

Protein Fv Produced during Viral Hepatitis Is an Endogenous Immunoglobulin Superantigen Activating Human Heart Mast Cells

Arturo Genovese^a Guglielmo Borgia^b Jean-Pierre Bouvet^c
Aikaterini Detoraki^a Amato de Paulis^a Marcello Piazza^b Gianni Marone^a

Divisions of ^aClinical Immunology and Allergy and ^bInfectious Disease, University of Naples Federico II, Naples, Italy; ^cUnité d'Immunopathologie Humaine, Institut National de la Santé et de la Recherche Médicale U430, Hôpital Broussais, Paris, France

Key Words

Cysteinyl leukotrienes · Histamine · IgE · Mast cells · Protein Fv · Superantigens

Abstract

Protein Fv, an endogenous protein produced in the liver, is released in biological fluids during viral hepatitis. Acute and chronic viral hepatitis can be associated with cardiovascular derangements. Protein Fv induced the release of histamine, tryptase and the de novo synthesis of prostaglandin D₂ and cysteinyl leukotriene C₄ from mast cells isolated from human heart tissue (HHMC). Protein Fv absorbed with protein A-Sepharose coated with polyclonal IgG did not induce histamine secretion. The maximal percent histamine secretion induced by protein Fv correlated ($r_s = 0.60$; $p < 0.05$) with that induced by anti-IgE, whereas there was no correlation between the release caused by proteins Fv and C5a. Preincubation of HHMC with protein Fv or anti-IgE caused complete cross-desensitization to subsequent challenge with heterologous stimulus. HHMC from which IgE had been dissociated no longer released histamine in response to anti-IgE and protein Fv. A human

monoclonal IgE blocked both anti-IgE- and protein Fv-induced release. Three human monoclonal IgM V_H3⁺ inhibited protein-Fv-induced secretion of histamine from HHMC, whereas monoclonal IgM V_H6⁺ did not inhibit the release induced by protein Fv. Protein Fv acts as an endogenous immunoglobulin superantigen by interacting with the V_H3 domain of IgE to induce the release of mediators from HHMC.

Copyright © 2003 S. Karger AG, Basel

Introduction

Protein Fv is a 175-kD dimeric sialoprotein produced in the human liver and released in biological fluids during viral hepatitis [1], which possesses unique immunoglobulin binding activity in vitro in various mammalian and non-mammalian species [2]. Protein Fv binds to the variable domain of the H chains of immunoglobulin, irrespectively of the immunoglobulin class, subclass, and light (L) chain type [3]. A single protein Fv molecule can bind six F(ab')₂ fragments [2], and human IgM, IgG, and IgE display binding activity [1–3]. Binding to protein Fv is mediated by the V_H region of immunoglobulin [2], and it

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2003 S. Karger AG, Basel
1018–2438/03/1324–0336\$19.50/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Gianni Marone
Divisione di Immunologia Clinica e Allergologia
Università di Napoli Federico II
Via S. Pansini 5, IT-80131 Napoli (Italy)
Tel. +39 081 7707492, Fax +39 081 7462271, E-Mail marone@unina.it

appears that protein Fv interacts with Fab external to the conventional antigen-binding pocket [1–3]. Although protein Fv is released in the digestive lumen of many patients affected by acute and chronic viral hepatitis B ($\approx 35\%$), C ($\approx 43\%$), A and E [1], its pathophysiologic role in viral hepatitis is unknown.

Acute and chronic viral hepatitis are associated with a myriad of hepatic and extrahepatic tissue injuries, but not all of them are directly related to the cytotoxic effects of HBV and HCV viruses [4–6]. The effects induced by viral hepatitis on the cardiovascular system are not widely recognized [7–10]. However, type II cryoglobulinemia, a systemic vasculitis caused by HCV infection [11, 12], can be associated with cardiovascular involvement [13]. The mechanism(s) by which viral infections impinge on these disorders are still largely obscure.

Mast cells and basophils, the only cells that express high-affinity receptors (Fc ϵ RI) for IgE and that synthesize histamine, are widely recognized as effector cells in several inflammatory disorders [14]. Fc ϵ RI⁺ cells play a fundamental role in the pathophysiology of inflammatory diseases. Indeed, they elaborate and release a myriad of proinflammatory [15, 16] and immunoregulatory molecules [17, 18], and they express a wide spectrum of surface receptors for cytokines and chemokines [19, 20]. Mast cells are found in the human heart [21, 22], around coronary arteries [23], and in the coronary intima [24]. Human heart mast cells (HHMC) have been implicated in the pathophysiology of myocarditis [25, 26], coronary artery diseases [23] and dilated cardiomyopathy [19]. Finally, mast cells, through the release of vasoactive mediators, are also involved in systemic vasculitis [27].

We have devised a technique for the dispersion of mast cells from human heart tissue and identified some of the immunological and non-immunological stimuli that induce HHMC to release vasoactive and proinflammatory mediators *in vitro* [22, 26].

The aim of the present study was to investigate the mechanism by which protein Fv activates HHMC to release preformed and *de novo* synthesized mediators.

Materials and Methods

Reagents

We purchased bovine serum albumin (BSA), human recombinant C5a, piperazine-N,N'-bis (2-ethanesulfonic acid; PIPES), hyaluronidase, collagenase type II, chymopapain, prostaglandin D₂ (PGD₂) and cysteinyl leukotriene C₄ (LTC₄) from Sigma (St. Louis, Mo., USA). Hanks' balanced salt solution and fetal calf serum (FCS) were from GIBCO (Gaithersburg, Md., USA). Deoxyribonuclease and pronase were from Calbiochem (La Jolla, Calif., USA). RPMI

1640 with 25 mM HEPES buffer, and Eagle's minimal essential medium were from Flow Laboratories (Irvine, UK). Percoll was from Pharmacia Fine Chemicals (Uppsala, Sweden). [³H]LTC₄ (168 Ci/mmol) and [³H]PGD₂ (210 Ci/mmol) were from New England Nuclear (Boston, Mass., USA). The rabbit anti-IgE was generously donated by Drs. T. and K. Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, Calif., USA). Dr. E. Kusner (Zeneca Pharmaceuticals, Philadelphia, Pa., USA) supplied the rabbit anti-LTC₄ and Dr. L.M. Lichtenstein (Johns Hopkins University, Baltimore, Md., USA) rabbit anti-PGD₂. The tryptase RIA kit (Pharmacia Tryptase RIACT 50) was generously donated by Pharmacia (Milan, Italy). The PIPES buffer used in these experiments was a mixture of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, pH 7.4 (referred to as P). PCG contains 2 mM CaCl₂ and 1 g/l dextrose in addition to P [26]. P-EDTA is P buffer containing 4 mM EDTA. PGMD is 0.25 g/l MgCl₂ × 6 H₂O, 10 mg/l DNase, and 1 g/l gelatin in addition to P, pH 7.4. The phosphate-buffered saline (PBS) contained 0.15 M NaCl and 0.06 M phosphate, pH 7.2. All other reagents were from Carlo Erba (Milan, Italy).

