Mechanism of Formation of Human IgE-binding Factors (Soluble CD23): III. Evidence for a Receptor (FcεRII)-associated Proteolytic Activity

By M. Letellier,* T. Nakajima,* G. Pulido-Cejudo,* H. Hofstetter,‡ and G. Delespesse*

*From the *Laboratory for Allergy Research, Notre-Dame Hospital, University of Montreal, Montreal, Quebec, Canada H2L 4M1; and ‡Ciba-Geigy Ltd., Department of Biotechnology, CH-4002 Basel, Switzerland*

Summary

There is mounting evidence that FcεRII (CD23) and its soluble fragments (IgE-binding factors [BFs] or soluble CD23) have pleiotropic activities. IgE-BFs are formed mainly by the proteolytic cleavage of surface FcεRII; they are first released as 37- and 33-kD unstable molecules that are subsequently transformed into 25-kD IgE-BFs. In this study, purified and radioiodinated 37-kD IgE-BFs as well as 45-kD FcεRII were used as substrates to identify the proteases leading to the formation of 25-kD IgE-BFs. These substrates generate 25-kD IgE-BFs when incubated with several FcεRII-bearing cells, including CHO1-7 cells (transfected with FcεRII cDNA); by contrast FcεRII$^-$ cells, including CHO control cells, have no effect. Highly purified unlabeled native 37-kD and recombinant 29-kD IgE-BFs also cleave labeled 45-kD FcεRII into 25-kD IgE-BFs. The proteolytic activity of these purified IgE-BFs is specifically removed by immunoprecipitation with an antibody against IgE-BFs. These data strongly suggest that FcεRII and some of its soluble fragments play an active role in the proteolytic mechanism generating IgE-BFs. They are supported by the observation that IgE-BFs released by CHO1-7 cells are cleaved exactly at the same sites as B cell–derived IgE-BFs. Taken collectively, the results are compatible with an autoproteolytic process.

The low affinity receptor for IgE (FcεRII) is implicated in protective immunity and in immunoregulation. The FcεRII on eosinophils, monocytes, and platelets is involved in IgE antibody–dependent cellular cytotoxicity against parasites (1, 2). On B lymphocytes, FcεRII is identical to CD23 (3, 4), a B cell differentiation antigen selectively expressed on sIgM/sIgD double-bearing lymphocytes. In a recent study on mouse B cells, FcεRII was shown to mediate IgE-dependent presentation of antigen to T cells (5). FcεRII is a 45-kD glycoprotein containing one N-linked carbohydrate chain of the complex type and several O-linked chains (5–9). The cDNA coding for the FcεRII from RPMI 8866 cells, a human lymphoblastoid B cell line, has been cloned (10–12) and used to express IgE-binding molecules on the surface of CHO cells (11). This receptor displays an unusual orientation with the COOH terminus being extracellular and also displays a striking homology to animal C-type lectins and to a new family of adhesion proteins (13).

The FcεRII is cleaved into biologically active soluble fragments, some of which retain the ability of binding to IgE, i.e., IgE binding factors (IgE-BFs)[1] (14, 15). Soluble FcεRII fragments were reported to have pleiotropic activities, including (*a*) the regulation of the in vitro synthesis of human IgE (15–18), (*b*) the control of B and T cell proliferation (19–22), (*c*) the induction in synergy with IL-1 of the differentiation of early thymocytes (23), and (*d*) the inhibition of monocyte migration (24). IgE-BFs are mainly produced by the cleavage of cell surface FcεRII (25). However, a small fraction of the IgE-BFs may also be formed intracellularly by proteolytic cleavage of newly synthetized FcεRII (26). IgE-BFs exist in different molecular forms with apparent M_r of 37,000, 33,000, and 25,000 (9, 27–30). The NH_2-terminal sequences of these IgE-BFs were determined and matched with the sequence deduced from the FcεRII cDNA in order to localize their respective proteolytic cleavage site (28). Recently we reported that IgE-BFs are first produced as 37-kD (and 33-kD) IgE-BFs which are then cleaved into 25-kD IgE-

[1] *Abbreviation used in this paper:* IgE-BFs, IgE binding factors.

