

SYNTHETIC Fc PEPTIDE-MEDIATED REGULATION OF THE IMMUNE RESPONSE

I. Characterization of the Immunomodulating Properties of a Synthetic 23-Amino Acid Peptide Derived from the Sequence of the CH₃ Domain of Human IgG₁*

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Passive in vivo administration of antibody has been shown to enhance as well as dampen specific immune responses upon subsequent injection of antigen (1-3). In addition to modulating specific immune responses, immune complexes have been shown to nonspecifically activate human (4, 5), rabbit (6), and murine (2, 7) lymphocytes. Fc fragments derived from enzymatic cleavage of human immunoglobulin (Ig) (2, 8-11) and aggregated human gamma globulin (AHGG)¹ (2, 12) have also been shown to induce human and murine bone marrow-derived (B) lymphocytes to proliferate and secrete polyclonal antibody. Moreover, Fc fragments possess the same immunoregulatory potential described for immune complexes in that both humoral (13, 14) and thymus-derived (T) cell-mediated (15, 16) responses are augmented. The underlying feature of lymphocyte activation by immune complexes, Fc fragments, and AHGG is that they all require the carboxyl terminal portion of the Fc region of Ig (17). The active fragment of Fc can be derived by macrophage-dependent enzymatic cleavage of Fc into biologically active 14-19,000 mol wt Fc subfragments (9, 11). The studies in this report demonstrate that a synthetic 23-amino acid peptide homologous to 335-357 in Eu IgG₁ (18) has the ability to nonspecifically activate lymphocytes and regulate ongoing immune responses.

Materials and Methods

Source of Peripheral Blood Lymphocytes (PBL). Venous blood was collected in heparinized Vacutainer tubes (Becton, Dickinson & Co., Rutherford, NJ) from healthy adult donors of

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Abbreviations used in this paper: AHGG, aggregated human gamma globulin; HPLC, high-performance liquid chromatography; PBL, peripheral blood lymphocyte; PFC, plaque-forming cell; NK, natural killer; *N-t*BOC, *N*-(*t*-butyloxycarbonyl); SRBC, sheep erythrocyte; TRF, T cell-replacing factor.

both sexes. Lymphocytes were separated by gradient centrifugation as previously described (11).

Animals. Male mice of the C57BL/6 strain were obtained from The Jackson Laboratory, Bar Harbor, ME. BALB/c mice were obtained from Scripps Clinic and Research Foundation, La Jolla, CA. All mice were between 8 and 15 wk old when used.

Solid Phase of Synthesis of IgG₁ Eu (335-357). Starting with *N*-(*t*-butyloxycarbonyl) (*N*-*t*-BOC)-cyclopentyl-L-glutamate resin ester, the appropriate amino acids (Aginomoto, Tokyo, Japan; Bach-Chem, Los Angeles, CA) were added stepwise as their *N*-*t*-BOC derivative using the standard Merrifield approach (19). However, residues Leu-351 and Pro-352 were added as the dipeptide, *N*-*t*-BOC-L-Leucyl-L-proline.

The deblocked peptide was purified by gel filtration on Sephadex G-25 Fine (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.2 M acetic acid, followed by preparative reverse-phase high-performance liquid chromatography (HPLC) on C₁₈-lipopolysaccharide-1 in 15% acetonitrile-0.05 M ammonium formate, pH 4.25. Finally, the peptide was chromatographed on Sephadex G-50 Fine (Pharmacia Fine Chemicals) in 0.2 M acetic acid, and lyophilized.

The final product was homogenous by analytical reverse-phase HPLC on an Altex Ultrasphere-ODS column (Altex Scientific Inc., Berkeley, CA) in acetonitrile-water-trifluoroacetic acid, and gave a satisfactory amino acid analysis. The sequence was confirmed by the automated Edman method. Samples used for biological tests were judged free of endotoxin by the limulus assay (20).

Preparation of Fc Fragments. A human IgG₁ myeloma protein (Fi) was a gift from Dr. Hans L. Spiegelberg, Research Institute of Scripps Clinic. Fc fragments were obtained by enzymatic digestion as previously described (8).

Isolation of Peripheral Blood B and T Cells. PBL suspensions were enriched for B and T cells by the neuraminidase-treated sheep erythrocyte (SRBC) rosette technique (11). The T cell populations were subjected to 2,000 rad (Gamma Cell 40; Atomic Energy of Canada, Ltd., Ottawa, Canada) before use in the *in vitro* polyclonal antibody response cultures (11).

