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Purification and characterization of a recombinant human IgE Fcε fragment lacking the C4 domain

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Complementary DNA of human IgE Fcε fragment (residues 226–480) lacking the Cε4 domain was expressed in *Escherichia coli* and the product was purified by immunoaffinity chromatography on a monoclonal antibody (E12 0.02)-Affi-Gel 10 column. About 1.8 mg of an apparent dimer and 5.9 mg of a monomer were obtained from 65 g *E. coli* cells with 9.3% recovery. The purified products were found to lack more than half of the COOH-terminal portion of the Cε3 domain. The apparent dimer showed high immunological specific activity (3.6×10^6 U/mg protein) comparable to that of natural human IgE when measured by commercial human IgE determination kits.

Recombinant IgE Fcε fragment; Immunoaffinity chromatography; *(Escherichia coli)*

1. INTRODUCTION

IgE, which binds to Fcε receptors (FcεR) on basophilic granulocytes and mast cells by the Fcε portion and sensitizes the cells for antigen-induced mediator release, is responsible for various hypersensitivity reactions of the immediate type [1]. The Fcε fragment of IgE, comprising the Cε2, Cε3 and Cε4 domains, is sufficient for binding to the FcεR and inhibits the antigen-induced mediator release from cells [2].

Recent progress in recombinant DNA technology has made it possible to obtain a large amount of the Fcε fragment. Gould and coworkers [3–5] reported that the recombinant human Fcε fragment synthesized in *E. coli* assembles spontaneously into a dimer and inhibits the sensitization of human lung mast cells in vitro and Prausnitz-Küstner reactions in vivo. We also succeeded in purifying the recombinant human Fcε fragment produced in mouse L cells [6], but did not succeed in obtaining the Fcε dimer from *E. coli* harbouring the recombinant plasmid, pGETtrp302 [7]. The Fcε fragment synthesized in *E. coli* did not form the dimer, but easily aggregated (unpublished). However, we found that Fcε fragments synthesized in *E. coli* harbouring the recombinant plasmid, pGETtrp712 [8], whose cDNA lacked the region coding the COOH-terminal 76 amino acid residues of the Fcε portion, show high immunological activity when measured by commercial human IgE determination kits. Thus, we have attempted to purify the human Fcε fragment from *E. coli*, 294/pGETtrp712 and this paper describes the purification and characterization of the purified protein.

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Abbreviations: FcεR, Fc receptor for IgE; PBS, phosphate-buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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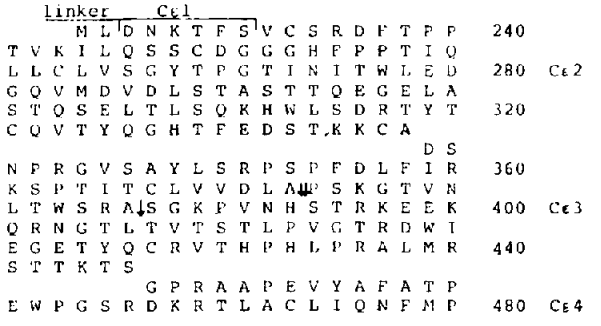

Fig.1. The amino acid sequence predicted from the DNA sequence of the insert region of plasmid pGETtrp712. Amino acids are expressed in the one-letter code as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. The linker (aspartic acid) of the COOH-terminal portion is omitted. (↓, ⇓) Cleavage sites predicted from the obtained fragments.

2. MATERIALS AND METHODS

Sephacryl S-200 and the PD-10 column were purchased from Pharmacia (Tokyo), DEAE-Toyopearl 650M from Toyo Soda (Tokyo), Affi-Gel 10 and M_r standards for SDS-PAGE from Bio-Rad (Richmond, CA), BCA protein assay reagent from Pierce (Rockford) and silver stain reagent from Wako (Osaka).

E. coli, 294/pGETtrp712 [8], was cultured in M9 medium [7]. The amino acid sequence predicted from the DNA sequence of the insert region of the plasmid is shown in fig.1.

The monoclonal anti-human IgE antibody, E12 0.02, which recognizes the Cε2 domain [8], was purified from ascites fluids and coupled to Affi-Gel 10. The detailed procedures were described in [9].

The concentration of Fcε fragment was determined by a radioimmunoassay using Pharmacia IgE RIA kits (Shionogi, Osaka). Protein was estimated by the BCA method [10] using crystalline bovine serum albumin as standard. SDS-PAGE was carried out as described by Laemmli [11]. The gels were stained with silver stain reagent according to the manufacturer's instructions. Protein chemical analyses were performed as in [6].