BFs. The proteolytic enzyme involved in this cleavage is expressed on FcεRII bearing cells but not on FcεRII$^-$ cells and it is inhibited by iodoacetamide (28). The present study shows that FcεRII itself, as well as some of its soluble fragments, plays an active role in the formation of 25-kD IgE-BFs.

Materials and Methods

Reagents. Carrier-free Na^{125}I was obtained from Amersham Corp. (Arlington Heights, IL); Affi-gel 10, SDS-PAGE standards (10,000–100,000 mol wt), Coomassie Brilliant Blue R-250, SDS, acrylamide, *N,N'*-methylene-bis-acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), Tris, glycine, ammonium persulfate, 2-ME, and molecular weight markers for gel filtration were purchased from Bio-Rad Laboratories (Richmond, CA); PMSF, benzamidine hydrochloride, ε-amino caproic acid, iodoacetamide, Hepes, NP-40, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 and HBSS were purchased from Flow Laboratories (McLean, VA). MEM, trypsin-EDTA, and FCS were obtained from Gibco Laboratories (Grand Island, NY); HB101 culture medium was from Hana Biologics (Berkeley, CA) and lactoperoxydase was from Calbiochem-Behring Corp. (San Diego, CA). Immobilon™-P transfer membranes were purchased from Millipore Corp. (Bedford, MA).

Cell Culture. RPMI 8866 and RPMI 8226 cells were cultured in RPMI 1640 medium supplemented with 5 mM L-glutamine, 10% FCS, 50 IU penicillin, and 50 mg/ml streptomycin. The CHO cells were maintained in MEM medium supplemented with 5% FCS, penicillin-streptomycin, and L-glutamine.

Antibody and Immunoadsorbents. The mAb against FcεRII used in this study has been described: 207.25.A.4.4/30: IgG1$_\kappa$: mAbER 30 (31). Affi-gel 10 was coupled to mAbER 30 at 10 mg/ml, according to Bio-Rad manual instructions.

Cell Surface Radioiodination and Membrane Solubilization. The RPMI 8866 cells were surface radioiodinated at room temperature, using lactoperoxydase as described elsewhere (8). Briefly, 3–4 × 10^7 cells were washed thrice with D-PBS (8 mM $Na_2HPO_4 \cdot 7H_2O$; 137 mM NaCl; 1.5 mM KH_2PO_4; 2.7 mM KCl, pH 7.3) and resuspended in 0.5 ml of the same solution. To this cell suspension, 5 μl of a 3.6 μM Na^{125}I (3.68 nmol/mCi) carrier-free solution was added. The reaction was started by adding 12 μl of lactoperoxidase (166 U/ml) and 12 μl of 9 mM H_2O_2. This reaction mixture was gently shaken and incubated during 1 min. Two further additions of lactoperoxidase (12 μl) and H_2O_2 (12 μl) were made at 1-min intervals. The cells were then washed three times with 10 ml of cold D-PBS and resuspended in HB101 serum-free medium at a density of 10^6 cells/ml for culture or in 1.0 ml of cold lysis buffer for membrane solubilization. The lysis buffer was made in D-PBS containing 0.5% NP-40, 1 mM PMSF, 10 mM benzamidine hydrochloride, 50 mM ε-amino caproic acid, and 20 mM iodoacetamide. The insoluble material was removed by centrifugation at 10,000 *g* for 30 min at 4°C.

