

Endogenous Superallergen Protein Fv Induces IL-4 Secretion from Human FcεRI⁺ Cells Through Interaction with the V_H3 Region of IgE¹

Vincenzo Patella,* Ada Giuliano,[†] Jean-Pierre Bouvet,[‡] and Gianni Marone^{2*}

We investigated the mechanism whereby protein Fv (pFv), a human sialoprotein found in normal liver and largely released in the intestinal tract in patients with viral hepatitis, induces mediator release from basophils and mast cells and evaluated whether it also induces IL-4 synthesis and secretion in basophils. pFv is a potent stimulus for histamine and IL-4 release from purified basophils. Histamine and IL-4 secretion from basophils activated by pFv was significantly correlated ($r_s = 0.70$; $p < 0.001$). There was also a correlation ($r_s = 0.58$; $p < 0.01$) between the maximum pFv- and anti-IgE-induced IL-4 release from basophils. The average $t_{1/2}$ for pFv-induced histamine release was lower (3.5 ± 1.5 min) than for IL-4 release (79.5 ± 8.5 min; $p < 0.01$). IL-4 mRNA, constitutively present in basophils, was increased after stimulation by pFv and was inhibited by cyclosporin A and tacrolimus. Basophils from which IgE had been dissociated by brief exposure to lactic acid no longer released IL-4 in response to pFv and anti-IgE. The response to an mAb cross-linking the α -chain of FcεRI was unaffected by this treatment. Three human V_H3⁺ monoclonal IgM concentration-dependently inhibited pFv-induced secretion of IL-4 and histamine from basophils and of histamine from human lung mast cells. In contrast, V_H6⁺ monoclonal IgM did not inhibit the release of IL-4 and histamine induced by pFv. These results indicate that pFv, which acts as an endogenous superallergen, interacts with the V_H3 domain of IgE to induce the synthesis and release of IL-4 from human FcεRI⁺ cells. *The Journal of Immunology*, 1998, 161: 5647–5655.

Protein Fv (pFv)³ is an Ig-binding protein present in normal liver and largely released in the intestinal tract in a significant percentage of patients affected by acute and chronic viral hepatitis B (HBV; ~35%), C (HBC; ~43%), A, and E (1, 2). pFv is an acidic sialoprotein (pI, 4.0) with a molecular mass of 175 kDa that dissociates into 85-kDa monomers during SDS-PAGE. pFv binds to Ig from various mammalian and nonmammalian species (3); it binds the variable domain of heavy chains (V_H), irrespective of Ig class, subclass, allotype, and light (L) chain type (4). Silverman and Bouvet recently demonstrated that the binding of this molecule involves mainly the human V_H3 family (5, 6). Because, like staphylococcal protein A (6), pFv binds outside the

Ag-binding site of Ab (4), it has been allocated to a new group of B cell superantigens (5, 7).

A wide spectrum of hepatic and extrahepatic tissue injuries, not necessarily directly related to the cytotoxic effects of HBV and HCV infections, are associated with acute and chronic viral hepatitis (8, 9). Skin rashes, urticarial reactions involving skin mast cells, and cutaneous and systemic vasculitis occur in patients with viral hepatitis (8–11). Human basophils and mast cells, through the release of vasoactive and proinflammatory mediators (histamine, etc.) (12, 13) and the elaboration of various cytokines (IL-4, etc.) (14–21), play a role in cutaneous and systemic vasculitis (12) and stimulate the collagen synthesis (22, 23) characteristic of the fibrotic response in chronic liver diseases (24).

Several laboratories have demonstrated that human basophils express and secrete such cytokines as IL-4 (17–19), IL-13 (19, 20), and macrophage inflammatory protein (MIP)-1 α (21). The striking correlation between IL-4 mRNA and protein secretion from basophils activated by IgE-mediated stimuli suggested that basophils are a major source of IL-4 (18). This important cytokine promotes isotype switching and induction of IgE synthesis by B cells (25–27), down-regulates the production of proinflammatory cytokines by monocytes and macrophages (28, 29), stimulates the expression of VCAM-1 on endothelial cells (30), and displays antitumor activity in vivo (31). IL-4 also regulates the cytokine profile and function of CD4⁺ T cells (26, 32).

We have previously demonstrated that pFv induces the release of proinflammatory mediators from human basophils and mast cells isolated from lung parenchyma and skin tissue, presumably by interacting with the IgE bound to FcεRI (33). This study has been undertaken to investigate the mechanism of the effects of pFv on the release of mediators from human basophils and mast cells and to evaluate whether this protein induces IL-4 synthesis and secretion in human basophils.

*Division of Clinical Immunology and Allergy, University of Naples Federico II, and [†]Istituto Nazionale Tumori Giovanni Pascale, Naples, Italy; and [‡]Unité d'Immunopathologie Humaine, Institut National de la Santé et de la Recherche Médicale U430, Hôpital Broussais, Paris, France

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² Address correspondence and reprint requests to Dr. Gianni Marone, Division of Clinical Immunology and Allergy, University of Naples Federico II, Via S. Pansini 5, 80131 Napoli, Italy. E-mail address: marone@unina.it

³ Abbreviations used in this paper: pFv, Fv fragment-binding protein; anti-FcεRI α , anti- α chain mAb of high affinity receptor for IgE; Anti-IgE, rabbit IgG anti-Fc fragment of human IgE; CsA, cyclosporin A; HBV, hepatitis B virus; HCV, hepatitis C virus; HLMC, human lung mast cells; ICS, internal calibration standard; L chain, light chain; rIL-3, recombinant human IL-3; P, PIPES buffer containing 25 mM PIPES, pH 7.37, 110 mM NaCl, and 5 mM KCl; PCG, PIPES buffer containing 2.0 mM CaCl₂ and 1 g/L D-glucose; TCF, Ca²⁺- and Mg²⁺-free Tyrode's buffer; TGMD, TCF buffer containing 0.25 g/L MgCl₂·6H₂O, 10 mg/L DNase, and 1 g/L gelatine.

Materials and Methods

Reagents

The following were purchased: 60% HClO₄ (Baker Chemical, Deventer, The Netherlands); PIPES, hyaluronidase, collagenase, chymopapain, and elastase type I (Sigma, St. Louis, MO); HBSS and FCS (Life Technologies, Grand Island, NY); deoxyribonuclease I (Calbiochem, La Jolla, CA); RPMI 1640 with 25 mM HEPES buffer, Eagle's MEM (Flow Laboratories, Irvine, Scotland); Dextran 70, Percoll, and protein A-Sepharose (Pharmacia, Uppsala, Sweden). Rabbit anti-human-Fc Ab was a generous gift from Drs. Teruko and Kimishige Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, CA). The mAb against the α -chain of Fc ϵ R1 was a generous gift of Dr. John Hakimi (Roche Research Center, Hoffmann-La Roche, Nutley, NJ).