Purification of Protein Fv

Stool extracts were obtained from patients (patients FAR, TER, LAR, and THU) suffering from HCV, as described previously [1]. These extracts contained high levels of protein Fv, which was purified by size permeation through a set of three 16 × 900-mm columns of S300 Sephacryl (Pharmacia), equilibrated with 1.16 mM sodium deoxycholate in PBS containing sodium azide. Under these conditions, protein Fv elutes in the 175-kD fraction as confirmed by HPLC with a calibrated Superose 12 column (Pharmacia) [1]. Purified fractions were extensively dialyzed against PBS to remove sodium deoxycholate and sodium azide, and stored at 4°C under sterile conditions.

Immunoglobulin Binding Test

Protein Fv preparations were assayed for immunoglobulin binding activity by a non-immune ELISA-like method [1]. Polyclonal normal IgG or F(ab')₂ fragments (2.5 µg/ml) were coated on Nunc plates. After three washes with 0.1% Tween 20 in PBS, the plates were saturated with PBS containing 1% BSA and 0.05% Tween 20. The extracts were left overnight at 4°C to bind the coated immunoglobulin. Immunoglobulin binding molecules were revealed by their non-immune binding to 0.5 µg/ml of peroxidase-labeled human IgG after a 1-hour 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 above 0.1 U at 492 nm and more than twofold the OD of a negative control. For each preparation of protein Fv, the activity giving 0.5 OD at 492 nm was obtained with the following dilutions: 1:200 (patient TER protein Fv), 1:128 (patient FAR protein Fv) [27].

Absorption Experiments

Nine volumes of sample were incubated twice with 1 volume of IgG-coated protein A-Sepharose beads under constant mixing at 37°C for 1 h. The supernatant was then checked for loss of protein Fv activity, extensively dialyzed, and stored at 4°C under sterile conditions. For immunoglobulin removal, 9 volumes of sample were incubated with 1 volume of anti-F(ab')₂ immunosorbent prepared by CNBr insolubilization of specific antibodies raised in sheep, a species unreactive with protein Fv [2]. In both cases, unabsorbed samples had been incubated with uncoated Sepharose 4B.

Purification of Human Monoclonal IgE and IgM and of Human Polyclonal IgG

IgE myeloma proteins were purified from the sera of 3 myeloma patients (PS, PP, and ADZ) by repeated gel filtration on Sephadex G-200, followed by elution through a Sepharose CL-4B column [27, 28]. Radioimmunoassay (RIA) revealed no IgG, IgM, or IgA contamination. Monoclonal IgM were purified from the sera of patients with Waldenström's macroglobulinemia by gel permeation [27, 28]. 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 framework regions [27]. Human polyclonal IgG was purified by precipitation of normal human serum with 50% saturated ammonium sulfate and ion-exchange chromatography [29].

Isolation and Partial Purification of Human Heart Mast Cells

The heart tissue used in this study was obtained from patients (31–61 years old) undergoing heart transplantation mostly for cardiomyopathy, and from donors without cardiovascular disease who had died in car accidents. The explanted heart was immediately immersed in cold (4°C) cardioplegic solution and processed within 5–18 h of removal. Fat tissue, large vessels, and pericardium were removed. The tissue was finely minced (2- to 5-mm fragments), suspended in PIPES (P) buffer (10 ml/g wet tissue) and washed three times by centrifugation [22]. After each centrifugation, the heart fragments were filtered through 150- μ m-pore Nytex cloth (Tetko, Elmsford, N.Y., USA). Fragments were incubated (15 min, 37°C) under constant stirring in P buffer containing 10 mg collagenase/g wet tissue. At the end of the first incubation, the cell suspension was filtered through 150- μ m-pore Nytex cloth. The residual tissue was weighed, and three further cycles of enzymatic digestion were performed, using a new preparation of collagenase each time. After the last enzymatic digestion, the cell suspension was centrifuged (150 g, 22°C, 8 min) and filtered first through 150- μ m-pore Nytex cloth and then through 60- μ m-pore Nytex cloth to remove large particles and large cells (mostly myocytes). Finally, cells were washed twice in PGMD (25 mM P, 110 mM NaCl, 1 mM Mg, 1 g/l gelatin, 20 mg/l Dnase, pH 7.37) by centrifugation (150 g, 22°C, 8 min). At this stage of the procedure, Alcian-blue-positive cells (mast cells) accounted for <0.1% of total cells [22, 26]. Cell pellets were resuspended in 250 ml of P buffer containing 2% BSA and centrifuged (25 g, 22°C, 2 min) to remove sedimented myocytes. Myocytes (>100 μ m long) were pelleted and discarded. HHMC were partially purified by flotation through a discontinuous Percoll gradient as detailed elsewhere [22]. The enzymatic dispersion of tissue yields $\approx 5 \times 10^4$ mast cells/g heart tissue. Short-term (≈ 16 h) cultures of HHMC were prepared by resuspending $2\text{--}5 \times 10^6$ cells/ml in a solution of RPMI 1640 containing 25 mM HEPES, 1% penicillin-streptomycin solution, 2 mM L-glutamine, and 10% FCS at 37°C in humidified 95% air/5% CO₂. The viability of mast cells, evaluated by trypan blue exclusion, was >95% [22, 26].

Histamine Release

Cells ($\approx 3 \times 10^4$ HHMC/tube) were resuspended in PCG, and 0.3 ml of the cell suspension was placed in 12 $\times$ 75 mm polyethylene tubes and warmed to 37°C; 0.2 ml of each prewarmed releasing stimulus was added, and incubation was continued at 37°C for 30 min [22]. At the end of this step, the reaction was stopped by centrifugation (1,000 g, 22°C, 2 min), and the cell-free supernatants were stored at –80°C for subsequent assay of histamine, tryptase, PGD₂

and LTC₄ contents. Histamine was assayed with an automated fluorometric technique [30]. To calculate histamine release as a percentage of total cellular histamine, the 'spontaneous' release from mast cells (3–10% of the total cellular histamine) was subtracted from both numerator and denominator [28]. The total histamine content in mast cells was obtained by cell lysis with 8% HClO₄ [22]. All values are based on the means of duplicate or triplicate determinations. Replicates differed from each other by less than 10% in histamine content.

RIA of PGD₂ and LTC₄

100- μ l fractions taken from the supernatant fluids were stored at –80°C. The PGD₂ and LTC₄ assays were carried out within 24 h of the experiments, to minimize degradation of the compounds, by a previously described RIA [22]. All determinations were run in duplicate against a standard curve run in duplicate. Duplicates differed from each other by less than 10%.

RIA of Tryptase

Tryptase was analyzed by a solid phase RIA (Pharmacia Tryptase RIACT 50); duplicate samples or tryptase standards were added to plastic tubes coated with mouse anti-tryptase monoclonal antibody. Then ¹²⁵I-radiolabeled anti-tryptase was added to the tubes, which were incubated for 18 h at 22°C. After washing (three times, 2 ml of saline, 22°C), bound radioactivity was counted and gave a linear concentration-response curve within a range of 2–50 μ g/l [22].

Lactate Dehydrogenase Assay

To test whether protein Fv exerts cytotoxic effects on HHMC, lactate dehydrogenase (LDH) was determined in HHMC supernatants. The cell-free supernatants were collected, and the concentrations of LDH were measured [22].