Depletion of T Lymphocytes. T lymphocytes were depleted from murine spleen cell preparations by treatment with anti-T cell serum (Microbiological Associates, Walkersville, MD) and complement as described previously (10).

Enrichment of T Lymphocytes. Murine spleen cell populations were enriched for T cells by filtration through nylon wool columns (Fenwal Laboratories, Morton Grove, IL) (10).

Depletion of Macrophages. Spleen cells were filtered through columns of Sephadex G-10 (Pharmacia Fine Chemicals) by the method of Ly and Mishell (21) as described previously (7, 8).

Murine Lymphocyte Response Assays

PROLIFERATIVE RESPONSE. The Fc fragment-mediated proliferative response was carried out as previously described (8).

POLYCLONAL ANTIBODY RESPONSE. The Fc fragment-mediated polyclonal antibody response was carried out as previously described (10, 22).

IN VITRO ANTI-SRBC RESPONSE. Spleen cells were removed from mice injected intraperitoneally 4-6 wk previously with 0.1 ml 10% suspension of SRBC. A modified Mishell-Dutton culture system was used for the generation of antibody-producing cells (13).

IN VIVO ANTI-SRBC RESPONSE. Mice were given 0.1 ml 0.1% suspension of SRBC intraperitoneally, followed by saline or Fc peptide intravenously. The spleens were assessed for IgM plaque-forming cells (PFC) to SRBC 4 d postimmunization. Results are expressed as mean PFC from spleens of four to five mice plus standard error of the mean.

CYTOTOXIC RESPONSE. Mice were injected with peptide and 5 d later their spleens were assessed for the ability to lyse the natural killer (NK)-sensitive Yac-1 cell line. Targets were prepared by labeling Yac-1 cells with ⁵¹Cr (250-500 mCi/mg; Amersham Corp., Arlington Heights, IL) as previously described (16). The cell mixtures were then incubated for 4 h at 37°C in 5% CO₂, and aliquots of cell-free supernatant were then taken and their ⁵¹Cr content determined. The cytolytic activity was determined by the equation: percent specific release = [(experimental ⁵¹Cr release - control ⁵¹Cr release)/(maximum ⁵¹Cr release - control ⁵¹Cr release)] × 100. Maximum release = 3 × freeze thaw.

Human Lymphocyte Response Assays

POLYCLONAL ANTIBODY RESPONSE TO FC FRAGMENTS. The protocol used has been described in detail previously (11). The number of Ig-secreting cells was determined 6 d later by the protein A plaque assay (23).

IN VITRO ANTI-SRBC RESPONSE. The primary anti-SRBC response was done as described by Misiti and Waldmann (24). 5×10^6 PBL were cultured in 1 ml RPMI 1640 supplemented with 2 mM L-glutamine, 1% basal medium Eagle vitamins, antibiotics, 5×10^{-5} 2-mercaptoethanol, and the SRBC-absorbed autologous human plasma was substituted for the fetal calf serum.

ANTIGEN-INDUCED PROLIFERATIVE RESPONSE. The proliferative response to tetanus toxoid was carried out as described by Broff et al. (25). Briefly, 1×10^5 PBL were cultured with 5 μ g tetanus toxoid/ml (Commonwealth of Massachusetts Dept. of Public Health, Boston). The response was assayed on day 8 of culture.

Results

In Vitro Polyclonal Activation by Fc Peptide. The 23-amino acid synthetic Fc peptide shown in Fig. 1 was synthesized and assessed for biological activity. Increasing amounts of Fc peptide were cultured with murine spleen cells, and the polyclonal antibody responses were measured. The results in Fig. 2 reveal that murine spleen cells are stimulated by Fc peptide to secrete polyclonal antibody in a dose-dependent fashion. Optimal stimulation occurred when 7.5×10^{-5} μ mol peptide/ml (0.17 μ g/ml) was added; this response was comparable to that induced by 1×10^{-3} μ mol Fc/ml (166 μ g/ml). Because Fc fragments have also been shown to stimulate human PBL to secrete high levels of polyclonal antibody (11), the Fc peptide was assessed for its ability to stimulate polyclonal antibody production by normal human PBL. The addition of Fc peptide to in vitro PBL cultures resulted in the induction of a significant polyclonal antibody response (Table I). The optimal response was achieved with 1.3×10^{-5} μ mol peptide/ml (0.03 μ g/ml). On a mole per mole basis the Fc peptide is more active in inducing polyclonal antibody production than Fc. In contrast to Fc fragments, Fc peptide was unable to induce B cell proliferation (data not shown).

