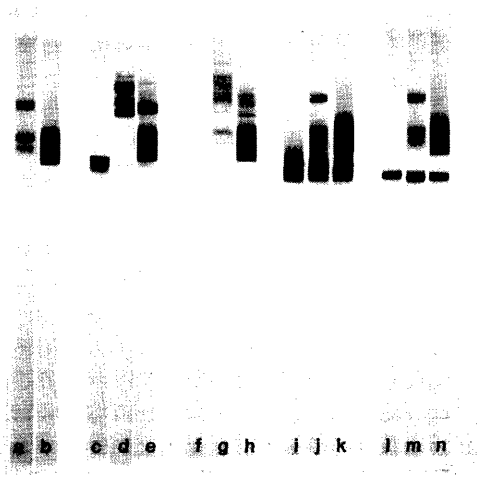

Fig. 5. Binding specificity of LIgG to toxins. The purified LIgG or IgG from CA12 ascites fluid was mixed with toxin and the mixture was electrophoresed. a, LIgG. b, IgG. c, CST toxin. d, LIgG and CST toxin. e, IgG and CST toxin. f, DSA toxin. g, LIgG and DSA toxin. h, IgG and DSA toxin. i, C6813 toxin. j, LIgG and C6813 toxin. k, IgG and C6813 toxin. l, D1873 toxin. m, LIgG and D1973 toxin. n, IgG and D1873 toxin.

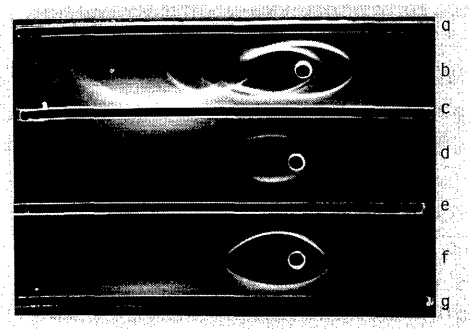

Fig. 4. Immunoelectrophoresis of the purified LIgG. Ten μl of sample was applied to a well and electrophoresed at a constant voltage of 6 v/cm. a, e (basins), anti-LIgG. b (well), mouse normal serum. c (basin), anti-mouse whole serum. d (well), purified LIgG. f (well), IgG from CA12 ascites fluid. g (basin), anti-mouse IgG.

to the toxin was assayed in controlled electrophoresis; when CA12-LIgG was mixed and electrophoresed with the toxin, all bands of LIgG disappeared and new four bands appeared (Fig. 5, lane d). The binding specificity of CA12-LIgG and CA12-IgG to toxin was identical. Both the CA12-LIgG and CA12-IgG bound to CST and DSA toxins, whereas they did not react with C6813 and D1873 toxins (Fig. 5).

Analysis of the molecular structure of LIgG: The purified LIgG showed an Mr 250,000 in the Sephadex G-200 gel filtration, while ten bands of Mr 245,000, 220,000, 188,000, 152,000, 135,000, 105,000, 75,000, 67,000, 52,000 and 25,000 were detected on SDS-PAGE without 2ME. In the presence of 2ME, seven bands of the Mr 120,000, 105,000, 82,000, 75,000, 67,000, 52,000 and 25,000 were detected. Among them, two bands of Mr 120,000 and 82,000 newly appeared and a band of Mr 67,000 increased density. On the other hand, normal IgG showed a single band of the Mr 152,000 in the absense of 2ME and two bands of Mr 52,000 and 25,000 in the presence of 2ME (Fig. 6).

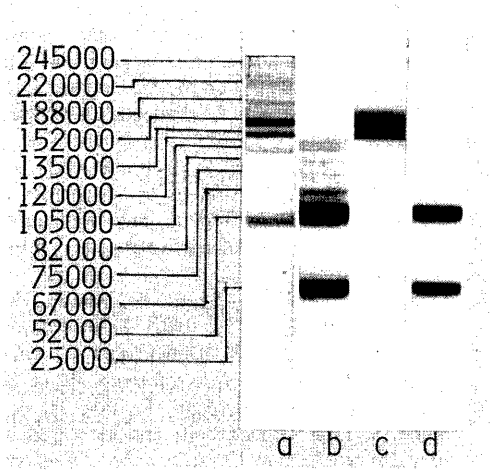

Fig. 6. SDS-PAGE analysis of components of LIgG and IgG. The purified LIgG and IgG were treated with 1% SDS with or without 2ME and electrophoresed on 5% gel. a, LIgG without 2ME. b, LIgG with 2ME. c, IgG without 2ME. d, IgG with 2ME.