3. RESULTS

3.1. *Purification of Fcε fragment*

All the purification procedures were carried out at 5°C.

(Step 1) Extraction: 65 g of frozen *E. coli* cells, 294/pGETtrp712, were mixed with 200 ml of 7 M guanidine-HCl/PBS (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4)/20 mM 2-mercaptoethanol (pH 7.4) and stirred for 4 h. After 267 ml PBS were added, the mixture was stirred again for 1 h and centrifuged at 30100 × *g* for 1 h. The supernatant was dialyzed against three changes of PBS, 4 l each, every 10 or 14 h. After dialysis, the resulting insoluble materials were removed by centrifugation.

(Step 2) Immunoaffinity chromatography on E12 0.02 Affi-Gel 10: The supernatant (156 ml) obtained in step 1 was applied to a monoclonal anti-human IgE mouse IgG-Affi-Gel 10 column (2.6 × 13.6 cm) equilibrated with PBS at a flow rate of 10 ml/h. The column was washed with the same buffer at a flow rate of 40 ml/h for 25 h. No Fcε fragment was detected in the pass-through and wash fractions. After the column was washed with 0.2 M sodium acetate/0.15 M NaCl (pH 7.2), the Fcε fragment bound to the column was eluted with 3 M urea/0.2 M sodium acetate/0.15 M NaCl (pH 5.0). The fractions containing the Fcε fragment were pooled and dialyzed against one change of 2 l PBS for 24 h. After dialysis, the resulting insoluble materials were removed by centrifugation.

(Step 3) Gel filtration chromatography on Sephacryl S-200: The supernatant obtained in step 2 was concentrated using a Diaflo YM-5 membrane and applied to a Sephacryl S-200 column (1.6 × 105 cm) equilibrated with PBS. The Fcε fragment was eluted with the same buffer at a flow rate of 10.8 ml/h. The fractions expected to be a dimer and a monomer of the Fcε fragment were separately pooled (fig.2,D,M).

(Step 4) Ion-exchange chromatography on DEAE-Toyopearl: The pooled fraction D obtained in step 3 was dialyzed against one change of 500 ml of 10 mM Tris-HCl (pH 8.2) for 24 h and applied to a DEAE-Toyopearl column (0.72 × 17 cm) equilibrated with the same buffer. The column was washed and then a linear NaCl gradient was applied to the column. The gradient was produced by

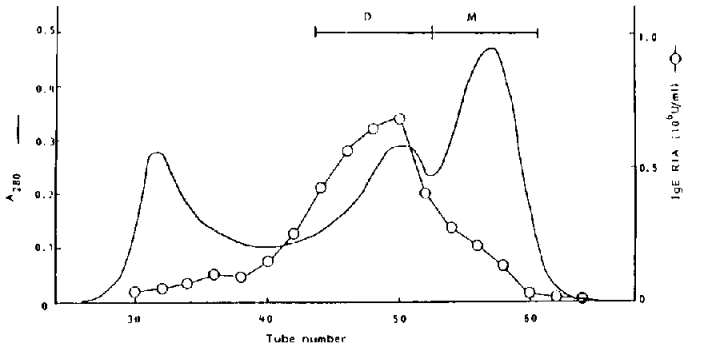

Fig.2. Gel-filtration chromatography on a Sephacryl S-200 column. The preparation from step 2 (see table 1 and text) was applied to the column (1.6 × 105 cm) equilibrated with PBS. The column was eluted with the same buffer at a flow rate of 10.8 ml/h. Fractions of 2.7 ml were collected. (——) Absorbance at 280 nm and (○) IgE RIA activity.

continuously adding 100 ml of 10 mM Tris-HCl (pH 8.2)/0.3 M NaCl to 100 ml of the equilibrating buffer. The proteins were eluted at about 110–170 mM NaCl. The fractions showing the highest specific activity (eluted at about 140 mM NaCl) were pooled (hereafter called pD). The pooled fraction M obtained in step 3 was also applied to the same column and eluted under the same conditions. The fractions containing the main peak (eluted at about 80 mM NaCl) were pooled (hereafter called pM).

A summary of the purification of the human Fcε fragment from 65 g of the *E. coli* cells is given in table 1.

