Isolation and identification of a biologically active peptide derived from the CH₃ domain of human IgG1

(Fc fragment/polyclonal antibody response/immunoregulation)

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ABSTRACT A 24-amino acid residue peptide has been isolated from the Fc region of a human IgG1 myeloma protein. The peptide has associated with it the same ability to induce murine B cells to polyclonally secrete antibody as does the intact Fc fragment. Amino acid composition of the peptide indicates that on a mole/mole basis the isolated peptide is identical to that published for residues 335–358 in the Eu IgG1 sequence. This peptide corresponds roughly to the first 24 amino acids of the CH₃ domain. It is believed that the immunoregulatory properties that have been ascribed to the Fc fragment are associated with this peptide.

Murine and human bone marrow-derived (B) lymphocytes proliferate and undergo polyclonal activation when cultured with Fc fragments derived from papain digestion of immunoglobulin (1–5), aggregated human gamma globulin (6), immune complexes (7), and fragments derived from plasmin digestion of human Ig (7). The unifying feature of these responses is that in each case a portion of the constant region of the Ig heavy chain (i.e., Fc portion) is present. Intact IgG1 F(ab')₂ and Fab fragments derived from human IgG possess no stimulatory activity (1).

These stimulating agents are unique when compared to most polyclonal B cell activators in that Fc-mediated lymphocyte activation requires the presence of both macrophages (2, 3) and thymus-derived (T) cells (4, 7). The polyclonal antibody response is mediated by two signals. The first or proliferative signal is mediated by a M_r 14,000 Fc subfragment derived from macrophage processing of the Fc fragments (3), and the second or differentiative signal is generated from the interaction of T cells with Fc subfragments (8–10).

In addition to being a polyclonal B cell activator, Fc fragments possess the capacity to act as a potent adjuvant. Fc fragments have been demonstrated to potentiate murine *in vivo*- and *in vitro*-specific antibody responses (11, 12) and such T cell responses as antigen- and alloantigen-induced proliferation (13) and cell-mediated cytolytic responses (14). Moreover, the adjuvant properties of Fc are not limited to the mouse, because human *in vitro*-specific antibody responses are also enhanced (15).

In the present study, we describe the isolation and characterization of a peptide derived from the Fc region of IgG1 that retains the ability to induce polyclonal antibody production. The biologically active peptide consists of 24 amino acids and has a composition identical to residues 335–358 of Eu IgG1.

MATERIALS AND METHODS

Animals. Inbred male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were 8–12 wk of age when used.

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Source of Purification of the IgG1 Myeloma Proteins. Two IgG1 myeloma proteins (Fi and Pet) (Gm^f) were kindly provided by Hans L. Spiegelberg (Scripps Clinic and Research Foundation). The IgG1 was purified by ammonium sulfate fractionation followed by DEAE cellulose chromatography with 0.01 M phosphate buffer at pH 8 used as the eluent.

Preparation of Fc Fragments. Fc fragments were obtained by digestion of IgG1 with papain (Sigma) in the presence of L-cysteine (Sigma) and EDTA (Baker) for 18 hr (16). After digestion, the material was chromatographed on Sephadex G-100 (Pharmacia) to remove any undigested IgG1. The Fc and Fab fragments were then separated from each other by DEAE cellulose chromatography (17).

Plasmin Digestion of IgG1. Pet IgG1 was cleaved with plasmin (Sigma) as described (7, 18). Briefly, 100 mg of IgG1 was incubated with 1.2 mg of plasmin at pH 7 for 24 hr at 37°C. The digested IgG1 was then separated on a Sephadex G-100 column (1.5 cm × 75 cm) with 0.01 M phosphate buffer at pH 7 used as the eluent.

CNBr Cleavage of Plasmin-Digested IgG1. The protein was cleaved at the methionine residues by CNBr (Baker) according to established procedures (19). The protein was dissolved in 70% formic acid (Baker) and a 100-fold excess (wt/wt) of CNBr was added. The reaction mixture was incubated for 24 hr at room temperature. The sample was subjected to repeated lyophilization to remove CNBr prior to analysis.

HPLC Analysis. Molecular exclusion HPLC was performed by using two protein I-60 columns (7.8 mm × 30 cm) (Waters Associates) in tandem and in line with a Waters HPLC apparatus. The mobile phase buffer consisted of 0.1 M NH₄HCO₃. Elution occurred with a flow rate of 1 ml/min and was monitored at A₂₁₀. Reverse-phase HPLC was performed with a TMS Zorbax (Du Pont) column (4.6 mm × 25 cm). Peptide elution was accomplished by using a linear gradient of 0–100% buffer B in buffer A developed for 45 min at a flow rate of 1 ml/min. Elution was monitored at A₂₁₀. Buffer A was composed of 5% CH₃CN (Waters) in 0.1% H₃PO₄ (Kluka-Garantie, Germany) and buffer B of 95% CH₃CN in 0.1% H₃PO₄.