Preparation of pFv

pFv was purified by size permeation through a set of three 16 $\times$ 900-mm columns of S300 Sephacryl (Pharmacia) from the stool extracts of two patients (FAR and TER) affected by HCV hepatitis. Under these conditions, pFv elutes in the 175-kDa fraction as confirmed by HPLC with a calibrated Superose 12 column (Pharmacia) (1). Purified fractions were extensively dialyzed against PBS to remove both sodium deoxycholate and sodium azide and were stored at 4°C under sterile conditions.

Ig-binding test

pFv preparations were assayed for Ig-binding activity by a nonimmune ELISA-like method (1). Polyclonal normal IgG (2.5 μ g/ml) or F(ab')₂ fragments were coated on Nunc plates. After three washes with 0.1% Tween 20 in PBS, the plates were saturated with a PBS solution containing 1% BSA and 0.05% Tween 20. The diluted extracts were left overnight at 4°C to bind the coated Ig. Ig-binding molecules were then revealed by their nonimmune binding to 0.5 μ g/ml of peroxidase-labeled human IgG or F(ab')₂ fragments after a 1-h incubation at 37°C in BSA-Tween PBS. This assay thus detected only bivalent or multivalent molecules. Results were considered positive when the OD was >0.1 U at 492 nm and more than twofold the OD of a negative control. For each preparation of pFv, the activity giving 0.5 OD at 492 nm was obtained with the following dilutions: 1:200 (TER pFv) and 1:128 (FAR pFv). The appropriate concentration of pFv as ponderal value was measured by this assay as described elsewhere (6).

Absorption experiments

For pFv absorption, 9 vol of sample were incubated twice with 1 vol of IgG-coated protein A-Sepharose beads under constant mixing at 37°C for 1 h. The supernatant was then checked for loss of pFv activity, extensively dialyzed, and stored at 4°C under sterile conditions. To remove Ig, 9 vol of sample were incubated with 1 vol of anti-F(ab')₂ immunosorbent. The latter was obtained by CNBr insolubilization of specific Abs raised in sheep, a species unreactive with pFv (3). In both cases, unabsorbed samples had been incubated with uncoated Sepharose 4B.

Buffers

The PIPES buffer (P) used in these experiments contains 25 mM PIPES, pH 7.37, 110 mM NaCl, 5 mM KCl. PCG buffer contains, in addition to P, 5 mM CaCl₂ and 1 g/L dextrose (33); pH was titrated to 7.4 with sodium bicarbonate. PBS contains (g/L): NaCl, 8.0; Na₂HPO₄·7H₂O, 2.89; KH₂PO₄, 0.2; KCl, 0.2, pH 7.3. TCF buffer contains (g/L): NaCl, 8.0; KCl, 0.2; NaH₂PO₄, 0.05; NaHCO₃, 0.28; D-glucose, 1.0, pH 7.3; TGMD contains, in addition to TCF, pH 7.3, 0.25 g/L MgCl₂·6H₂O, 10 mg/L DNase, and 1 g/L gelatin (34).

Purification of human monoclonal IgE and IgM

IgE myeloma proteins were purified from the sera of two myeloma patients (PP and ADZ) by repeated gel filtration on Sephadex G-200, followed by elution through a Sepharose CL-4B column. RIA showed no IgG, IgM, or IgA contamination (35). Monoclonal IgM were purified from the sera of patients with Waldenström's macroglobulinemia by gel permeation in a diluted buffer as described (36). Variable regions of these monoclonal IgM were determined using a well-characterized panel of primary sequence-dependent V_H and V_K family-specific reagents that identify previously described framework regions (6).

Purification of peripheral blood basophils

Basophils were purified from peripheral blood cells of normal subjects, ages 21 to 39 yr (mean age, 32.4 $\pm$ 4.1 yr), undergoing hemapheresis. "Buffy

coat" cell packs from healthy volunteers, provided by the Immunohematology Service at the University of Naples Federico II, were reconstituted in PBS containing 0.5 g/L human serum albumin and 3.42 g/L sodium citrate and loaded onto a countercurrent elutriator (model J2-21, Beckman Instruments, Fullerton, CA). Several fractions were collected, and fractions containing basophils in large numbers (>20 $\times$ 10⁶ basophils) and of improved purity (>15%) were further enriched by discontinuous Percoll gradients (37). Yields by this technique ranged from 3 to 10 $\times$ 10⁶ basophils with a purity from 74–98%, as assessed by basophil staining with Alcian blue and counting in a Spiers-Levy eosinophil counter (38).

Isolation and purification of human lung mast cells (HLMC)

Macroscopically normal lung tissue obtained from patients undergoing thoracotomy and lung resection, mostly for lung cancer, was dissected free from pleura, bronchi, and blood vessels, minced into 3- to 8-mm fragments and dispersed into single-cell suspension as described (34). Yields with this technique ranged between 3–20 $\times$ 10⁶ mast cells, and mast cell with a purity ranged between 1 and 8%. The cells were resuspended and incubated overnight in RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine, 1% gentamicin, and 10% FCS as previously described (13). Mast cells isolated from lung parenchyma were fractionated in a Beckman elutriator fitted with an elutriation apparatus; elutriation fractions with the highest purity mast cells were pooled and were further purified by flotation in Percoll density gradients as described (34). The fractions rich in mast cells were then counted by Alcian blue staining (38).

Histamine release assay

Basophils (~6 $\times$ 10⁴ basophils/tube) or mast cells (~3 $\times$ 10⁴ mast cells/tube) cells resuspended in PCG, and 0.1 ml of the cell suspension were placed in 12 $\times$ 75-mm polyethylene tubes and warmed to 37°C; 0.1 ml of each prewarmed stimulus for release was added, and incubation was continued at 37°C for 45 min (histamine release) or 4 h (IL-4 secretion). At the end of this step, the reaction was stopped by centrifugation (1000 $\times$ g, 22°C, 2 min), and the cell-free supernatants were stored at -20°C for subsequent assay of histamine and IL-4 content. The cell-free supernatants were assayed for histamine with an automated fluorometric technique (39). Total histamine content was assessed by lysis induced by incubation of cells with 2% perchloric acid before centrifugation. To calculate histamine release as a percentage of total cellular histamine, the "spontaneous" release of histamine from basophils (0–10% of the total cellular histamine) and mast cells (5–20% of the total cellular histamine) was subtracted from both numerator and denominator. All values are based on means of duplicate or triplicate determinations. Replicates differed from each other in histamine content by <10%.