3.2. *Purity and molecular forms*

pD and pM were examined by SDS-PAGE to check their homogeneity. pM gave almost one band that migrated at an apparent M_r of about 18000 under non-reducing and reducing conditions although a very minor band was present (fig.3, lanes 2,4). pD gave a main band that migrated at an apparent M_r of 26000 and very heterogeneous minor bands that migrated at apparent M_r values of 25000–54000 under non-reducing conditions (fig.3, lane 1). When reduced with dithiothreitol and analyzed by SDS-PAGE, it gave mainly two bands that migrated at apparent M_r values of 20000 and 18000 (fig.3, lane 3).

3.3. *Protein chemical properties*

The NH_2-terminal amino acid sequences of pD and pM were determined by a gas-phase sequencer. At the first step, both pD and pM gave only

Table 1

Summary of purification of recombinant Fcε fragment from the extract of *E. coli*, 294/pGETtrp712

Purification step	Volume (ml)	Total protein (mg)	IgE RIA activity (10^6 U)	Spec. act. (10^6 U/mg)	Recovery (%)
(1) Extract	470	2115	98.7	0.047	(100)
(2) E12 0.02-Affi-Gel 10	241	31.3	55.4	1.8	56
(3) Sephacryl S-200					
D	24.7	9.4	19.3	2.1	20
M	22.3	9.8	3.7	0.38	3.7
(4) DEAE-Toyopearl 650M					
pD	17.2	1.8	6.5	3.6	6.6
pM	19.6	5.9	2.7	0.46	2.7

65 g of *E. coli* cells were used for purification

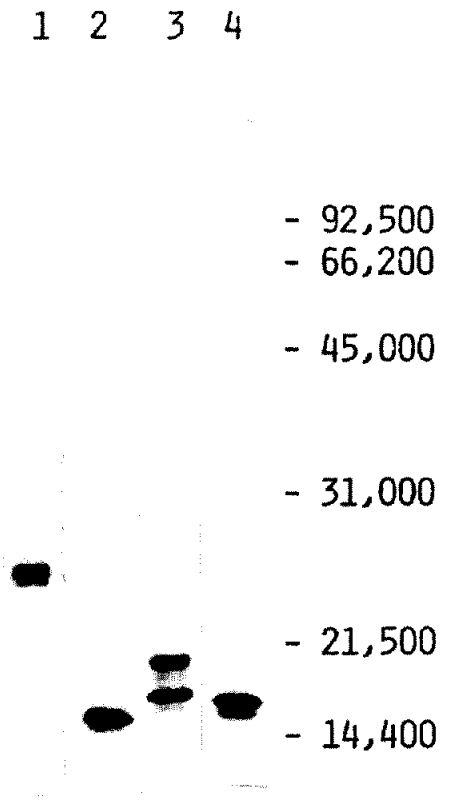

Fig.3. SDS-PAGE of the purified dimer and monomer fractions. SDS-PAGE was carried out in 12.5% acrylamide slab gel. pD (lanes 1,3) and pM (lanes 2,4) (see table 1 and text) were treated in a boiling water bath for 5 min in the absence (lanes 1,2) and presence (lanes 3,4) of 2% dithiothreitol. Migration of M_r markers is shown on the right.

methionine (recovery 29 and 40%, respectively) and the amino acid sequences up to position 15 were consistent with those deduced from the DNA sequence. The COOH-terminal amino acid of pD and pM was analyzed by hydrazinolysis and only alanine was detected with 23 and 28% recovery, respectively. These results and the M_r values estimated from SDS-PAGE suggested that our preparations deleted the COOH-terminal portion. The species of M_r 20000 and 18000 may be terminated at the position shown by the single and double arrows, respectively, in fig.1. The amino acid compositions were consistent with those deduced from the sequence shown in fig.1 except for several amino acids (not shown).

4. DISCUSSION

The COOH-terminal portion was deleted from recombinant Fcϵ fragments produced in and purified from *E. coli*, 294/pGETtrp712. We chose guanidine-HCl, which decreases the proteolytic activity, to extract the peptide from *E. coli*. For example, human recombinant γ-interferon was obtained as the intact molecule from *E. coli* when guanidine-HCl was used for extraction but not when other methods were used [13]. Nevertheless, we could not obtain the intact molecule. Since our purpose is to obtain the small fragment having binding activity to FcϵR, no further efforts to obtain the intact molecule were made. The predicted cleavage sites are shown in fig.1. It is unknown why two kinds of fragment were obtained from pD and only one fragment from pM under reducing conditions.