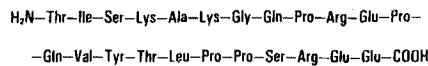


FIG. 1. Synthetic Fc peptide based on EU IgG₁ sequence 335-357.

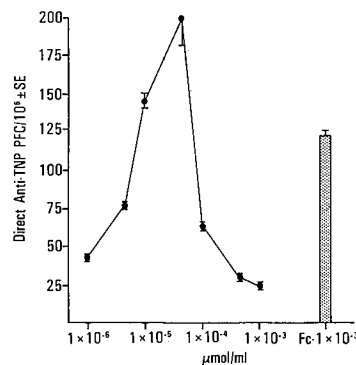


FIG. 2. Comparison of the ability of Fc peptide and Fc fragments to induce an in vitro polyclonal antibody response in murine spleen cell cultures.

TABLE I
Ability of a 23-Amino Acid Synthetic Fc Peptide to Induce a Polyclonal Antibody Response by Human PBL*

Stimulator	$\mu\text{mol/ml}$	Ig-secreting cells/ 10^6 B cells \pm SE
—	—	230 \pm 20
23 Peptide	1.3×10^{-6}	1,040 \pm 7
	1.3×10^{-5}	1,642 \pm 19
	1.3×10^{-4}	450 \pm 37

* Polyclonal antibody response was measured on day 6 of culture.

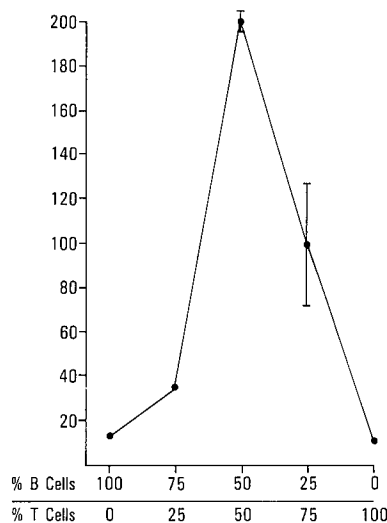


FIG. 3. Requirement for T cells in the Fc-peptide-induced murine polyclonal antibody response. Varying ratios of T/B cells were assessed for their ability to make polyclonal antibody in response to Fc peptide stimulation. 7.5×10^{-5} μmol (0.17 μg) Fc peptide/ml was used. Direct anti-TNP PFC \pm SE were measured.

The Fc fragment-mediated polyclonal antibody response has been shown to have a strict requirement for accessory T cells (10, 11, 26) and macrophages (10, 11). To ascertain the role of T cells in the Fc peptide-induced polyclonal antibody response, cell-mixing experiments were performed. Spleen cell populations depleted of T cells were found to be unable to produce polyclonal antibody in response to Fc peptide stimulation (Fig. 3), thus indicating the need for T cells. Restoration of the polyclonal antibody response was accomplished by the addition of nylon wool-purified T cells to culture (Fig. 3). A mixture of $\sim 50\%$ B cell-enriched and 50% T cell-enriched spleen cells produced the optimal antibody response.

To assess the need for macrophages, spleen cell preparations were filtered through columns of Sephadex G-10 before stimulation with Fc peptide. The results reveal that Sephadex G-10 filtration had no effect on the level of polyclonal antibody produced (Fig. 4). However, as previously reported (10, 11), G-10 filtration severely diminished the polyclonal antibody response induced by Fc.