Purification of ^{125}I-labeled FcεRII. A total of 1 ml of cell lysate from radioiodinated RPMI 8866 cells was mixed with 200 μl of mAbER 30-Affi-gel; after overnight rotation at 4°C, the gel was washed with 30 ml of D-PBS containing 0.5% NP-40 and with 30 ml of D-PBS containing 200 μg/ml BSA. Bound material was eluted with 0.2 M glycine-HCl, pH 2.3, containing 200 μg/ml BSA. The eluted material was neutralized with 2 M Tris, pH 9.0, and dialyzed against D-PBS.

Purification of ^{125}I-labeled 37-kD IgE-BFs. Briefly, 3 × 10^7 RPMI 8866 cells were surface radioiodinated as described before and cultured for 2 h (10^6 cells/ml) in HB101 medium in the presence of iodoacetamide (10 mM). At the end of the incubation, the culture supernatant was recovered and 1 M Tris-HCl, pH 7.3; 1% NaN_3 was added to a final concentration of 66 mM Tris-HCl, 0.06% NaN_3. This supernatant was subsequently filtered on a Whatman filter paper No. 4 and adsorbed on 300 μl of mAbER 30 Affigel at a flow rate of 2 ml/h. The gel was then washed and the bound material was eluted as described for the purification of ^{125}I-FcεRII.

Electrophoresis and Autoradiography. SDS-PAGE was done on 1.5-mM 12.0% slab gels according to Laemmli (32). Samples containing 5% (vol/vol) 2-ME were boiled for 2 min and applied on gels. The gels were stained with 0.25% (wt/vol) Coomassie Brilliant Blue, destained, and dried. Autoradiography was performed at −70°C by using XAR-5 x-ray film (Eastman Kodak Co., Rochester, NY).

Purification of 29- and 37-kD IgE-BFs. For the purification of 37-kD IgE-BFs, the supernatant from RPMI 8866 cells cultured for 4 h in serum-free HB101 medium at 2 × 10^6 cells/ml in the presence of iodoacetamide (20 mM) was adsorbed on 1 ml of mAbER 30-Affi-gel at a flow rate of 2 ml/h. The gel was then washed with 50 ml of D-PBS containing 0.05% NP-40 and 5 mM iodoacetamide. The bound material was eluted with 3.5 M $MgCl_2$, pH 4.8, made 0.05% NP-40, and dialyzed against PBS containing 0.05% NP-40 and iodoacetamide (5 mM). The dialyzed material was further purified on 1 ml of freshly prepared mAbER 30-Affi-gel. This gel was washed with D-PBS containing 0.05% NP-40 and the 37-kD IgE-BFs were eluted with 3.5 M $MgCl_2$, pH 4.8, made 0.05% NP-40. The eluted material was subsequently dialyzed against D-PBS containing 0.05% N-P40 and concentrated by centrifugation at 6,000 *g* on an IEC refrigerated centrifuge using Centricon 10 microconcentrators (Amicon Corp., Danvers, MA). This procedure was also used to isolate 29-kD and 25-kD IgE-BFs from the supernatant of CHO-29 and CHO 20.3 cells, respectively, with the exception that iodoacetamide was omitted in every step. In some experiments, the affinity-purified 29-kD IgE-BFs were further purified by HPLC ion-exchange chromatography exactly as previously described (27).

Fractionation of IgE-BFs by Gel Filtration on HPLC. 25 μl of concentrated culture supernatants were applied on a BIOSIL TSK 250 column (Bio-Rad) and eluted with a buffer containing 0.05M Na_2SO_4 and 0.02M NaH_2PO_4, pH 6.8, at a flow rate of 0.5 ml/min. The column was calibrated by using the Bio-Rad gel filtration standards. Fractions (0.25 ml) were tested by RIA for IgE-BFs as described elsewhere (14). The results are expressed in units/milliliter by reference to a standard preparation. 1 U of IgE-BFs corresponds to 150 pg of 25-kD IgE-BFs.