Statistical Analysis

Results are means $\pm$ SEM. Student's t test with Bonferroni's correction was used for multiple comparisons between groups [31]. When the data were subjected to linear correlation analysis, correlations were calculated using the Spearman rank coefficient (r_s) [32]. The level of statistical significance was $p < 0.05$.

Results

Effects of Protein Fv on Histamine Release from Human HHMC

We first evaluated the effects of increasing concentrations of protein Fv obtained from 4 patients suffering from HCV on histamine release from HHMC. Low concentrations of protein Fv from patients FAR, TER, and LAR (0.3–30 ng/ml) concentration-dependently released a significant percent of histamine from HHMC isolated from 6 donors (fig. 1). Protein Fv from patient THU also induced a significant percent release of histamine, but the release was consistently lower with respect to the other protein Fv preparations. Protein Fv did not cause LDH release in the supernatants of HHMC after incubation for 30 min at 37°C (data not shown).

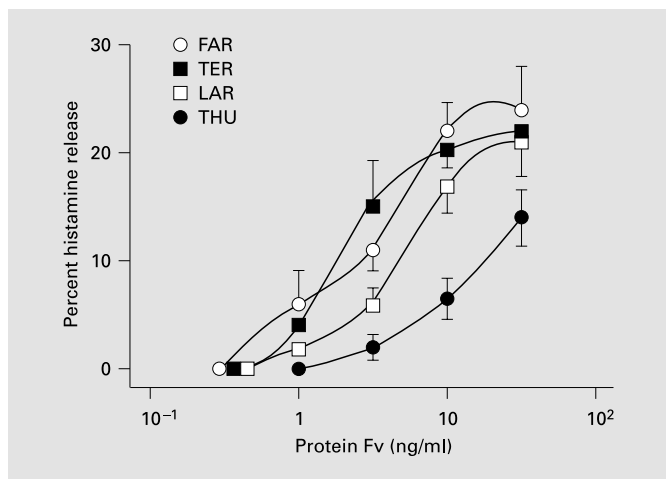


Fig. 1. Effect of increasing concentrations of protein Fv from donors FAR, TER, LAR, and THU on histamine release from HHMC obtained from 6 donors. Cells were challenged with protein Fv for 30 min at 37°C. Each point is the mean $\pm$ SEM. Error bars are not shown when graphically too small.

Effects of Protein Fv and Anti-IgE on Histamine Release from HHMC

We next compared the histamine-releasing activity of increasing concentrations of protein Fv (0.3–30 ng/ml) from patients FAR and TER and anti-IgE (10^2 to 3×10^3 ng/ml) on histamine release from HHMC. Protein Fv (0.3–30 ng/ml) from both patients concentration-dependently released histamine from HHMC obtained from 7 donors. The concentration-response curves induced by the two preparations of protein Fv paralleled that induced by anti-IgE. However, protein Fv was approximately 100 times more potent than anti-IgE (fig. 2). The same preparations of protein Fv (FAR and TER) adsorbed with protein A-Sepharose coated with human polyclonal IgG, which were negative for immunoglobulin binding, did not induce histamine release. Consequently, protein Fv was responsible for the mediator secretion from HHMC, presumably by binding to immunoglobulin.

In 13 experiments, we challenged HHMC with protein Fv (0.3–30 ng/ml), anti-IgE (0.1–3 μ g/ml), or C5a (10^{-7} to 10^{-6} M), and found a significant correlation between the maximum percent histamine release induced by protein Fv and that induced by anti-IgE ($r_s = 0.60$; $p < 0.05$). This suggests that protein Fv might induce histamine release from HHMC by interacting with IgE. In contrast, there was no correlation between the release caused by protein Fv and C5a ($r_s = 0.14$; NS).

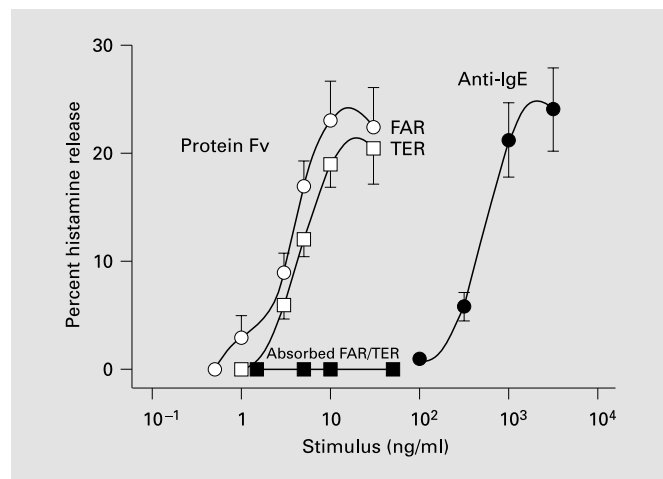


Fig. 2. Effects of increasing concentrations of proteins Fv and anti-IgE on histamine release from HHMC from 7 donors. Protein Fv from donors FAR and TER was adsorbed or non-adsorbed with protein A-Sepharose coated with polyclonal IgG. Cells were challenged with protein Fv or anti-IgE for 30 min at 37°C. Values are expressed as means $\pm$ SEM. Error bars are not shown when graphically too small.

Correlation between Histamine and Tryptase Release from HHMC Induced by Protein Fv

HHMC secretory granules contain tryptase as well as histamine [22]. We investigated whether histamine release was correlated to the secretion of tryptase induced by protein Fv and found a significant correlation between the maximum histamine and tryptase release ($r_s = 0.82$; $p < 0.01$). These findings indicate that protein Fv releases tryptase in parallel with histamine from HHMC.

Effect of Protein Fv on PGD₂ and LTC₄ Synthesis from HHMC

HHMC challenged with anti-IgE synthesize de novo PGD₂ and LTC₄ [22, 26], two proinflammatory mediators with vasoactive and biological properties [33–36]. Protein Fv acted as complete secretagogue because it caused also the de novo synthesis of both PGD₂ and LTC₄ by HHMC. There was a significant correlation between the percent histamine secretion and the release of PGD₂ ($r_s = 0.76$; $p < 0.01$) and LTC₄ ($r_s = 0.75$; $p < 0.01$) from HHMC activated by protein Fv (fig. 3).