IL-4 ELISA

The harvested supernatants were assayed for IL-4 by using the IL-4 Quantikine High sensitivity kit (R&D Systems, Minneapolis, MN). The standard curve for this kit was run in the same RPMI 1640 medium used for the release experiments (18).

Isolation of cellular mRNA

RNA was isolated by harvesting the basophils from culture wells and centrifuging for 30 s at 10,000 $\times$ g. After removing the supernatants, the cell pellet was extracted with RNeasy B (Tel-test, Friendswood, TX), which is a modified guanidinium thiocyanate single-step procedure. In this procedure, RNA is extracted from an aqueous phase after the addition of CHCl₃. The isopropanol precipitated RNA was washed once with 70% ethanol, dried, resuspended in 25 μ L of diethylpyrocarbonate-treated water, and stored at -80°C.

RT-PCR and quantitative PCR

An aliquot of total cellular mRNA was reversed transcribed to cDNA and PCR expanded using the GeneAmp RNA PCR Core Kit (Perkin-Elmer, Nieuwerkerk, The Netherlands) according to the manufacturer's protocol. In this protocol, RT was performed on 2 μ L of the RNA extract (10–20% of the total RNA extracted). The RT mix (5 mM MgCl₂) was incubated for 20 min in a Perkin-Elmer Cetus thermocycler followed by 2 min at 95°C to inactivate the reverse transcriptase. Buffers, dNTPs (final concentration, 0.4 mM each), Amplitaq polymerase (1 U/50 μ L reaction), and paired primers (0.5 μ M each) were added to RT tubes (bringing MgCl₂ to 2 mM) and the PCR reaction cycled according to the following protocol: denaturation at 95°C for 15 s, annealing at 60°C for 15 s and at 72°C for 30 s. IL-4 was cycled 56 times before a 15-min incubation at 72°C for a final extension. The primers for IL-4 and IFN- γ were performed and synthesized by a commercial source (Life Technologies Italia, Milan, Italy) based on the

known cDNA sequences of the following cytokines: IL-4, 5' primer (5'-ATG GGT CTC ACC TCC CAA CTG CT) and 3' primer (5'-GTT TTC CAA CGT ACT CTG GTT GGC); IFN- γ , 5' primer (5'-ATG AAA TAT ACA AGT TAT ATC TTG GCT TT) and 3' primer (5'-GAT GCT CTT CGA CCT CGA AAC AGC AT).

An aliquot (10 μ l) of the reaction product was visualized on 2% agarose in buffer containing 0.5 μ g/ml of ethidium bromide. As a negative control, an aliquot of each RNA sample was subjected to PCR amplification without the reverse transcription step.

In some experiments, an aliquot of total cellular mRNA was reverse-transcribed to cDNA and PCR expanded for quantitative PCR using a Cytopress detection kit (BioSource, Camarillo, CA). This technique is a competitive PCR in which a known copy number of an exogenously synthesized DNA, known as the internal calibration standard (ICS), is mixed with the sample cDNA prior to amplification. The ICS was constructed to contain PCR primer binding sites identical to the IL-4 cDNA and a unique capture binding site that allows the resulting ICS amplicon to be distinguished from the IL-4 amplicon. The Cytopress kit contains IL-4 primers, one of which is biotinylated, to be included in the PCR mix. During amplification, biotin-labeled primer is incorporated into both ICS and IL-4 amplicons. After PCR, the amplicons are denatured and hybridized to either ICS or IL-4 sequence-specific capture oligonucleotides. Capture oligonucleotides are prebound to microtiter wells. The captured sequences are detected and quantified by the addition of an enzyme-streptavidin conjugate followed by substrate. The signal generated in the reaction is proportional to the amount of amplicon present. Since the ICS is amplified at an efficiency identical to the IL-4 cDNA, it can serve as a standard for IL-4 cDNA quantitation. The number of copies of IL-4 in each PCR reaction is calculated from the ratio of the total OD for the IL-4-specific well to the total OD for the ICS well and the input copy number of the ICS. The following formula is used to calculate the starting copies of IL-4 cDNA in the PCR reaction: $[(\text{total IL-4}/\text{total ICS OD}) \times (2 \times \text{ICS input copy number})] = \text{starting copy number of IL-4 cDNA}$. Factor 2 is used to correct for dsDNA ICS. The copy number has been adjusted for any dilution done on cDNA before amplification according manufacturer's protocol.

Statistical analysis

The results are the mean $\pm$ SEM. The data subjected to linear regression were calculated by the least squares method ($y = a + bx$) in which a was the y-axis intercept and b the slope of the line. The correlations were calculated by Student's t test (40).

Results

Comparative effects of pFv and anti-IgE on histamine release from human basophils

In a first series of experiments, we compared the histamine-releasing activity of increasing concentrations of pFv purified from patients FAR and TER on histamine release from human basophils. Low concentrations of pFv (0.1–1 ng/ml) concentration-dependently released histamine from basophils purified (64–93%) from seven donors. pFv was at least 100 times more potent than anti-IgE (Fig. 1). pFv had no effect on basophil viability (data not shown). The same preparations of pFv (FAR and TER) absorbed with protein A-Sepharose coated with human polyclonal IgG, which were negative for Ig binding, did not induce histamine release. This result indicates that pFv was responsible for mediator secretion from basophils by binding to Ig.

Effects of pFv on IL-4 release from human basophils

In a second series of experiments, we evaluated the effects of increasing concentrations of pFv (FAR and TER) on histamine and IL-4 release from human basophils. Fig. 2 shows that pFv (0.1–1 ng/ml) concentration-dependently induced the release of histamine and IL-4 from purified (60–96%) basophils. The same preparations of pFv (FAR and TER) absorbed with protein A-Sepharose coated with normal IgG did not induce histamine or IL-4 release in any subject studied.