IgE-BFs Amino Acid Sequence Analysis. IgE-BFs were isolated from the culture supernatant of CHO1-7 cells by affinity chromatography and fractionated by PAGE in the presence of SDS as described. The gel was then electroeluted to Immobilon™P transfer membrane according to the Millipore tech protocol TP006 and TP017. Briefly, the gels were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol [vol/vol], pH 8.3) for 30 min and the Immobilon membrane was pre-wet in 100% methanol and soaked in transfer buffer for 30 min. The gel was sandwiched between the Immobilon membrane and sheets of filter paper, assembled into a Bio-Rad transblot cell apparatus and electroeluted for 5 h at 50 V in transfer buffer. The membrane was stained with 0.2% Coomassie brilliant blue R-250 in 45% methanol, 10% acetic acid, and destained in 90% methanol, 7% acetic acid. The stained bands were excised from the membrane and loaded into a sequencer cup of a gas-phase sequencer (No. 470A; Applied Biosystems, Inc., Foster City, CA). The phenylthiohydantoin derivatives of the amino

acids obtained by Edman degradation were identified by reverse-phase HPLC using an Altex 5 μm ultrasphere-ODS column (33).

Expression of FcεRII, 37-, 29-, and 25-kD IgE-BFs in CHO Cells. The construction of the expression plasmid pSVd-ER, and the selection of cell line CHO (1–7) expressing human FcεRII have been described (11). In order to express 37-, 29-, and 25-kD IgE-BFs, plasmid pSVd-ER was modified. First, a 2.3-kb Dhfr selection marker, derived from plasmid pSVneo2911 (34) by digestion with SalI and XhoI, was integrated into the SalI site of pSVd-ER next to the ampicillin gene. Second, the SV40 enhancer/promoter of pSVd-ER was replaced by the 540-bp AccI-HindIII fragment of the SV40-MCMV virus P1 (35). Third, direct secretion of 37-, 29-, and 25-kD IgE-BFs was achieved by removal of the leader and NH_2-terminal coding sequences of FcεRII cDNA up to amino acid residues 75, 118, and 147, respectively, and subsequent replacement by an artificial DNA segment coding for the signal sequence of the avian influenza virus hemagglutinin (36). These modifications yielded the following plasmids: pCAL-12Mdhfr, pCAL-8Mdhfr and pCAL-10Mdhfr, which were used to transfect $Dhfr^-$ CHO DUKX-B1 cells. After 14 d of Dhfr selection, single colonies were picked and expanded, and colonies that produce 37-kD IgE-BFs (CHO-37), 29-kD IgE-BF (CHO-29), and 25-kD IgE-BFs (CHO-20.3) at high level were selected. These cell lines were maintained in MEM medium lacking ribonucleosides and deoxyribonucleosides.

Cleavage of ^{125}I-labeled 45-kD FcεRII by Purified Recombinant 29-kD IgE-BFs. In typical experiments 60 pmol of recombinant 29-kDa IgE-BFs (in 50 μl D-PBS, 0.05% NP40) was incubated with 50 μl (5,000 cpm) of radioiodinated 45-kD FcεRII dissolved in D-PBS in a final volume of 100 μl. After 17–24 h of incubation at 37°C, samples were taken into 80 μl of SDS-PAGE electrophoresis buffer consisting of 63 nM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% 2-ME, and were boiled for 2 min. In control samples the ^{125}I-labeled 45-kD FcεRII was incubated with D-PBS containing 0.05% NP-40. SDS-PAGE was then performed in 12% polyacrylamide gels at 10 mA during 17 h.

Results

Purified ^{125}I-labeled FcεRII Is Cleaved into 25-kD IgE-BFs after Interaction with FcεRII-bearing Cells. In the course of our studies on the degradation of 37-kD IgE-BFs into 25-kD IgE-BFs we noticed that affinity-purified and radiolabeled 45-kD FcεRII was degraded into 25-kD IgE-BFs when incubated with $FcεRII^+$ cells (RPMI 8866, HC-EBV transformed cell lines, U937 macrophage cell line), whereas the same preparation remained stable when incubated with $FcεRII^-$ cells (RPMI 8226, U266 myeloma cells). One such experiment is shown in Fig. 1 *a* where ^{125}I-labeled 45-kD FcεRII was incubated with an NP-40 lysate of either $FcεRII^+$ (RPMI 8866 cells) or $FcεRII^-$ cells (RPMI 8226). As seen, the 45-kD labeled FcεRII was cleaved into a 25-kD molecule only by the NP-40 lysate of RPMI 8866 cells.