Amino Acid Composition Analysis. Amino acid compositions were determined by using a Beckman model 121M amino acid analyzer. Samples were hydrolyzed for 24 hr in evacuated ampules containing 0.5 ml of 5.5 M HCl, 1 μl of 88% phenol, and 1 μl of 2-mercaptoethanol.

Polyclonal Antibody Response. For the generation of the polyclonal antibody response, spleen cells were suspended to a concentration of 6 × 10⁶ per ml in RPMI 1640 (Flow Laboratories, Rockville, MD) supplemented with 2 mM L-glutamine, 1% BME vitamins (GIBCO), 100 units of penicillin and 100 μg of streptomycin (Microbiological Associates, Bethesda, MD), 5 × 10⁻⁵ M 2-mercaptoethanol, and 7.5% fetal calf serum.

Abbreviations: TNP, 2,4,6-trinitrophenyl; pfc, plaque-forming colonies.

(GIBCO). Duplicate cultures of 6×10^5 cells per 0.3 ml were incubated in microtiter plates (3042 Microtest II, Falcon) at 37°C in an atmosphere of 5% CO₂ in air. Various amounts of stimulator were added and the response was measured on day 3 of culture.

Plaque-Forming Response. The *in vitro* polyclonal antibody response was measured by a plaque-forming response to 2,4,6-trinitrophenyl (TNP)-conjugated sheep erythrocytes by the slide modification of the Jerne plaque assay (20). Heavily TNP-conjugated sheep erythrocytes were prepared according to the method of Kettman and Dutton (21). Guinea pig serum (Pel-Freez) was the source of complement to develop the direct or IgM plaques. Results of the assay are expressed as mean ($\pm$ SEM) plaque-forming colonies (pfc) per 10^6 original cells of duplicate cultures.

RESULTS

Polyclonal Antibody Production of Plasmin-Digested IgG1. Digestion of human IgG1 with the enzyme plasmin generates a fragment that has the capacity to induce murine splenic B cells to secrete polyclonal antibody. Chromatographic separation of the IgG1 digest on Sephadex G-100 reveals that the biological activity is associated with material in pool 2 (Fig. 1). The majority of the protein elutes as high molecular weight material (pool 1) which has no polyclonal activity.

Purification of the Polyclonal Inducer Activity from Plasmin-Digested IgG1. To further define the biologically active plasmin fragment, it was subjected to CNBr cleavage of the methionine residue with subsequent separation of the cleavage products by HPLC with a gel permeation I-60 column. The results of such a separation are shown in Fig. 2a. Two components, pools 1 and 2, were resolved after HPLC separation of the CNBr digest. These fractions were then assayed for their ability to induce a polyclonal antibody response. The results depicted in Fig. 2b show that only the material in pool 2 contained the polyclonal activity. The polyclonal antibody response induced by the material in pool 2 was similar in magnitude to the Fc fragment-induced response (151 pfc vs. 135 pfc). To determine if the pool 2 material derived from gel-permeation HPLC separation was a single or multiple component, this

FIG. 1. Pet IgG1 was digested with plasmin and the mixture was resolved by Sephadex G-100 chromatographic separation into two pools. Each pool was assessed for the ability to induce a polyclonal antibody response. The vertical bars represent the magnitude of the response.

FIG. 2. The biologically active small molecular weight pool from the plasmin digestion of IgG1 was cleaved with CNBr. The digest was separated on molecular exclusion HPLC by employing two I-60 columns in tandem (a) and the pooled material was assayed for the ability to induce polyclonal antibody production (b). The vertical bars represent the magnitude of the response.

material was subjected to reverse-phase HPLC separation on a TMS Zorbax column. The chromatographic profile generated by reverse-phase separation of pool 2 is shown in Fig. 3a. Four pools (A-E) were collected on the basis of optical density and each was assessed for the ability to induce murine splenic B cells to secrete polyclonal antibody. The results in Fig. 3b reveal that only the material in pool A retained the capacity to induce polyclonal antibody production. The magnitude of the response by pool A was not significantly different from the response obtained with Fc fragments (98 pfc vs. 106 pfc).

Amino Acid Composition of Peptides in Pools A and B Obtained After Reverse-Phase HPLC. Amino acid compositions of material in pools A and B from reverse-phase HPLC separation were determined. The results indicate that on a mole/mole basis the amino acid composition of the peptide in pool A derived from the IgG1 myeloma protein is identical to that published for residues 335–358 in Eu IgG (22). The peptide in pool B corresponds to residues 429–446 Eu IgG1 (Table 1). It can be concluded from these analytical results that the fragment of Fc region IgG1 that is responsible for inducing polyclonal antibody production consists of residues 335–358 from the CH₃ domain (Fig. 4).