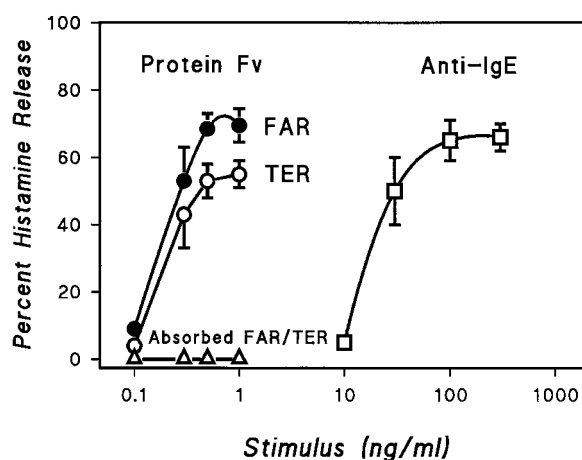


FIGURE 1. Effect of increasing concentrations of anti-IgE and pFv obtained from donors FAR and TER absorbed with protein A-Sepharose coated with polyclonal IgG, which does not bind Ig, and was not absorbed on histamine secretion from purified (64%–93%) human basophils ($n = 7$). Values are expressed as the mean $\pm$ SEM. Error bars are not shown when details are too small.

Correlation between IL-4 and histamine release from human basophils induced by pFv

In eight experiments, using basophils enriched to 95–98% purity, cells were challenged with a wide range of concentrations of pFv (0.3–1 ng/ml) obtained from patients FAR and TER (Fig. 3). pFv concentration-dependently induced both IL-4 and histamine release from basophils. A significant correlation was found between the percent histamine release and IL-4 secretion induced by pFv ($r_s = 0.70$; $p < 0.001$). These results are in agreement with the hypothesis that pFv induces IL-4 secretion from basophils.

Kinetics of pFv-induced IL-4 and histamine release from human basophils

Fig. 4 compares the kinetics of histamine and IL-4 release from basophils challenged with an optimal concentration (0.5 ng/ml) of pFv. Histamine release induced by maximal stimulation was complete within 5 min, whereas IL-4 release was complete in 2–3 h.

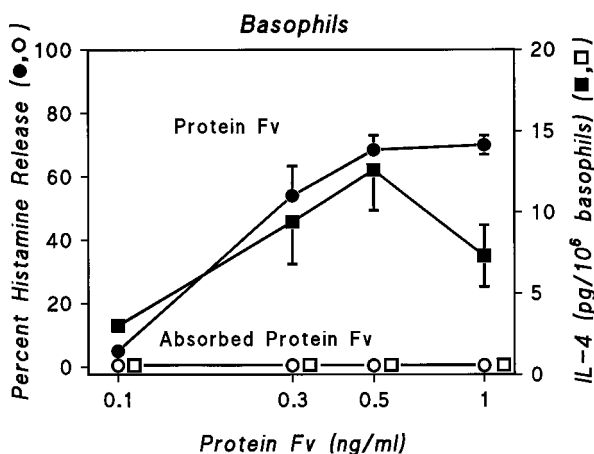


FIGURE 2. Effect of increasing concentrations of pFv on histamine and IL-4 secretion from human basophils obtained from normal donors ($n = 5$). pFv (FAR and TER) was absorbed with protein A-Sepharose coated with polyclonal IgG or was not absorbed. Values are expressed as the mean $\pm$ SEM. Error bars are not shown when details are too small.

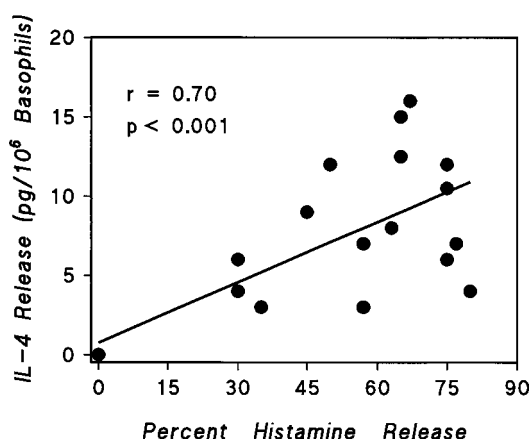


FIGURE 3. Correlation between the maximum percentage of histamine release and IL-4 secretion caused by pFv from human basophils ($n = 8$). Each point represents the mean of duplicate determinations from separate experiments.

The average $t_{1/2}$ for histamine release was 3.5 ± 1.5 min, whereas the average $t_{1/2}$ for IL-4 release was 79.5 ± 8.5 min ($p < 0.001$).

Effect of pFv on IL-4 mRNA expression in human basophils

The studies described above support the hypothesis that human basophils secrete IL-4 after stimulation with pFv. Further evidence in favor of this hypothesis comes from a series of measurements of the IL-4 mRNA present in resting and in pFv-stimulated basophils. In these experiments, highly purified basophils (97–99%) were cultured with recombinant human IL-3 (rhIL-3; 10 ng/ml) for 16 h, washed, and challenged with anti-IgE (0.3 μ g/ml) or pFv (0.5 ng/ml) in the presence of rhIL-3 (10 ng/ml). We measured IL-4 mRNA expression in basophils by RT-PCR. In three experiments, one of which is illustrated in Fig. 5, we found that IL-4 mRNA is constitutively expressed in human basophils (Fig. 5A); panel A also shows that pFv induced an apparent increase in IL-4 mRNA similar to the previously reported stimulation with anti-IgE (18). In these experiments in which IL-4 mRNA was detected by RT-PCR, we were unable to find any IFN- γ mRNA expression in control basophils or in pFv- and anti-IgE-stimulated basophils (Fig. 5B). Therefore, IL-4 mRNA was extracted from purified preparations of basophils uncontaminated by IFN- γ -synthesizing leukocytes (e.g., lymphocytes and monocytes).

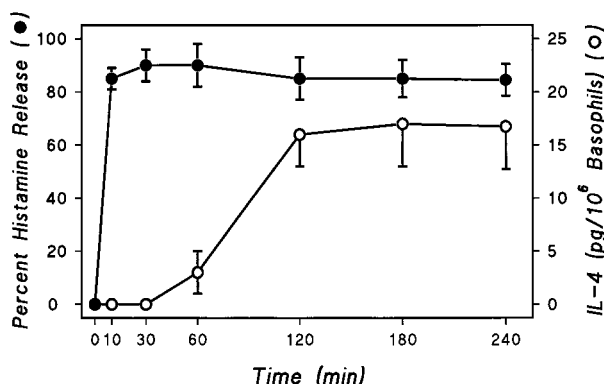


FIGURE 4. Kinetics of IL-4 and histamine secretion from human basophils induced by pFv (0.5 ng/ml). Each point represents the mean $\pm$ SEM obtained from three experiments. Error bars are not shown when details are too small.