Augmentation of In Vitro and In Vivo Humoral Immune Responses. To study whether the Fc peptide has the capacity to act as an adjuvant, it was added to the in vitro spleen

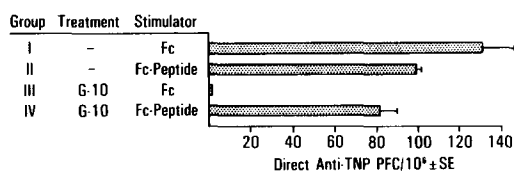


FIG. 4. The requirement for macrophages in the Fc-peptide-induced murine polyclonal antibody response was assessed by Sephadex G-10 filtration of the spleen cells. Cultures received either $7.5 \times 10^{-5} \mu\text{mol}$ ($0.17 \mu\text{g}$) Fc peptide/ml or $1 \times 10^{-3} \mu\text{mol}$ ($166 \mu\text{g}$) Fc/ml.

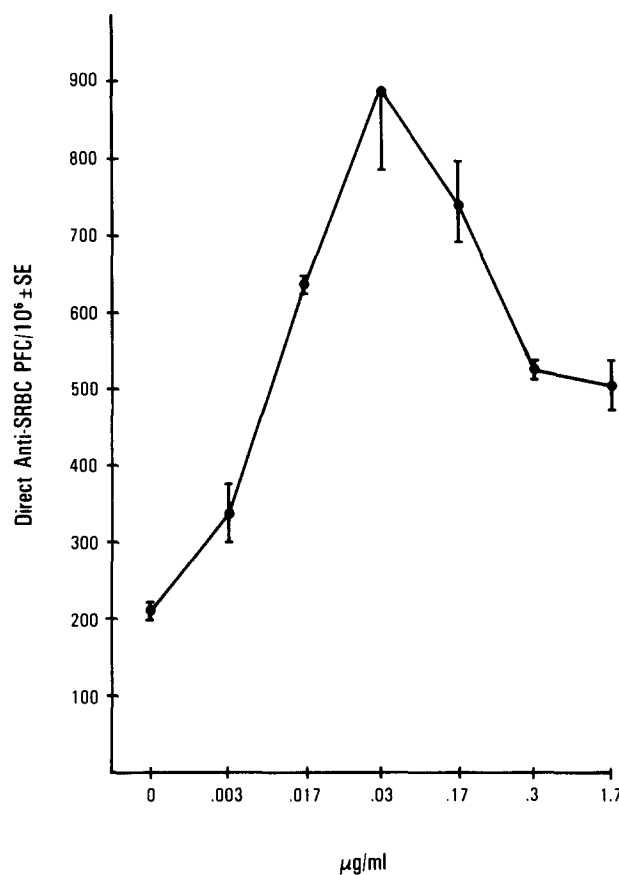


FIG. 5. The ability of Fc peptide to augment the murine in vitro anti-SRBC was assessed by culturing spleen cells with Fc peptide and 0.1 ml of a 1% suspension of SRBC.

cell cultures containing SRBC and the responses were measured. The data presented in Fig. 5 show that the addition of Fc peptide to murine spleen cell cultures along with low doses of antigen results in a significant augmentation of the response. Maximal enhancement (4–5-fold) occurred when $0.03 \mu\text{g}$ Fc peptide/ml was used in culture. In addition to the effects of Fc peptide on the murine in vitro response, the primary human anti-SRBC response was analyzed. Co-culture of SRBC and Fc peptide in PBL cultures produced an enhanced (5–6-fold) anti-SRBC response as compared with cultures receiving only SRBC (Fig. 6). To further explore the adjuvant properties of the Fc peptide in vivo, mice were injected with peptide and SRBC and

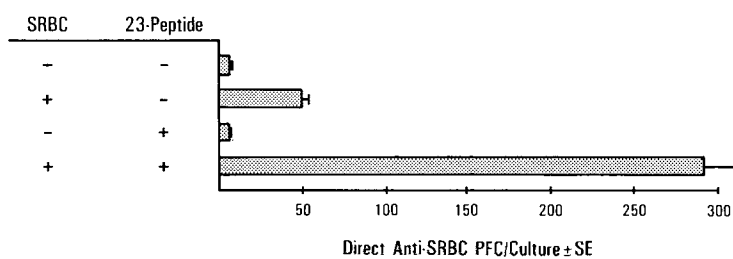


FIG. 6. The ability of Fc peptide to augment the human in vitro anti-SRBC response was assessed by culturing human PBL with 1.3×10^{-5} μ mol (0.03 μ g) Fc peptide/ml and 1×10^{-6} SRBC/ml. The response was measured on day 11 of culture.