Cross-Desensitization between Protein Fv and Anti-IgE

We further examined the relationship between anti-IgE and protein Fv using cross-desensitization between these two stimuli. Anti-IgE activates mast cells to release

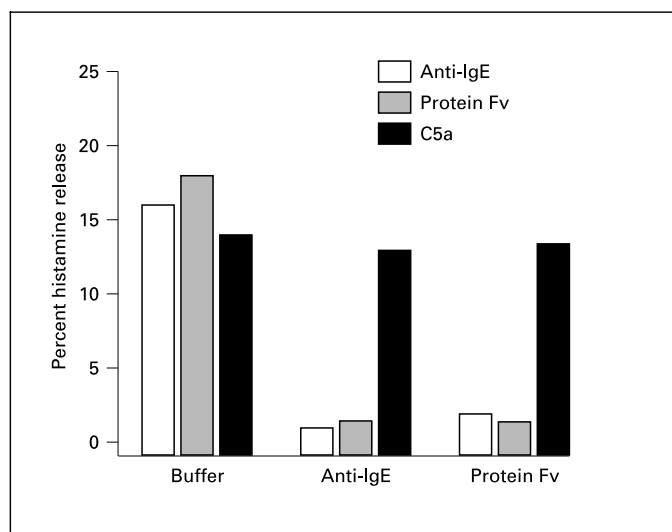
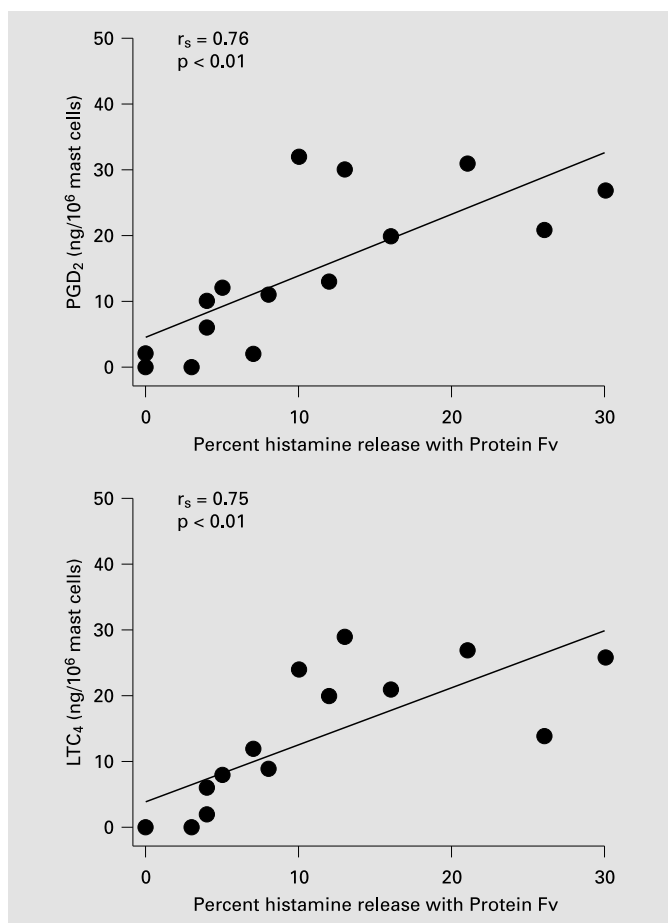


Fig. 3. Correlations between the maximum percent of histamine secretion and PGD_2 and LTC_4 release induced by protein Fv from HHMC. Each point is the mean of duplicate determinations from separate experiments.

Fig. 4. Effect of desensitization to one stimulus on the response to a second stimulus. Cells were desensitized to anti-IgE (1 $\mu\text{g}/\text{ml}$) or to protein Fv (10 ng/ml) by preincubation with the stimuli or buffer alone in Ca^{2+} -free PIPES buffer containing 4 mM EDTA (P-EDTA) for 30 min at 37°C . Cells were then washed twice at 4°C , resuspended in PCG, and challenged with anti-IgE (1 $\mu\text{g}/\text{ml}$) or protein Fv (10 ng/ml) for 30 min at 37°C . Each bar shows the mean of duplicate determinations of a typical experiment. Similar results were obtained in two other experiments.

histamine by binding to the $\text{Fc}\epsilon$ portion of the IgE molecule on the cell membrane [37]. HHMC were treated with anti-IgE (1 $\mu\text{g}/\text{ml}$) or protein Fv (10 ng/ml) in P-EDTA for 30 min at 37°C . At the end of incubation, cells were washed and suspended in PCG. Cells preincubated with P buffer and then challenged with anti-IgE released histamine, whereas cells preincubated with anti-IgE released less than 5% histamine. Cells desensitized to protein Fv released $\approx 90\%$ less histamine than control cells. In the reverse experiment, cells were preincubated with protein Fv or anti-IgE in P-EDTA before challenge with protein Fv. As expected, when protein-Fv-pretreated cells were challenged with protein Fv they had lost their ability to release the homologous stimulus (fig. 4). HHMC desensitized with anti-IgE or protein Fv continued to respond to C5a, which activates a specific receptor on the mast cell [22]. Similar results were obtained in two other experiments. Thus, it appears that the releasing property of protein Fv is mediated principally by interaction with IgE present on the mast cell surface.

Effect of IgE Stripping on Protein-Fv-Induced Histamine Release from HHMC

A second line of evidence that protein Fv induces histamine release by binding to IgE comes from the finding that protein Fv does not induce histamine release from HHMC stripped of IgE from $\text{Fc}\epsilon\text{RI}$ by brief exposure to low pH [22]. Lactic-acid-induced dissociation of IgE from mast cells completely eliminated both anti-IgE and protein-Fv-induced release from HHMC (fig. 5). In contrast, this treatment did not affect the response to a monoclonal antibody cross-linking the α -chain of $\text{Fc}\epsilon\text{RI}$ [38].

Interaction between Protein Fv or Anti-IgE with Different IgE Myeloma Proteins

It has been shown that protein Fv binds specifically to the V_H domain of immunoglobulin [3, 27, 39], and that six sites on protein Fv bind immunoglobulin, making this natural protein similar to the divalent anti-IgE antibodies. To evaluate the mechanism of activation of HHMC by

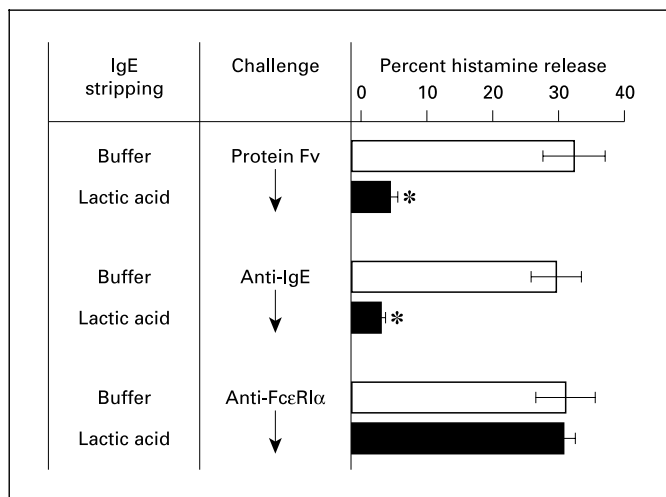


Fig. 5. Effect of lactic acid treatment on histamine release from HHMC induced by protein Fv, anti-IgE and anti-FcεRIα. HHMC were either untreated (buffer) or treated with lactic acid (0.01 M, pH 3.9, 5 min, 22°C) and washed twice. Cells were then challenged with protein Fv (10 ng/ml), anti-IgE (1 μg/ml) or anti-FcεRIα (1 μg/ml) for 30 min at 37°C. Each bar shows the mean ± SEM of triplicate determinations. * $p < 0.01$ vs. cells preincubated with buffer.

protein Fv, protein Fv or anti-IgE were preincubated with two IgE myelomas that possess different L chains (patient PP IgE λ, and patient ADZ IgE κ), or with human polyclonal IgG [28, 40]. In three experiments, one of which is illustrated in figure 6, we found that IgE from patient ADZ (IgE κ) and human polyclonal IgG markedly reduced the releasing activity of protein Fv. In contrast, PP IgE λ did not affect the histamine-releasing activity of protein Fv. In similar experiments, IgE from patients PP and ADZ blocked the releasing activity of anti-IgE, whereas polyclonal IgG had no inhibitory effect (data not shown).