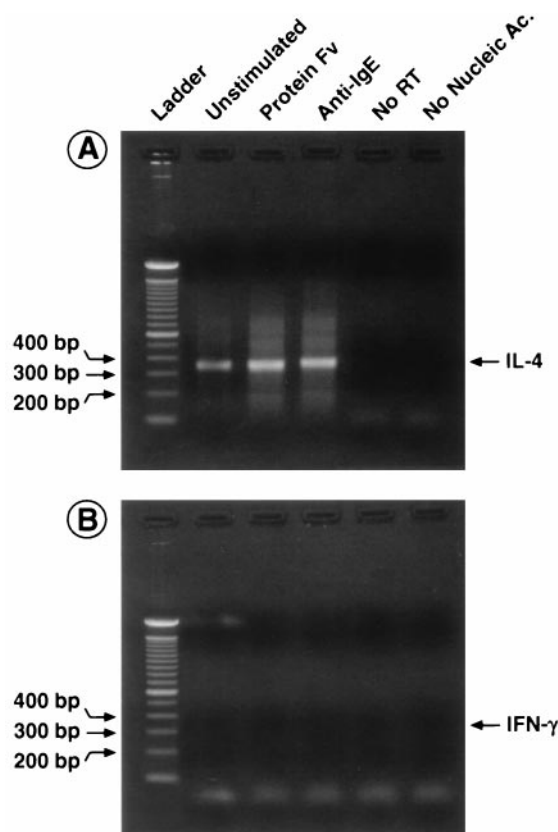


FIGURE 5. RT-PCR analysis of expression of IL-4 mRNA in human peripheral blood basophils stimulated via Fc ϵ RI. Total cellular RNA (2.5 μ g) was extracted from highly purified (~98%) basophils after a 16-h preincubation with rhIL-3 (10 ng/ml), washed, and then incubated with rhIL-3 (10 ng/ml) in the absence or presence of anti-IgE (0.3 μ g/ml) or pFv (0.5 ng/ml) at 37°C for 4 h. mRNA for IL-4 (A) and IFN- γ (B) was amplified by RT-PCR, subjected to electrophoresis, and visualized by ethidium bromide. In the absence of cDNA (No RT) and nucleic acid (No Nucleic Ac.), no PCR product is visualized.

In a second series of experiments, we used a quantitative PCR to confirm that pFv induced an increase in IL-4 mRNA similar to the stimulation with anti-IgE. Fig. 6 shows a representative result of three experiments in which we examined the effects of anti-IgE and pFv on the secretion of histamine, on the extracellular protein levels of IL-4, and on the specific IL-4 mRNA in unstimulated purified (~97%) basophils and cells activated by anti-IgE (0.3 μ g/ml) or pFv (0.5 ng/ml). These experiments confirmed the presence of constitutive expression of IL-4 mRNA and demonstrated that pFv and anti-IgE increased specific IL-4 mRNA copies (Fig. 6A). The results shown in panels A and B demonstrate that pFv and anti-IgE stimulated the release of IL-4 in parallel to the secretion of histamine from basophils.

Cyclosporin A (CsA) and tacrolimus inhibit pFv-induced IL-4 secretion from human basophils

Two immunophilin-binding drugs, CsA and tacrolimus (FK-506), are potent inhibitors of the IgE-dependent release of proinflammatory mediators from human basophils and mast cells (34, 41, 42). We compared the effects of preincubation of low concentrations of CsA (8–800 nM) and tacrolimus (1–100 nM) on the release of histamine and on the secretion of IL-4 from purified basophils activated by pFv. CsA concentration-dependently inhibited pFv-induced release of histamine and of IL-4 from basophils at concentrations as low as 240 nM (Fig. 7A). The inhibition of IL-4

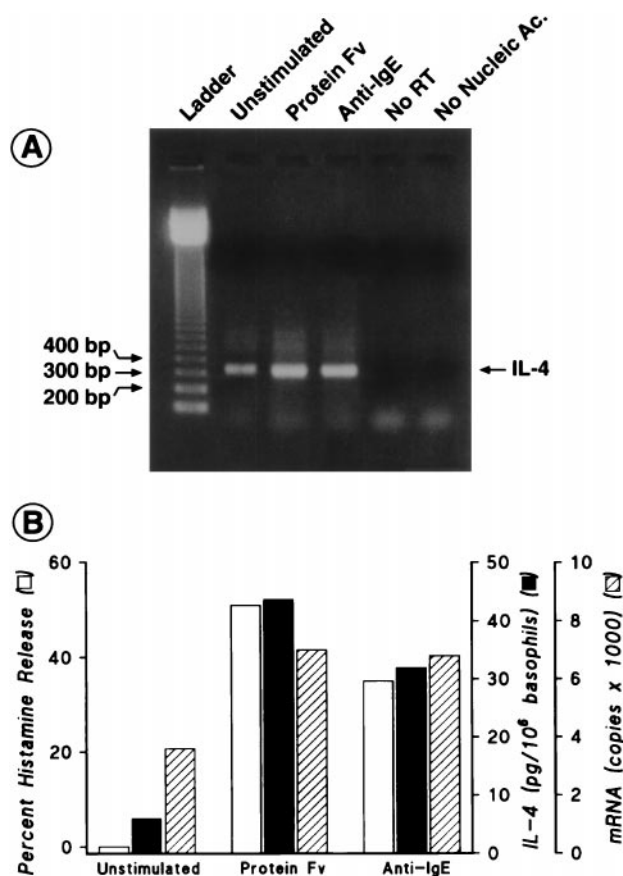


FIGURE 6. A, RT-PCR quantitative analysis of expression of IL-4 mRNA in human basophils stimulated with anti-IgE (0.3 μ g/ml) and pFv (0.5 ng/ml). Highly purified basophils (~97%) were preincubated (16 h at 37°C) with rhIL-3 (10 ng/ml), washed, and then incubated with rhIL-3 (10 ng/ml) in the absence or presence of anti-IgE or pFv for 4 h at 37°C. IL-4 mRNA was amplified by a quantitative PCR, as reported in *Materials and Methods*. The cDNA subjected to electrophoresis was visualized by ethidium bromide. In the absence of cDNA (No RT) and nucleic acid (No Nucleic Ac.), no PCR product is visualized. B, Effects of anti-IgE and pFv on the intracellular levels of IL-4 mRNA copies, on the extracellular protein levels of IL-4, and on the release of histamine from basophils compared with unstimulated cells. The same preparation of purified basophils was used for the experiments reported in A and B.

release ranged from ~10% at 8 nM to 90–95% at 800 nM, with a median inhibitory concentration (IC_{50}) of 12.1 ± 2.1 nM, similar to that calculated from the corresponding inhibition of histamine release (15.1 ± 5.7 nM). The effects of tacrolimus on the release of histamine and on the secretion of IL-4 from human basophils were similar to those observed with CsA (Fig. 7B). However, tacrolimus was ~10 times more potent than CsA; i.e., for IL-4 release, it had an IC_{50} of 1.8 ± 0.6 nM, similar to that calculated from the corresponding inhibition of histamine release (1.5 ± 1.1 nM).