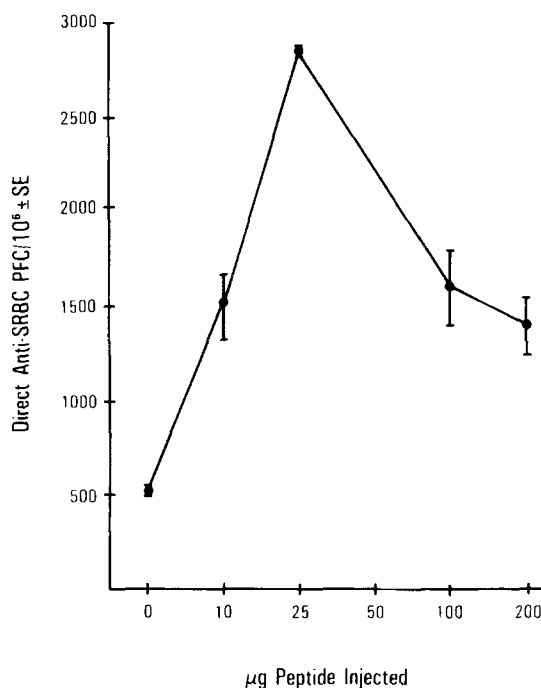


FIG. 7. The ability of Fc peptide to augment the in vivo anti-SRBC response was assessed by injecting mice with increasing amounts of peptide and a constant amount of SRBC (0.1 ml of 1% suspension of SRBC). The response was measured on day 5.

the antibody response was measured. The results indicate that in vivo administration of the Fc peptide produces a significant augmentation (5-6-fold) of the primary anti-SRBC response (Fig. 7). Optimal stimulation was observed when 25 μ g Fc peptide per mouse was given intravenously. The results in Fig. 8 were obtained when antigen and peptide were both injected on day 0.

Augmentation of In Vitro T Cell-mediated Proliferative Responses. In addition to potentiating humoral immune responses (13, 14), Fc fragments have been shown to enhance murine (15, 16) and human (27) T cell-mediated responses. To ascertain whether the synthetic Fc peptide maintained this capacity, the tetanus toxoid-induced T cell proliferative response was examined. Stimulation of human PBL with tetanus toxoid in the presence of Fc peptide resulted in a pronounced enhancement of the prolifer-

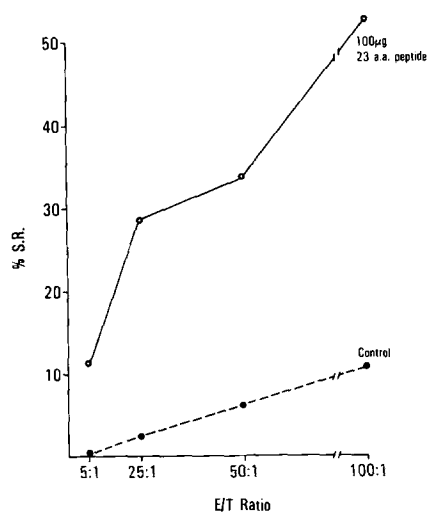


FIG. 8. The ability of Fc peptide to augment NK cell activity. Mice were injected with 100 µg Fc peptide and their spleens were used as the source of NK cells 5 d later. NK activity was measured by the ability of spleen cells to lyse ^{51}Cr -labeled Yac-1 target cells in a 4h assay. a.a., amino acid; E/T ratio, effector/target cell ratio; S.R., specific release.

TABLE II
Ability of the 23-Amino Acid Peptide to Augment Tetanus Toxoid-induced Proliferation in Human PBL Cultures

Cells	Tetanus toxoid (3 µg/ml)	23 Peptide (0.017 µg/ml)	[^3H]Thymidine \pm SE
+	-	-	5,133 \pm 2,069
+	+	-	41,770 \pm 372
+	+	+	101,730 \pm 320
+	-	+	5,911 \pm 1,391

ative response (Table II). These results indicate that Fc peptide, like Fc fragments, can potentiate both humoral and T cell-mediated immune responses.

Augmentation of NK Responses. Because in vivo administration of Fc fragments and synthetic Fc peptide results in the potentiation of immune responses, experiments were conducted to assess whether Fc peptide was capable of enhancing NK cell activity. As shown in Fig. 8, spleen cells from mice receiving Fc peptide were superior in their ability to lyse the NK-sensitive Yac-1 cell compared with those from untreated control mice.