DISCUSSION

Antibody molecules with larger size than usual IgG could be separated by gel filtration and affinity chromatography. The purified material differ from other immunoglobulins known in some properties, Mr, electric charge and antigenicity. Mr 250,000 does not correspond to any of the five classes of immunoglobulins known [13]. Electric charge of LIgG is more negative since LIgG migrated at α_2-β_1 position whereas other immunoglobulins known migrated at β_2-γ position in immunoelectrophoresis. Antigenicities of LIgG and IgG are slightly different. However, we consider that LIgG belongs to IgG class for following reasons; 1) LIgG contains the normal size of IgG molecule as an essential structure, 2) antigenicities of LIgG and IgG are essentially similar but slightly different, 3) antibody activities (recognizing an epitope and neutralizing toxicity) of LIgG and IgG are identical, 4) LIgG and IgG are produced in the same clone.

The molecular structure of LIgG is implicated. In SDS-PAGE, ten protein bands (Mr 245,000–25,000) appeared in the absence of 2ME, whereas five bands above Mr 135,000 disappeared and three bands newly appeared in the presence of 2ME. Since the molecular weight of LIgG in gel filtration is 250,000, LIgG molecules are considered to be constituted by the interchain disulfide bonds and noncovalent forces between the protein components. And three types of molecular structure of LIgG are possible; 1) LIgG molecule is constituted by the interchain disulfide bonds between the components as observed in the molecule with Mr 245,000, 2) LIgG molecules are constituted by the interchain disulfide bonds and noncovalent forces between the components as observed in the association of components having Mr below 220,000, 3) LIgG molecules are constituted by only noncovalent forces as observed in the association of components having Mr below 105,000.

The components of IgG molecule, heavy (H) and light (L) chains, are separately synthesized and linkaged by disulfide bonds in the process of H, $H \rightarrow H_2 \rightarrow H_2L \rightarrow H_2L_2$ (3). In this process, hybridoma cells may partially produce the heavy and light chains which have the unusual conformation without the active sulfhydryl group or with the extra of active sulfhydryl group to form LIgG molecules. Although the unusual disulfide bonded structure of mouse IgA has been discussed [8], the structural similarity to LIgG is not clear.

The origins of the components except for the heavy and light chains of LIgG molecules are still obscure. Among them, a component with Mr 67,000 is similar to secretory component of IgA [7] and serum albumin in the molecular size, but neither anti-mouse IgA nor anti-mouse albumin reacted with LIgG. However, it may be unable to exclude the possibility that LIgG molecule contains some of serum proteins

since LIgG and anti-mouse whole serum formed a precipitin line distinguishable from that of IgG in immunoelectrophoresis.

In other clones of hybridoma cells, the existences of LIgG were also observed. These observations and considerations mentioned above led us to conclude that two kinds of molecules, LIgG and normal IgG, are produced in hybridoma cells.

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要　　約

別種の構成成分を含む大分子 IgG の存在：首藤文栄[1)]・村山　識[1)]・小熊恵二[2)]・久保周一郎[1)]（[1)]北海道大学獣医学部，[2)]札幌医科大学）—— 細胞融合によるボツリヌス毒素に対する単クローン抗体作製の過程で，大分子量の抗体存在が予想されたので，これを製精して性状を調べた．単クローン抗体を含む腹水を Sephadex G-200 でゲルろ過し，IgG・IgM 間に溶出される画分を，抗原（毒素）を結合させたカラムに負荷して，目的物質を分離製精した．製精標品は，disc 電気泳動で移動率の異なる 3 バンドを示し，すべての成分が抗体活性を有していた．SDS 電気泳動では，245,000，22,000，188,000，152,000，135,000，105,000，75,000，67,000，52,000，および25,000のバンドが検出された．2—メルカプトエノタール処理により，135,000以上のバンドは消失し，通常 IgG の重鎖と軽鎖の他に，120,000，82,000および67,000のバンドが検出された．標品はゲル内沈降反応で抗 IgG 抗体と反応したが，抗 IgM 抗体および抗 IgA 抗体とは反応しなかった．以上の結果から，細胞融合では通常 IgG の他に，大分子 IgG も産生されていることが示唆された．