Correlation between the maximum pFv- and anti-IgE-induced IL-4 release from human basophils

In eight experiments, basophils of a purity from 76–96% were challenged with a range of concentrations of pFv from patients FAR and TER (0.1–1 ng/ml) and anti-IgE (0.03–1 μ g/ml). A significant correlation was found between the maximum IL-4 release induced by anti-IgE and that induced by pFv ($r_s = 0.58$; $p < 0.01$), in agreement with the hypothesis that pFv might induce IL-4 release from basophils by interacting with IgE (data not shown).

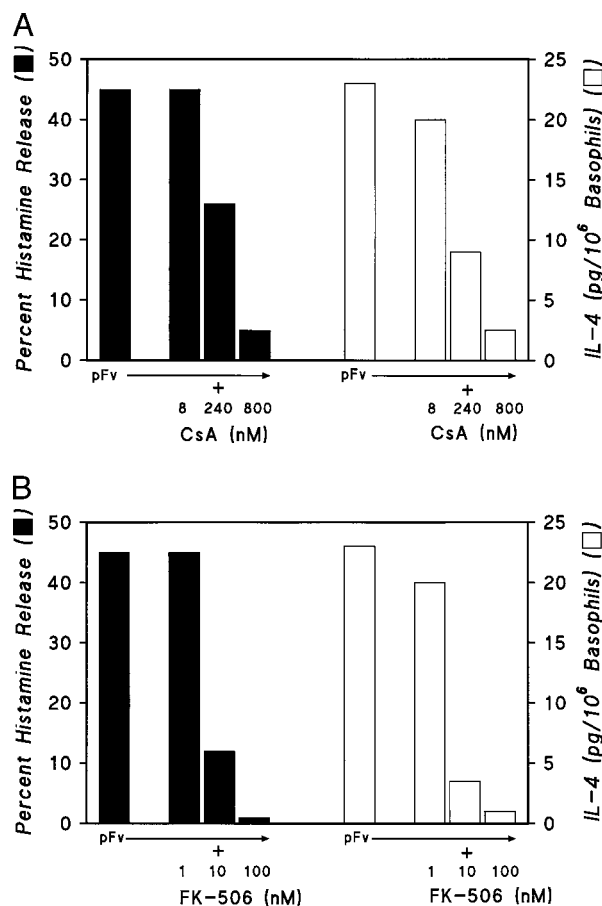


FIGURE 7. A, Effects of various concentrations of CsA on histamine and IL-4 release from human basophils. Cells were preincubated for 15 min at 37°C with the indicated concentrations of CsA and then challenged (4 h at 37°C) with pFv (0.5 ng/ml). Each bar represents the mean of duplicate measurements. B, Effects of various concentrations of FK-506 on histamine and IL-4 release from human basophils. Cells were preincubated for 15 min at 37°C with the indicated concentrations of CsA and then challenged (4 h at 37°C) with pFv (0.5 ng/ml). Values are the mean of duplicate measurements in a typical experiment. The same preparation of purified basophils was used for the experiments reported in A and B.

Effect of IgE stripping on pFv-induced IL-4 release from human basophils

Brief exposure to low pH removes most of the IgE bound on basophils, thus markedly reducing the activating properties of IgE-mediated stimuli (35, 43). Fig. 8 illustrates the representative results of three experiments showing that brief exposure to lactic acid completely blocks the effect of pFv and of anti-IgE on IL-4 secretion from basophils. In contrast, the response to a mAb cross-linking the α -chain of Fc ϵ RI (anti-Fc ϵ RI α) (33, 44) was not affected by this treatment. As a control, the same preparation of absorbed pFv, which is negative for Ig binding, did not induce IL-4 release.

Interaction between pFv with different IgM myeloma proteins

To evaluate the mechanism whereby pFv activates basophils, the protein was preincubated with monoclonal IgM of different V_H families (7). In a group of three experiments, one of which is illustrated in Fig. 9A, preincubation of basophils with three preparations of monoclonal IgM (M3, M11, and LAN), which possess a V_H3 domain (7), concentration-dependently inhibited the histamine releasing activity of pFv. In contrast, a monoclonal IgM

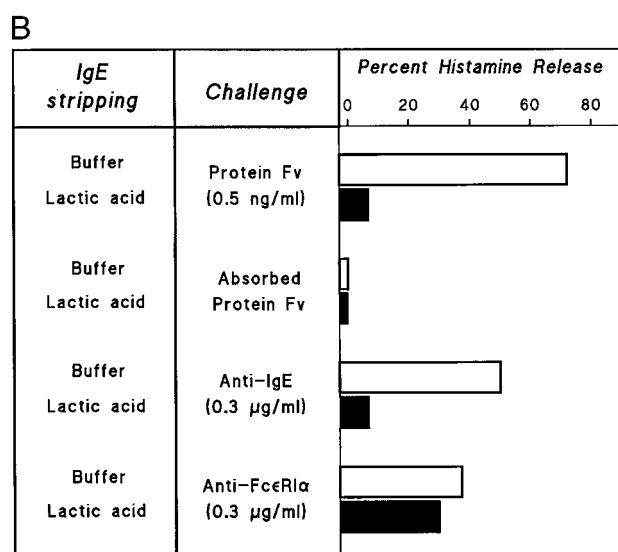
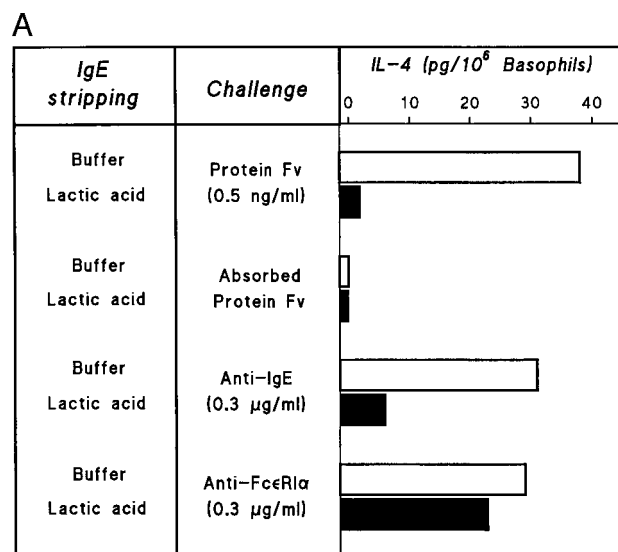


FIGURE 8. Effect of lactic acid treatment on IL-4 (A) and histamine release (B) from human basophils induced by pFv, anti-IgE, and anti-FcεRI. Leukocytes were either untreated (buffer) or treated with lactic acid (0.01 M, pH 3.9, 5 min, 22°C) and then washed twice. Leukocytes were then challenged (4 h at 37°C) with pFv (0.5 ng/ml), anti-IgE (0.3 µg/ml), or anti-FcεRIα (0.3 µg/ml). Each bar represents the mean of duplicate determinations.