The Fcϵ fragment (Cϵ2–Cϵ4 domains) synthesized in *E. coli*, 294/pGETtrp302 easily formed aggregates, and a stable monomer or dimer having high reactivity against anti-human IgE antibodies has not been obtained in our laboratory (unpublished). On the other hand, the present results show that the Fcϵ fragment lacking the Cϵ4 domain and approximately the terminal half of the Cϵ3 domain formed only small aggregates; the majority of the fragment was stable. pD apparently seemed to be a dimer because a main band giving an apparent M_r of 26000 on SDS-PAGE under non-reducing conditions gave two bands of lower M_r values of 18000 and 20000 under reducing conditions. It is curious, however, that the observed M_r of the dimer is far smaller than double that of the monomer. At the moment, the reason for this is unknown. At any rate, it is noteworthy that these short molecules retained the ability to bind to anti-human IgE antibodies comparable to that of natural IgE. These results suggest that the Cϵ4 domain and the terminal half of the Cϵ3 domain play an important role in the instability of IgE and Fcϵ. Ishizaka et al. [14] observed an increase in the number of oligomeric forms during the storage of the Fcϵ fragment (Cϵ2–Cϵ4 domains) synthesized in *E. coli* at 0°C for several weeks. No polymer or aggregates were detected in either pD or pM during

storage at 4°C for 1 month (not shown). These results support the idea that the Fcε fragment lacking the COOH-terminal portion was more favourable than the Fcε fragment when *E. coli* were used as host cells to produce active Fcε molecules.

It was reported very recently that cDNA containing parts of the Cε2 and Cε3 domains was expressed in *E. coli* and that the product inhibited passive cutaneous anaphylaxis in human skin [15]. However, no protein-chemical characterization was carried out for the peptide produced in *E. coli* in that report. The specific activity of our pD was 3.6×10^6 U/mg protein (table 1). That of the recombinant Fcε fragment (Cε2–Cε4 domains) produced in and purified from mouse L cells was 0.96×10^6 U/mg protein [6] and that of the L cell derived Fcε fragment stored at 4°C for 6 months was 1.5×10^6 U/mg protein (not shown; the concentration of protein was also reestimated by the BCA method). The M_r values of pD and L cell derived Fcε fragment calculated from their amino acid compositions are 17830 and 38480 (values as monomer), respectively. Therefore, the activity per molecule is nearly the same. These results indicate that the dimers lacking the Cε4 domain and half of the Cε3 domain synthesized in *E. coli* have full binding ability to anti-human IgE antibodies in the IgE RIA kits. The biological activity of these fragments remains to be compared.

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REFERENCES

[1] Ishizaka, T. and Ishizaka, K. (1975) Prog. Allergy 19, 60–121.
[2] Stanworth, D.R., Humphrey, J.H., Bennich, H. and Johansson, S.G.O. (1968) Lancet 2, 17–18.
[3] Kenten, J., Helm, B., Ishizaka, T., Cattini, P. and Gould, H. (1984) Proc. Natl. Acad. Sci. USA 81, 2955–2959.
[4] Geha, R.S., Helm, B. and Gould, H. (1985) Nature 315, 577–578.
[5] Coleman, J.W., Helm, B.A., Stanworth, D.R. and Gould, H.J. (1985) Eur. J. Immun. 15, 966–969.
[6] Ikeyama, S. (1987) Mol. Immun., in press.
[7] Kurokawa, T., Seno, M., Sasada, R., Ono, Y., Onda, H., Igarashi, K., Kikuchi, M., Sugino, Y. and Honjo, T. (1983) Nucleic Acids Res. 11, 3077–3085.
[8] Ichimori, Y., Kurokawa, T., Ikeyama, S., Sasada, R. and Tsukamoto, K. (1985) Hybridoma 4, 47–52.
[9] Ikeyama, S., Nakagawa, S., Arakawa, M., Sugino, H. and Kakinuma, A. (1986) Mol. Immun. 23, 159–167.
[10] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Liu, F.-T., Albrandt, K.A., Bry, C.G. and Ishizaka, T. (1984) Proc. Natl. Acad. Sci. USA 81, 5369–5373.
[13] Kung, H.-F., Pan, Y.-C.E., Moschera, J., Tsai, K., Bekesi, E., Chang, M., Sugino, H. and Honda, S. (1986) Methods Enzymol. 119, 204–210.
[14] Ishizaka, T., Helm, B., Hakimi, J., Niebyl, J., Ishizaka, K. and Gould, H. (1986) Proc. Natl. Acad. Sci. USA 83, 8323–8327.
[15] Gould, H.J., Helm, B.A., Marsh, P.J. and Geha, R.S. (1987) Int. Arch. Allergy Appl. Immunol. 82, 392–393.