The interactions between human immunoglobulin and protein Fv were also analyzed by preincubating two monoclonal IgG1 κ preparations from the sera of two myeloma patients (patients ZEG and CHE). ZEG IgG1 κ, which binds protein Fv [1], inhibited the releasing activity of protein Fv, whereas CHE IgG1 κ did not. Figure 7 shows the results of one of three experiments demonstrating that preincubation with ZEG IgG1 κ inhibited the releasing activity of protein Fv, whereas CHE IgG1 κ had no effect. Neither monoclonal IgG modified the histamine-releasing activity of anti-IgE.

Since the two monoclonal IgG possess the same L chain type (κ), these results support the hypothesis that

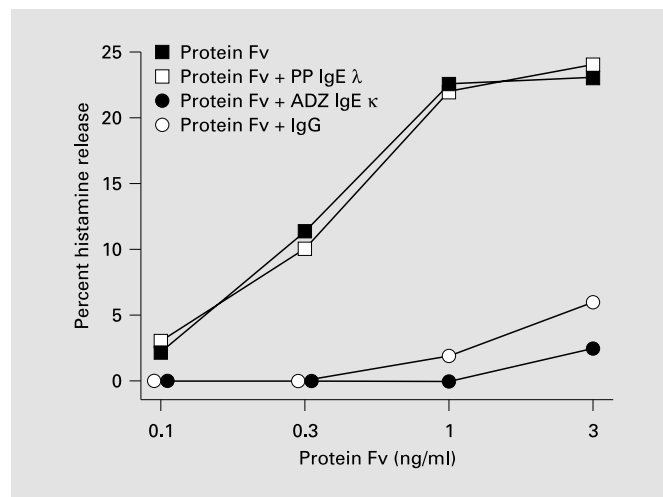


Fig. 6. Effect of preincubation of protein Fv with human monoclonal IgE or human polyclonal IgG on histamine release from HHMC. Protein Fv (10 ng/ml) or anti-IgE (1 μg/ml) were preincubated for 15 min at 37°C with PP (1 μg/ml) or ADZ (1 μg/ml) human polyclonal IgE, or human polyclonal IgG (10 μg/ml). HHMC were then added, and incubation continued for another 30 min at 37°C. Each bar shows the mean of duplicate determinations of a typical experiment. Similar results were obtained in two other experiments.

protein Fv binds to variable regions of the V_H domain and not to the L chain. This explains why some individual IgG and IgE do not react with protein Fv and do not inhibit their releasing activity.

Protein Fv Induces Mediator Release from HHMC by Interaction with the V_H3 Domain of Immunoglobulins

We further examined the structural basis of the interaction between human immunoglobulins and protein Fv. The specificity of binding site(s) of protein with human immunoglobulins is encoded by the germline sequences of many of the commonly expressed V_H3 genes [39, 41]. To assess the mechanism by which protein Fv activates HHMC, the protein was preincubated with monoclonal IgM of different V_H families [28]. In three experiments, preincubation of HHMC with three preparations of monoclonal IgM (M3, M11, and LAN), which possess a V_H3 domain, concentration-dependently inhibited the histamine-releasing activity of protein Fv (fig. 8). In contrast, a monoclonal IgM (M14), which has a V_H6 domain, had no such effect. These results suggest that binding to the V_H3 domain inhibits the binding of protein Fv to IgE bound to FcεRI on HHMC.

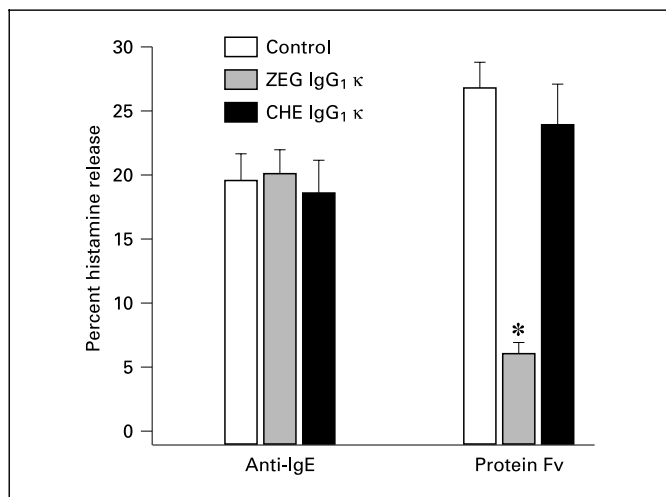


Fig. 7. Effect of preincubation of protein Fv and anti-IgE with monoclonal ZEG IgG₁ κ or CHE IgG₁ κ on histamine release from HHMC. Protein Fv (10 ng/ml) or anti-IgE (1 μg/ml) were preincubated for 15 min at 37 °C with human monoclonal ZEG IgG₁ κ (10 μg/ml) or CHE IgG₁ κ (10 μg/ml). HHMC were then added, and incubation continued for another 30 min at 37 °C. Each bar shows the mean ± SEM of triplicate determinations. * $p < 0.01$ vs. control activated by protein Fv.

Discussion

Here we show that protein Fv, an endogenous sialoprotein present in normal liver and released in biologic fluids during viral hepatitis [1, 2], is a potent inducer of mediator release from HHMC. The releasing activity of protein Fv appears to be mediated by interaction with the V_H3 region of IgE present on human mast cells. This represents the first evidence that a human protein induced by viral infections can act as an endogenous immunoglobulin superantigen, i.e. it causes the release of several vasoactive and immunoregulatory mediators from HHMC.

We excluded the possibility that the releasing activity of purified protein Fv preparations could be due to contamination by natural anti-Id, anti-L chain, or anti-ε antibodies reacting with IgE, rather than to protein Fv itself. In fact, the releasing activity was totally abolished when protein Fv was removed with insoluble immunoglobulin, and it remained unchanged after immunoglobulin absorption with an immunosorbent anti-F(ab')₂. The activation of human inflammatory cells that possess FcεRI appears to be mediated by interaction of protein Fv with membrane-bound IgE. This hypothesis is supported by the observations that: (1) protein Fv binds with high affinity all immunoglobulin isotypes [3, 39]; (2) the maximal mediator release induced by anti-IgE is significantly cor-