(M14), which possesses a V_H6 domain, had a marginal effect. Similarly, the three preparations of monoclonal V_H3⁺ IgM concentration-dependently inhibited IL-4 secretion from basophils, whereas IgM M14 had no effect (Fig. 9B). These results confirm that the binding to V_H3 domain inhibits the binding of pFv to IgE bound to FcεRI on basophils.

pFv induces mediator release from HLMC by interaction with the V_H3 region of IgE

Lung involvement has been documented in patients with type II cryoglobulinemia (45), which is associated with HCV infection (46, 47). pFv from donors FAR and TER concentration-dependently induced histamine release from HLMC. In contrast, the absorbed preparations of pFv from FAR and TER, which no longer bind Ig, failed to induce histamine secretion from HLMC (data not shown). Fig. 10 shows the mean ± SEM of three experiments in

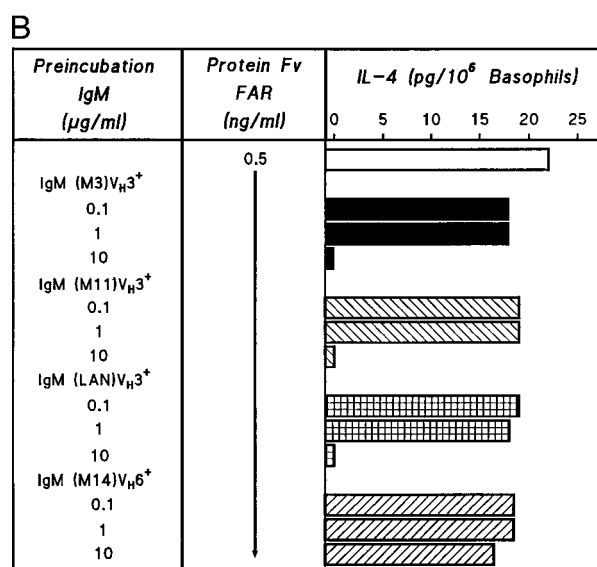
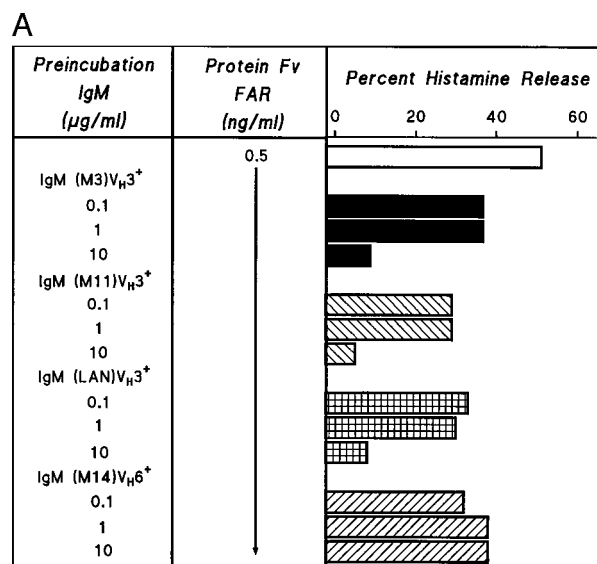


FIGURE 9. A, Effect of preincubation of pFv with monoclonal IgM on histamine release from human basophils. pFv (0.5 ng/ml) was preincubated 15 min at 37°C with human monoclonal IgM M3 (0.1–10 µg/ml), IgM M11 (0.1–10 µg/ml), IgM LAN (1–10 µg/ml), or IgM M14 (0.1–10 µg/ml). Leukocytes were then added and the incubation was continued for an additional 45 min at 37°C. Each bar represents the mean histamine release from duplicate incubations. B, Effect of preincubation of pFv with monoclonal IgM on IL-4 release from human basophils. pFv (0.5 ng/ml) was preincubated 15 min at 37°C with human monoclonal IgM M3 (0.1–10 µg/ml), IgM M11 (0.1–10 µg/ml), IgM LAN (1–10 µg/ml), or IgM M14 (0.1–10 µg/ml). Leukocytes were then added, and the incubation was continued for an additional 4 h at 37°C. Each bar represents the mean histamine release from duplicate incubations. The same preparation of purified basophils was used for the experiments reported in A and B.

which HLMC were challenged with pFv from patient TER. Preincubation with the three V_H3⁺ monoclonal IgM concentration-dependently inhibited the release of histamine from HLMC. In contrast, the V_H6⁺ IgM M14 had no effect on pFv-induced activation of HLMC.

Discussion

This study shows that pFv, an endogenous sialoprotein present in the normal liver and released largely in the digestive tract during

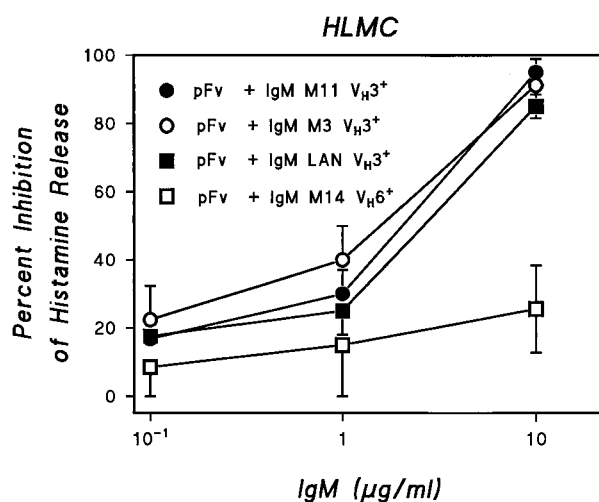


FIGURE 10. Effect of preincubation of pFv with monoclonal IgM on histamine release from human lung mast cells. pFv (0.5 ng/ml) was preincubated 15 min at 37°C with human monoclonal IgM M3 (0.1–10 μg/ml), IgM M11 (0.1–10 μg/ml), IgM LAN (1–10 μg/ml), or IgM M14 (0.1–10 μg/ml). HLMCs were then added, and the incubation was continued for an additional 45 min at 37°C. Values are expressed as the mean ± SEM of three experiments in which pFv induced the release of 26.3 ± 4.8% of the total histamine content. Error bars are not shown when details are too small.

viral hepatitis (1), is a potent inducer of IL-4 synthesis and release from human basophils. The releasing activity of pFv appears to be mediated by interaction with the V_H3 region of IgE present on human basophils and mast cells. This is the first indication that a human gut-associated protein induced by viral infections can act as an endogenous superallergen causing the release from human FcεRI⁺ cells of such an important cytokine as IL-4 that possesses several immunoregulatory functions.