To further examine whether the ability of a given cell line to cleave ^{125}I-labeled FcεRII into IgE-BFs is related to the expression of surface FcεRII, we compared CHO1-7 cells (transfected with FcεRII cDNA) with CHO control cells. As seen, the CHO1-7 cell line cleaves both the 45-kD FcεRII (Fig. 1 *b*) and the 37- and 33-kD IgE-BFs (Fig. 1 *c*) into 25-kD IgE-BFs while CHO control cells have no effect. These experiments indicate that the expression of FcεRII is required for a given cell line to cleave ^{125}I-FcεRII. This may be ex-

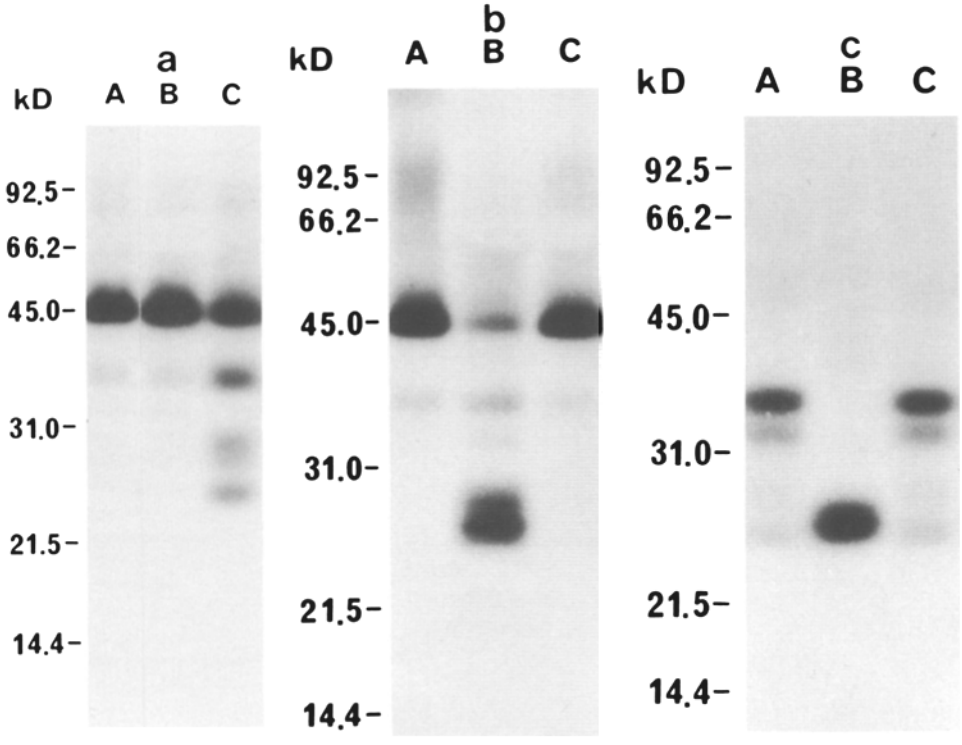

Figure 1. (*a*) Cleavage of FcεRII by RPMI 8866 cells NP-40 extract. SDS-PAGE analysis and autoradiography of ^{125}I-labeled FcεRII incubated with PBS (lane *A*), NP-40 cell lysates from RPMI 8226 (lane *B*) and 8866 cells (lane *C*). 50 μl of ^{125}I-labeled FcεRII (5,000 cpm; about 6 pmol) were incubated for 24 h at 37°C with either 50 μl of PBS or 50 μl of NP-40 cell lysates. The cell lysates were prepared as described in Materials and Methods with the exception that protease inhibitors were omitted. The lysate of RPMI 8866 cells contained 50 nM of FcεRII as estimated by RIA. The molecular weight markers are indicated at the side.