Discussion

A synthetic 23-amino acid peptide derived from the CH_3 domain of human IgG_1 was found to be a potent adjuvant as well as being a polyclonal activator. These responses are reminiscent of the immunoregulatory activities that have been described for Fc fragments (7-11), AHGG (12), and immune complexes (2, 7).

The synthetic Fc peptide, on a mole per mole basis, is more potent than native Fc fragments in inducing polyclonal antibody production and potentiating immune

responses. The reason for this difference is unknown and a matter of speculation. Two possibilities that could account for the gain in activity are: (a) that Fc peptide has a higher affinity for receptors on lymphocytes, and (b) that a portion of the Fc molecule responsible for suppressing immune responses is not associated with the synthetic peptide.

Accessory T cells are required to generate the Fc peptide-induced polyclonal antibody response. This finding is identical to what is observed when Fc fragments are used. The role of the T cell in the Fc fragment-mediated polyclonal antibody response is the production of a soluble T cell-replacing factor (TRF), FcTRF (26). Incubation of Fc fragments with Lyt-1⁺2⁻ T cells results in the secretion of FcTRF, which acts directly on the B cells, inducing them to differentiate to plasma cells and secrete antibody (27). The generation of polyclonal antibody by Fc peptide is independent of macrophages. These results are in contrast to Fc fragment stimulation of murine and human lymphocytes (10, 11). Fc peptide behaves in a fashion similar to Fc subfragments (10) and plasmin-derived fragments (7). The role of the macrophage is to enzymatically digest the Fc fragment into biologically active 14–19,000-mol wt Fc subfragments that are responsible for inducing the polyclonal antibody response (8–11). Likewise, the plasmin fragment can stimulate lymphocytes without macrophage processing (7). Both Fc subfragment and plasmin Fc are capable of inducing B cell proliferation (7), which contrasts with the results obtained with Fc peptide. One possibility for this finding is that B cell proliferation requires another portion of the CH₃ domain. Alternatively, a portion of the CH₃ domain in conjunction with this peptide is required for proliferation.

The immunomodulatory ability of the Fc fragment apparently lies in the Fc peptide since this peptide has the capacity to enhance both humoral and cell-mediated immune responses. Injection of Fc peptide along with antigen or the addition of peptide with antigen to *in vitro* cultures produces a pronounced augmentation of the specific antibody response. As described for the Fc fragment-induced adjuvant effect, the dose of antigen used is critical. Fc peptide can induce the greatest enhancement when suboptimal antigen doses are used. In addition to Fc fragments and Fc peptide, antigen concentration has been shown to play a critical role in the adjuvanticity of other agents (28). In addition to potentiating antibody responses, Fc peptide can enhance antigen-induced T cell proliferation. We have previously shown that the addition of Fc fragments to *in vitro* human PBL cultures enhances the proliferative response to tetanus toxoid (17).

Fc peptide can also modulate immune cytotoxicity mediated by NK cells. Intravenous injection of Fc peptide renders the spleen cells of C57BL/6 mice more reactive to the NK-sensitive Yac-1 cell line. *In vivo* and *in vitro* administration of agents that induce an increase in interferon also enhance NK-mediated lysis (29). It has been postulated that the enhancement of NK activity by interferon-inducing agents occurs through an increase in the lytic potential of effector cells as opposed to clonal expansion. The mechanism by which Fc peptide augments NK lytic activity is unknown. Thus, experiments are currently in progress to determine if Fc peptide induces an increase in interferon levels.

The observation that a synthetic peptide derived from the CH₃ domain of human Ig can modulate homologous and heterologous immune responses opens up the possibility of using the synthetic Fc peptide as a potential human adjuvant. An ideal

adjuvant would be a nonimmunogenic substance that could potentiate immune reactivity and yet not produce nonspecific inflammatory reactions.

Summary

A synthetic 23-amino acid peptide derived from the CH₃ domain of human IgG₁ was found to be a potent adjuvant as well as a polyclonal activator. The Fc peptide was found to enhance human and murine humoral, and T cell-mediated immune responses. Moreover, in vivo administration of Fc peptide enhanced murine natural killer cell activity. The synthetic Fc peptide was found to be more potent, on a molar basis, than native Fc fragments in inducing polyclonal antibody production and potentiating immune responses.

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