The IL-4-releasing activity of purified pFv preparations is due to pFv itself and not to contamination by natural anti-Id, anti-L chain, or anti-ε Abs reacting with IgE. Indeed, this activity was fully abolished by removal of pFv with insoluble Ig, and it remained unchanged after Ig absorption with an immunosorbent anti-F(ab')₂. The mechanism of activation of human inflammatory cells possessing FcεRI appears to be mediated by interaction of pFv with the V_H3 region of membrane-bound IgE. This hypothesis is supported by the observations that: 1) pFv binds with high affinity all Ig isotypes (4, 33); 2) there is a highly significant correlation between the maximal mediator release induced by anti-IgE and that induced by pFv; and 3) basophils from which IgE had been dissociated by brief exposure to lactic acid no longer released IL-4 in response to pFv and anti-IgE. These findings agree with the notion that pFv has six distinct functional Fab binding sites (48) that enable binding by at least 50% of human F(ab')₂ fragments (5). Therefore, pFv, which has been shown to function as an endogenous B cell superantigen (5–7), can also function as an endogenous superallergen, i.e., it reproduces the releasing activity of specific allergens and of anti-IgE on human FcεRI⁺ cells (49, 50).

This study provides insight into the mechanism of interactions between pFv and IgE bound on the basophil/mast cell membrane. Three different monoclonal IgM with a V_H3 domain inhibited the release of histamine and of IL-4 induced by pFv from basophils, whereas an IgM with a V_H6 domain had no effect. This confirms that the releasing activity of pFv depends on the binding to an Ig

structure not located in the CHε constant regions but in the V_H3 domain, a fragment common to all Ig classes and subclasses. Since pFv does not bind free or combined L chains (4), the Ig structure in question must be the V_H domain of IgE, as demonstrated by direct evidence using Ig fragments (4). Structural variations between V_H families explain why the V_H6⁺ IgM M14 failed to inhibit the releasing effect exerted by pFv on FcεRI⁺ cells, whereas the three V_H3⁺ IgM exerted a concentration-dependent inhibition.

Our results provide the first evidence that an endogenous superallergen, pFv, largely released during viral infections, can induce the synthesis and secretion of such an important cytokine as IL-4 from human basophils. Interestingly, the activity of pFv is extremely potent, which raises the possibility that it exerts important effects in vivo. The activity of IL-4 has been linked to several immunoregulatory functions including the synthesis of IgE by B lymphocytes (25–27), adherence and selective eosinophil and basophil endothelial transmigration (30), antitumor activity in vivo (31), and development of T lymphocytes expressing a Th2 phenotype (25–27). Given the biologic importance of IL-4, our findings could have biologic relevance. pFv, found in normal liver (1), occurs as a free molecule in the gut lumen of patients with hepatitis A, B, C, and E (2). It might be envisioned that pFv released in the blood of patients with acute and chronic infection with HCV and HBV contributes, through the release of IL-4 from FcεRI⁺, to the increased serum IgE levels found under these conditions (51–53).

Our data represent the first indication that viruses can induce in vivo the release of an endogenous superallergen (pFv) that acts on human FcεRI⁺ cells to release cytokines. This novel finding might be of interest not only to students of allergic diseases in which basophils, mast cells, and their mediators play a key pathogenetic role (14, 16, 34). There is increasing evidence that human basophils (17–21) and mast cells (14–16, 54–55) synthesize a growing list of cytokines and chemokines, thus playing a more complex immunoregulatory role than previously anticipated (56). Therefore, the mechanism of basophils/mast cell activation by pFv represents a new model for a pathogenetic link between viral infections, FcεRI⁺ cell activation, and cytokine release.

Our previous in vitro (34, 41) and in vivo (42) studies demonstrated that immunophilin-binding drugs (i.e., CsA and tacrolimus) exert anti-inflammatory effects by inhibiting the IgE-dependent release of proinflammatory mediators from human FcεRI⁺ cells. Here, we demonstrate that CsA and tacrolimus also concentration-dependently inhibit the pFv-induced release of IL-4 from human basophils. Based on these findings, it would appear that some of the effects of this class of anti-inflammatory/immunosuppressive drugs exerted on basophil-dependent inflammatory reactions (57, 58) reflect actions of these drugs also on the secretion of IL-4 from FcεRI⁺ cells.

Besides an increase of the secretory “immune exclusion” (48), the pathophysiologic role of pFv is largely unknown. One may speculate that pFv released in the digestive tract provides a stimulus for IL-4 secretion from FcεRI⁺ cells, which triggers B cells to produce IgE. IgE bound to mast cells might play a role in the elimination of helminthic infections (59, 60), thus providing an explanation of a new ancestral role of such an endogenous superallergen as pFv in defense mechanisms. Indeed, the possibility of a parallel anti-infectious role of a pFv-related superantigen bound to natural Abs has been raised (2, 61).

The concept that superallergens from diverse biologic origins can induce IL-4 secretion from human basophils is both without precedent and of potential interest. *Staphylococcus aureus* protein A (62) and the HIV-1 retrovirus envelope protein (gp120) have a V_H3 family-directed specificity (63, 64) that may contribute to the immunodeficient state present in AIDS patients, presumably

through release of IL-4 (65, 66). It might, therefore, be important to investigate whether gp120 can modulate IL-4 secretion from human basophils.

In conclusion, we demonstrate that pFv can activate human FcεRI⁺ cells to release cytokines, probably through interaction with the V_H3 region of IgE. This finding supports the hypothesis that the in vivo release of pFv, previously recognized as a B cell superantigen (5, 7) and here identified as a superallergen, might be pathophysiologically relevant.

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