Cleavage of FcεRII (*b*) and 37-kDa IgE-BFs (*c*) by CHO1-7 cells. ^{125}I-labeled FcεRII (*b*) and ^{125}I-labeled 37-kD IgE-BFs (*c*) were incubated with HB101 culture medium (lanes *A*), CHO1-7 cells (lanes *B*) and CHO control cells (lanes *C*). CHO cells were cultured in 96-well plates until confluence was reached. The culture supernatants were then removed from the wells and replaced with 50 μl of HB101 medium and 50 μl of either ^{125}I-labeled FcεRII or ^{125}I-labeled 37-kD IgE-BFs (5,000 cpm; about 6 pmol). After a 24-h incubation at 37°C, the supernatants were analyzed by SDS-PAGE and autoradiography.

plained either by a receptor-associated proteolytic activity of FcεRII or by the FcεRII-dependent activation of an unknown protease. The latter should be ubiquitous since it is expressed on several human as well as hamster cells.

Moreover, several serine protease or thiolesterase (SH-protease) inhibitors failed to inhibit this cell-associated proteolytic activity. Indeed, with the exception of iodoacetamide, none of the following inhibitors could inhibit the formation of 25-kD IgE-BFs by RPMI 8866 cells: ε-amino caproic acid 50 mM, benzamidine hydrochloride (10 mM), PMSF (1 mM), leupeptin (100 μg/ml), cystatin (10 μg/ml), soybean trypsin inhibitor (100 μg/ml), pepstatin (10 μg/ml), TLCK (4 mM), TPCK (4 mM), $HgCl_2$ (10 μM), 2-ME (1 mM), and EDTA (10 mM). In these experiments, RPMI 8866 cells were incubated for 2 h in the presence of the protease inhibitors. The IgE-BFs in the culture supernatants were measured by RIA and their molecular weight was determined by HPLC gel filtration. In keeping with our previous studies, cells cultured in the presence of iodoacetamide released a 37-kD instead of 25-kD IgE-BFs.

The Proteolytic Activity Is Associated with the 37-kD IgE-BFs. To further analyze the cleavage of 37-kD IgE-BFs into 25-kD IgE-BFs by FcεRII-bearing cells, a functional recombinant 37-kD IgE-BFs was expressed in CHO cells after stable transformation with a mammalian expression vector containing the coding region of FcεRII corresponding to amino acids 77–321. Surprisingly, the 24-h culture supernatant of these transformed CHO cells (CHO-37) contained a 25-kD IgE-BFs and not a 37-kD IgE-BFs as expected. The latter was detected only when CHO-37 cells were cultured in the presence of iodoacetamide. The instability of 37-kD IgE-BFs suggested that these may be endowed with the proteolytic activity ascribed to the intact FcεRII. In the next series of experiments we examined the ability of several native or recombinant IgE-BFs to cleave ^{125}I-labeled 45-kD FcεRII. Fig. 2 shows that both purified native 37-kD IgE-BFs as well as a recombinant 29-kD IgE-BFs are capable of cleaving the labeled receptor into 25-kD IgE-BFs. The 29-kD IgE-BFs were purified from the supernatant of CHO cells transfected with a cDNA coding for the amino acids 120–321 of the FcεRII. Contrary to the recombinant 37-kD IgE-BFs, the 29-kD IgE-BFs are rather stable in the absence of iodoacetamide. In several similar experiments, we could hardly detect any proteolytic activity of either native or recombinant 25-kD IgE-BFs. The possibility that the proteolytic activity associated with the purified IgE-BFs might be due to a contaminating protease was not supported by the following experiments. First, immunoprecipitation of the 37-kD IgE-BFs by anti-FcεRII mAb completely abrogates the proteolytic activity (Fig. 2 *c*). Second, 25-kD and 37-kD IgE-BFs were purified from parallel culture supernatants of RPMI 8866 cells by using identical purification procedure. The only difference was that RPMI 8866 cells were supplemented with iodoacetamide for the preparation of 37-kD IgE-BFs. When reacted with radioiodinated 45-kD FcεRII, only the 37-kD IgE-BFs have proteolytic activity (data not shown).