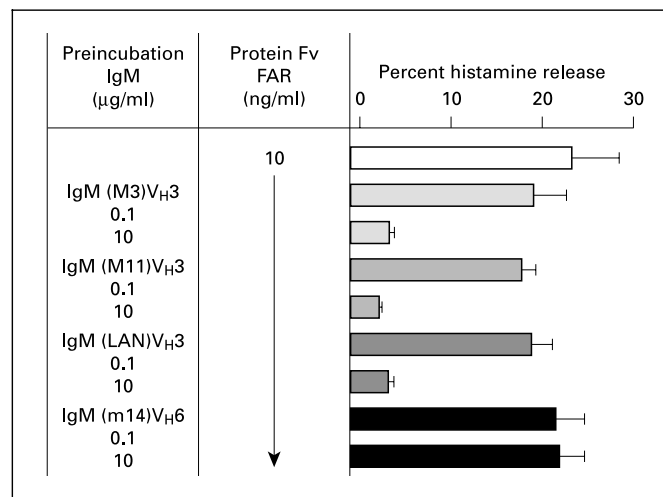


Fig. 8. Effect of preincubation of protein Fv with V_H3⁺ or V_H6⁺ monoclonal IgM on histamine release from HHMC. Protein Fv (10 ng/ml) was preincubated for 10 min at 37 °C with increasing concentrations (0.1–10 μg/ml) of V_H3⁺ (M3, M11, and LAN) or V_H6⁺ (M14) human monoclonal IgM. HHMC were then added, and incubation was continued for another 30 min at 37 °C. Each bar shows the mean ± SEM of triplicate determinations.

related with that induced by protein Fv; (3) preincubation of HHMC with either protein Fv or anti-IgE resulted in complete cross-desensitization, and (4) HHMC from which IgE had been dissociated by brief exposure to lactic acid did not release histamine in response to protein Fv and anti-IgE. These findings reinforce the concept that protein Fv, which has six distinct functional Fab binding sites [41], can be bound by about 50% of human F(ab')₂ fragments [39]. Therefore, protein Fv functions as an endogenous immunoglobulin superantigen and reproduces the releasing activity of specific antigens and of anti-IgE on human FcεRI⁺ cells [28, 37].

The results of this study provide clues as to the mechanism of interaction between protein Fv and IgE bound on the mast cell membrane. An IgE myeloma (patient PP) did not block the release of histamine induced by protein Fv from mast cells, but it did suppress the release induced by anti-IgE. In contrast, IgE myeloma from patient ADZ and human polyclonal IgG inhibited the releasing activity of protein Fv.

Consequently, given these results, it is clear that the histamine-releasing activity of protein Fv depends on the binding to an immunoglobulin structure not located in the C_H domains but in the V_H region, a fragment common to all immunoglobulin classes and subclasses [39]. Since protein Fv does not bind free or combined L chains and binds

free H chains [3], the latter must be the V_H domain of IgE, as has been demonstrated in studies with immunoglobulin fragments [3]. In fact, three V_H3⁺ monoclonal IgM prevented the release of histamine induced by protein Fv from HHMC, whereas an IgM with a V_H6 domain had no effect. Structural variations between V_H families explain why one of our two monoclonal IgE and one of our two monoclonal IgG failed to bind this sialoprotein, and why the V_H6⁺ monoclonal IgM did not inhibit the releasing effect exerted by protein Fv, unlike the three V_H3⁺ monoclonal IgM, which exerted a concentration-dependent inhibition.

Our results provide the first evidence that an endogenous superantigen, protein Fv, released during viral infections, can induce the *in vitro* synthesis and secretion of several important mediators from HHMC. The activity of protein Fv is extremely potent, which raises the possibility that it exerts some effects *in vivo*. Protein Fv, found in normal liver [1], occurs as a free molecule in the gut lumen of patients with hepatitis A, B, C and E [41] and in monkeys experimentally infected with HCV and E viral hepatitis [42]. Although there are no data about the serum concentrations of protein Fv in different pathophysiological conditions, it is not inconceivable that this protein synthesized by the liver in patients with acute and chronic infection with HCV, HAV, and HBV contributes, through the release of mediators from FcεRI⁺, to some of the cardiovascular manifestations observed in some of these patients [7–10].

We have previously shown that bacterial [43, 44] and viral [45] immunoglobulin superantigens can activate human basophils and mast cells *in vitro*. These observations raise the possibility that protein Fv is contaminated by HCV or bacterial peptides. However, this hypothesis is unlikely for several reasons. First, protein Fv can be detected also in normal subjects and in uninfected animals [46]. Second, the protein has been isolated in biological samples of patients with hepatitis due to virus A, B and E [47] and not only in HCV patients. Concerning the bacterial origin, protein Fv contains a carbohydrate chain [48] and sialoglycoproteins cannot be synthesized by bacteria.

Protein Fv is a complete secretagogue, i.e. it is capable of releasing preformed mediators (histamine and tryptase) and of inducing the *de novo* synthesis of LTC₄ and PGD₂ from HHMC. Given the biological importance of mast-cell-derived mediators such as histamine [14, 49], tryptase [50], PGD₂ [33, 36] and cysteinyl leukotrienes [34, 35] in heart pathophysiology, our findings might explain the association between hepatitis viruses and cardiovascular damage in some patients with these infections. Therefore, protein Fv activation of mast cells represents a new model for a pathogenetic link between viral

hepatitis infections and FcεRI⁺ cell activation. More generally, the mechanism of FcεRI⁺ cell activation by protein Fv represents a new pathogenetic cascade involving viral infection, endogenous immunoglobulin superantigen production, activation of human FcεRI⁺ cells and tissue injury. This raises the possibility that additional endogenous immunoglobulin superantigens induced by viruses can cause tissue injury through this mechanism that involves human FcεRI⁺ cell activation.

Acute and chronic urticaria can be associated with HAV, HBV and HCV infection [51–54]. In addition, basophil activation occurs in patients with type II cryoglobulinemia [55] that have concomitant HCV infection [11–13]. There is also evidence that serum IgE levels are increased in patients with viral hepatitis [56–58]. We have previously shown that protein Fv activates human basophils to release proinflammatory mediators [59].

Hepatitis C virus has been associated with many extra hepatic manifestations [4–6], including myocarditis and cardiomyopathy [60, 61]. Histologic and functional observations have led to the hypothesis that mast cells play an important role in many fibrotic reactions [62–64]. Potential mast cell-derived mediators of the fibrotic response include histamine, tryptase and TGF-β₁, which stimulate fibroblast proliferation and collagen synthesis [65–67]. By producing these factors, chronic activation of HHMC by protein Fv may induce the heart fibrosis found in some patients with HCV infection [60, 61].

Taken together, these findings emphasize the possibility that protein Fv is a relevant molecule *in vivo* during certain viral infections.

Our data demonstrate that an endogenous protein produced in the liver can activate HHMC by acting as an immunoglobulin superantigen through interaction with V_H3⁺ IgE. The mechanism of HHMC activation by immunoglobulin-binding endogenous superantigens could represent a model to explain a pathogenetic link between viral infections, IgE-mediated activation of HHMC and cardiovascular diseases.

Acknowledgments

This work was supported by grants from the 'Istituto Superiore Sanità' (AIDS project 40.B.64 and 40.B1), 'Ministero della Salute', 'Sviluppo di strategie diagnostiche e terapeutiche innovative nella epatite cronica da HCV nell'adulto e nel bambino' and 'Alzheimer Project', 'Consiglio Nazionale delle Ricerche' (Targeted Project Biotechnology No. 01.000191.PF31 and 01.00295.PF49) and 'Ministero dell'Istruzione, Università e Ricerca' (Rome, Italy). We thank Bärbel Lamparter-Schummert for providing human heart specimens. G.M. is the recipient of the Esculapio Award 2002 (Accademia Tiberina, Rome, Italy).