DISCUSSION

The lymphocyte-activating properties of the Fc region of human IgG1 are associated with a 24-amino acid peptide derived from the CH₃ domain of the IgG1 molecule. Fc fragments derived from papain digestion of Ig have been shown to induce a wide variety of immunological functions which include B cell proliferation (1–3) and polyclonal antibody production (4, 5). Moreover, the Fc fragment represents a potent adjuvant in that marked enhancement of humoral (11, 12) and cell-mediated (13, 14) immune responses occurs in the presence of Fc. In addition to Fc fragments, digestion of human Ig with the enzyme plasmin results in a fragment that induces murine and human B cells to secrete polyclonal antibody (7). Connell and Painter (18) first reported that digestion of rabbit IgG with plasmin resulted in the generation of a large fragment (Fab_c) that contains all but the terminal CH₃ domain and a small molecular weight fragment consisting of the free CH₃ domain. The results reported here demonstrate that human IgG1 is also susceptible to plasmin that cleaves the molecule into fragments similar to those obtained from rabbit IgG.

FIG. 3. The biologically active (pool 2, Fig. 2) material derived from molecular exclusion HPLC was subjected to reverse-phase HPLC separation by increasing the percentage of buffer B with a TMS Zorbax column. Material was pooled on the basis of OD₂₁₀ (a) and assayed for the ability to induce polyclonal antibody production (b). The vertical bars represent the magnitude of the response.

Activation of lymphocytes by Fc fragments to secrete polyclonal antibody requires accessory T cells and macrophages (2–4, 7). Macrophages are responsible for enzymatically cleaving Fc into biologically active Fc subfragments that are responsible for lymphocyte activation. Fc subfragments stimulate T cells to secrete a factor, (Fc)/TRF, which induces B cells to differentiate to antibody-secreting cells (8–10). Polyclonal activation by plasmin Fc fragments (7) and peptides derived from the plasmin Fc fragments (unpublished observations) requires T cells but does not require macrophages. This finding can be accounted for by the fact that plasmin cleavage resembles the cleavage produced by macrophages, thus exposing the active binding site on the molecule.

Human IgG subclasses can be distinguished on the basis of allotype. The appearance of certain amino acid residues at defined positions confers the allotype. The IgG1 protein (Pet) employed in these studies bears the Gm^f allotype markers with a

methionine at position 358 (23). An additional methionine is also present at position 428 (23). Cleavage of the biologically active plasmin-derived fragment of IgG1 with CNBr results in production of three peptides (Fig. 4)—the biologically active TMS Zorbax pool A peptide corresponding to the first 24 amino acid residues from the amino terminus (335–358), the inactive TMS Zorbax pool B peptide corresponding to the carboxy-terminal 18 amino acid residues (429–446) of the CH₃ domain, and the intervening biologically inactive peptide consisting of 70 amino acid residues (359–428), which is probably found in the high molecular weight pool 1 recovered from the HPLC gel-permeation column (Fig. 2). It can be concluded from these results that the ability of Fc to induce a polyclonal antibody response is mediated by a limited region of primary structure located at the amino-terminal end of the CH₃ domain. In addition to the biological activity associated with the CH₃ domain as described in this report, the CH₃ domain of IgG1 has been associated with the portion of the Fc region that binds to the Fc receptor (24). The exact location of the binding site in the Fc region remains a matter of controversy (25–29) and whether it includes the sequence described here is currently unknown.

It is now feasible to synthetically produce the Fc peptide and explore the active site of the molecule. On the basis of the re-

Table 1. Amino acid composition of TMS Zorbax fractions

Amino acid	Pool A, mole/mole	Eu IgG1 residues 335–358*	Pool B, mole/mole	Eu IgG1 residues 429–446*
Lysine	1.97	2.0	1.07	1.0
Histidine	0.11	0	3.0	3.0
Arginine	2.04	2.0	0	0
Aspartic acid	0	0	1.06	1.0
Threonine	2.02	2.0	1.0	1.0
Serine	1.98	2.0	2.79	3.0
Glutamic acid	5.01	5.0	2.21	2.0
Proline	4.41	4.0	1.13	1.0
Glycine	1.08	1.0	1.21	1
Alanine	1.01	1.0	1.06	1
Valine	0.97	1.0	0	0
Methionine [†]	—	1	—	0
Isoleucine	0.98	1	0	0
Leucine	1.07	1	2.98	3
Tyrosine	0.57	1	1.27	1.0
Phenylalanine	0	0	0	0

* Published sequence (22).

[†] Methionine was converted to homoserine lactone by the CNBr and was not quantitated by these analyses.

FIG. 4. A schematic representation of residues 334–445 of the Eu IgG1 protein. Plasmin cleaves at residue 334 and upon treatment with CNBr three peptides are produced. Pool A consists of residues 335–358 and contains all the biological activity. Pool B consists of the carboxy-terminal peptide, residues 429–445, and is unable to induce a polyclonal antibody response.

sults reported here, a 23-residue synthetic peptide based on the sequence of residues 335–357 (Fig. 4) was produced and found to exhibit all the activities of the natural peptide (unpublished data).

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