References

- Bouvet J-P, Pirès R, Lunel-Fabiani F, Crescenzo-Chaigne B, Maillard P, Valla D, Opolon P, Pillot J: Protein F: A novel F(ab)-binding factor, present in normal liver, and largely released in the digestive tract during hepatitis. *J Immunol* 1990;145:1176–1180.
- Bouvet J-P, Pirès R, Charlemagne J, Pillot J, Iscaki S: Non-immune binding of human protein Fv to immunoglobulins from various mammalian and nonmammalian species. *Scand J Immunol* 1991;34:491–496.
- Bouvet J-P, Pirès R, Quan C, Iscaki S, Pillot J: Non-immune VH-binding specificity of human protein Fv. *Scand J Immunol* 1991;33:381–386.
- Lee WM: Hepatitis B virus infection. *N Engl J Med* 1997;337:1733–1745.
- Willson RA: Extrahepatic manifestations of chronic viral hepatitis. *Am J Gastroenterol* 1997;92:4–17.
- Lauer GM, Walker BD: Hepatitis C virus infection. *N Engl J Med* 2001;345:41–52.
- Bell H: Cardiac manifestations of viral hepatitis. *JAMA* 1971;218:387–391.
- Kanda T, Kobayashi I, Suzuki T, Murata K, Radio SJ, McManus BM: Elevation of ALT to AST ratio in patients with enteroviral myocarditis. *J Med* 1995;26:153–162.
- Nagaratnam N, DeSilva D, Gunawardene K: Myocardial involvement in infectious hepatitis. *Postgrad Med J* 1971;47:785–788.
- Ursell PC, Habib A, Sharma P, Mesa-Tejada R, Lefkowitz JH, Fenoglio JJ Jr: Hepatitis B virus and myocarditis. *Hum Pathol* 1984;15:481–484.
- Agnello V, Chung RT, Kaplan LM: A role for hepatitis C virus infection in type II cryoglobulinemia. *N Engl J Med* 1992;327:1490–1495.
- Dammacco F, Sansonno D: Antibodies to hepatitis C virus in essential mixed cryoglobulinemia. *Clin Exp Immunol* 1992;87:352–356.
- Gorevic PD, Kassab HJ, Levo Y, Kohn R, Meltzer M, Prose P, Franklin EC: Mixed cryoglobulinemia: Clinical aspects and long-term follow-up of 40 patients. *Am J Med* 1980;69:287–308.
- Marone G, Lichtenstein LM, Galli SJ: Mast Cells and Basophils. San Diego, Academic Press, 2000.
- Ochensberger B, Tassera L, Bifare D, Rihs S, Dahinden CA: Regulation of cytokine expression and leukotriene formation in human basophils by growth factors, chemokines and chemotactic agonists. *Eur J Immunol* 1999;29:11–22.
- Lantz CS, Boesiger J, Song CH, Mach N, Kobayashi T, Mulligan RC, Nawa Y, Dranoff G, Galli SJ: Role for interleukin-3 in mast cell and basophil development and in immunity to parasites. *Nature* 1998;392:90–93.
- Bradding P, Feather IH, Howarth PH, Mueller R, Roberts JA, Britten K, Bews JPA, Hunt TC, Okayama Y, Heusser CH, Bullock GR, Church MK, Holgate ST: Interleukin 4 is localized to and released by human mast cells. *J Exp Med* 1992;176:1381–1386.
- Ochensberger B, Daepf G-C, Rihs S, Dahinden CA: Human blood basophils produce interleukin-13 in response to IgE-receptor-dependent and -independent activation. *Blood* 1996;88:3028–3037.
- Patella V, Marinò I, Arbustini E, Lamparter-Schummert B, Verga L, Adt M, Marone G: Stem cell factor in mast cells and increased mast cell density in idiopathic and ischemic cardiomyopathy. *Circulation* 1998;97:971–978.
- Romagnani P, de Paulis A, Beltrame C, Annunziato F, Dente V, Maggi E, Romagnani S, Marone G: Tryptase-chymase double-positive human mast cells express the eotaxin receptor CCR3 and are attracted by CCR3-binding chemokines. *Am J Pathol* 1999;155:1195–1204.
- Bankl HC, Radaszkiewicz T, Klappacher GW, Glogar D, Sperr WR, Grossschmidt K, Bankl H, Lechner K, Valent P: Increase and redistribution of cardiac mast cells in auricular thrombosis. Possible role of kit ligand. *Circulation* 1995;91:275–283.
- Patella V, Marinò I, Lamparter B, Arbustini E, Adt M, Marone G: Human heart mast cells. Isolation, purification, ultrastructure, and immunologic characterization. *J Immunol* 1995;154:2855–2865.
- Kalsner S, Richards R: Coronary arteries of cardiac patients are hyperreactive and contain stores of amines: A mechanism for coronary spasm. *Science* 1984;223:1435–1437.
- Kaartinen M, Penttilä A, Kovanen PT: Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation* 1994;90:1669–1678.
- Estensen RD: Eosinophilic myocarditis: A role for mast cells? *Arch Pathol Lab Med* 1984;108:358–359.
- Patella V, de Crescenzo G, Marinò I, Genovese A, Adt M, Gleich GJ, Marone G: Eosinophil granule proteins activate human heart mast cells. *J Immunol* 1996;157:1219–1225.
- Silverman GJ, Pirès R, Bouvet J-P: An endogenous sialoprotein and a bacterial B cell superantigen compete in their VH family-specific binding interactions with human Igs. *J Immunol* 1996;157:4496–4502.
- Genovese A, Bouvet J-P, Florio G, Lamparter-Schummert B, Björck L, Marone G: Bacterial immunoglobulin superantigen proteins A and L activate human heart mast cells by interacting with immunoglobulin E. *Infect Immun* 2000;68:5517–5524.
- Marone G, Tamburini M, Giudizi MG, Biagiotti R, Almerigogna F, Romagnani S: Mechanism of activation of human basophils by *Staphylococcus aureus* Cowan 1. *Infect Immun* 1987;55:803–809.
- Siraganian RP: An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal Biochem* 1974;57:383–394.
- Snedecor GW, Cochran WG: Statistical Methods. Ames, Iowa State University Press, 1980.
- Scheffler WC: Statistics for Health Professionals. Reading, Addison-Wesley, 1984.
- Pugliese G, Spokas EG, Marcinkiewicz E, Wong PYK: Hepatic transformation of prostaglandin D₂ to a new prostanoid, 9α,11β-prostaglandin F₂, that inhibits platelet aggregation and constricts blood vessels. *J Biol Chem* 1985;260:14621–14625.
- Vigorito C, Giordano A, Cirillo R, Genovese A, Rengo F, Marone G: Metabolic and hemodynamic effects of peptide leukotriene C₄ and D₄ in man. *Int J Clin Lab Res* 1997;27:178–184.
- Samuelsson B: The discovery of the leukotrienes. *Am J Respir Crit Care Med* 2000;161:S2–S6.
- Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takano S, Nagata K: Prostaglandin D₂ selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* 2001;193:255–261.
- MacGlashan DW Jr, Peters SP, Warner J, Lichtenstein LM: Characteristics of human basophil sulfidopeptide leukotriene release: Releasability defined as the ability of the basophil to respond to dimeric cross-links. *J Immunol* 1986;136:2231–2239.
- Riske F, Hakimi J, Mallamaci M, Griffin M, Pilson B, Tobkes N, Lin P, Danho W, Kochan J, Chizzonite R: High affinity human IgE receptor (FcεRI). Analysis of functional domains of the α-subunit with monoclonal antibodies. *J Biol Chem* 1991;266:11245–11251.
- Silverman GJ, Roben P, Bouvet J-P, Sasano M: Superantigen properties of a human sialoprotein involved in gut-associated immunity. *J Clin Invest* 1995;96:417–426.
- Sala P, Tonutti E, Ruscio M, Colle R, Antonutto G, Falconieri G: IgE myeloma. Report of a new case and review of the literature. *Haematologica* 1981;66:788–795.
- Bouvet J-P, Pirès R, Iscaki S, Pillot J: Nonimmune macromolecular complexes of Ig in human gut lumen. Probable enhancement of antibody functions. *J Immunol* 1993;151:2562–2572.
- Sharma MD, Maillard P, Vratì S, Mukherjee R, Rajagopalan MS, Poynard T, Talwar GP, Pillot J: Serial passage of west-European sporadic non-B hepatitis in rhesus monkeys by inoculation with fecal extracts. *J Med Virol* 1990;30:36–41.
- Genovese A, Bouvet J-P, Florio G, Lamparter-Schummert B, Björck L, Marone G: Bacterial immunoglobulin superantigen proteins A and L activate human heart mast cells by interacting with immunoglobulin E. *Infect Immun* 2000;68:5517–5524.
- Genovese A, Borgia G, Björck L, Petraroli A, de Paulis A, Piazza M, Marone G: Immunoglobulin superantigen protein L induces IL-4 and IL-13 secretion from human FcεRI⁺ cells through interaction with the κ light chains of IgE. *J Immunol* 2003;170:1854–1861.

- 45 Patella V, Florio G, Petraroli A, Marone G: HIV-1 gp120 induces IL-4 and IL-13 release from human FcεRI⁺ cells through interaction with the V_H3 region of IgE. *J Immunol* 2000; 164:589–595.
- 46 Guihard AI, Pirès R, Bouvet J-P: An endogenous superantigen in the rat gut lumen as a model to study the role of human protein-Fv. *Scand J Immunol* 1997;45:36–42.
- 47 Bouvet J-P: Role of a liver F(ab')₂-binding molecule (protein Fv) in mucosal immunity; in Tsuchiya M, Yodoi J, Hibi T, Miura S (eds): *Current Topics in Mucosal Immunology*. Amsterdam, Elsevier, 1994, pp 169–174.
- 48 Andrieux C, Pirès R, Moreau MC, Bouvet J-P: Release of the soluble co-receptor (protein Fv) of secretory immunoglobulins after colonization of axenic rats by the human gut microflora. *Scand J Immunol* 1998;48:192–195.
- 49 Vigorito C, Poto S, Picotti GB, Triggiani M, Marone G: Effect of activation of the H₁ receptor on coronary hemodynamics in man. *Circulation* 1986;73:1175–1182.
- 50 Schwartz LB, Kawahara MS, Hugli TE, Vik D, Fearon DT, Austen KF: Generation of C3a anaphylatoxin from human C3 by human mast cell tryptase. *J Immunol* 1983;130:1891–1895.
- 51 Segool RA, Lejtenyi C, Taussig LM: Articular and cutaneous prodromal manifestations of viral hepatitis. *J Pediatr* 1975;87:709–712.
- 52 Weiss TD, Tsai CC, Baldassare AR, Zuckner J: Skin lesions in viral hepatitis. *Am J Med* 1977; 64:269–273.
- 53 Popp JW Jr, Harrist TJ, Dienstag JL, Bhan AK, Wands JR, LaMont JT, Mihm MC: Cutaneous vasculitis associated with acute and chronic hepatitis. *Arch Intern Med* 1981;141: 623–629.
- 54 Vaida GA, Goldman MA, Bloch KJ: Testing for hepatitis B virus in patients with chronic urticaria and angioedema. *J Allergy Clin Immunol* 1983;72:193–198.
- 55 Miadonna A, Tedeschi A, Leggieri E, Invernizzi F, Zanussi C: Basophil activation in idiopathic mixed cryoglobulinemia. *J Clin Immunol* 1982;2:309–313.
- 56 Heiner DC, Rose B: Elevated levels of γ-E (IgE) in conditions other than classical allergy. *J Allergy* 1970;45:30–42.
- 57 Joske RA, Turner KJ, Murphy BP: Serum IgE levels in patients with liver disease. *Med J Aust* 1976;2:555–557.
- 58 Vanepps E, Husby R, Williams RC Jr, Strickland RG: Liver disease – A prominent cause of serum IgE elevation. *Clin Exp Immunol* 1976;23:444–450.
- 59 Patella V, Bouvet J-P, Marone G: Protein Fv produced during viral hepatitis is a novel activator of human basophils and mast cells. *J Immunol* 1993;151:5685–5698.
- 60 Matsumori A, Matoba Y, Nishio R, Shioi T, Ono K, Sasayama S: Detection of hepatitis virus RNA from the heart of patients with hypertrophic cardiomyopathy. *Biochem Biophys Res Comm* 1996;222:678–682.
- 61 Matsumori A, Yutani C, Ikeda Y, Kawai S, Sasayama S: Hepatitis C virus from the hearts of patients with myocarditis and cardiomyopathy. *Lab Invest* 2000;80:1137–1142.
- 62 Hawkins RA, Claman HN, Clark RAF, Steigerwald JC: Increased dermal mast cell population in progressive systemic sclerosis: a link in chronic fibrosis? *Ann Intern Med* 1985;102: 182–186.
- 63 Broide DH, Smith CM, Wassermann SI: Mast cells and pulmonary fibrosis. Identification of a histamine releasing factor in bronchoalveolar lavage fluid. *J Immunol* 1990;145:1838–1844.
- 64 de Paulis A, Valentini G, Spadaro G, Lupoli S, Tirri G, Marone G: Human basophil releasability in patients with scleroderma. *Arthritis Rheum* 1991;34:1289–1296.
- 65 Hatamochi A, Fujiwara K, Ueki H: Effects of histamine on collagen synthesis by cultured fibroblasts derived from guinea pig skin. *Arch Dermatol Res* 1985;277:60–64.
- 66 Ruoss SJ, Hartmann T, Caughey GH: Mast cells tryptase is a mitogen from cultured fibroblasts. *J Clin Invest* 1991;88:493–499.
- 67 Pennington DW, Lopez AR, Thomas PS, Peck C, Gold WM: Dog mastocytoma cells produce transforming growth factor beta₁. *J Clin Invest* 1992;90:35–41.