Iodination Procedure of FcεRII Destroys the Proteolytic Activity. The above experiments were made possible because, in contrast to the unlabeled molecules, radiolabeled 45-kD FcεRII or 37-kD IgE-BFs are stable when incubated for 24 h at 37°C. Since the labeled molecules may still be cleaved, it may be predicted that the iodination procedure destroys the proteolytic activity. To examine this possibility, purified 37-kD IgE-BFs were iodinated with cold iodine (0.1 mM)

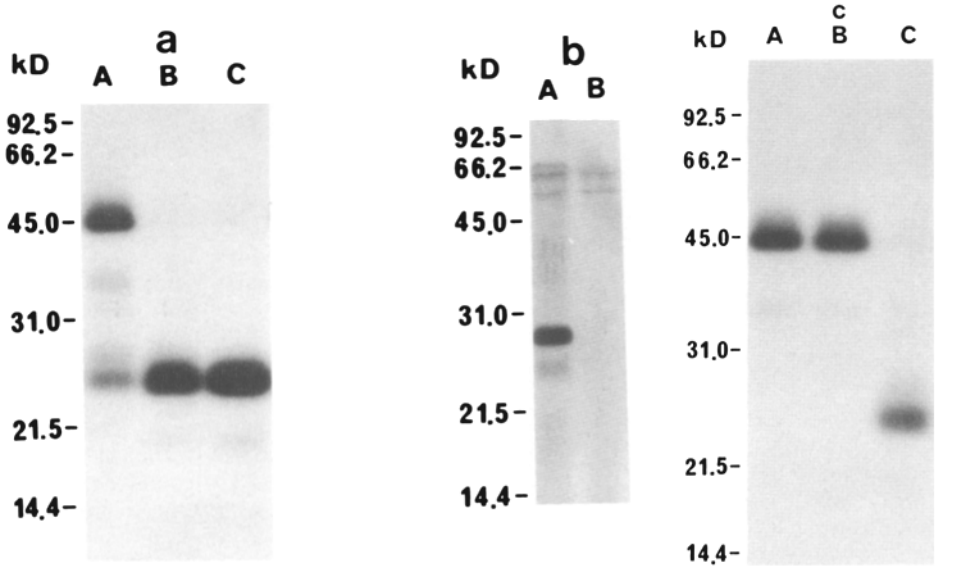

Figure 2. (*a*) Cleavage of FcεRII by purified 37-kD and 29-kD IgE-BFs. 50 μl of ^{125}I-labeled FcεRII (5,000 cpm; about 6 pmol) were incubated for 24 h at 37°C with 50 μl of PBS (lane *A*), 50 μl of affinity chromatography purified 37-kD IgE-BFs (0.3 nmol) (lane *B*), and 50 μl of recombinant 29-kD IgE-BFs (0.3 nmol) purified by affinity chromatography followed by HPLC ion-exchange chromatography (lane *C*). The mixtures were analyzed by SDS-PAGE and autoradiography. SDS-PAGE molecular weight markers are indicated at the side. (*b*) SDS-PAGE analysis of the IgE-BFs (29-kD) purified from the culture supernatant of CHO-29 cells (lane *A*). Lane *B* is an empty well. The gel was stained with silver nitrate. (*c*) ^{125}I-labeled FcεRII were incubated with PBS (lane *A*), supernatants from 37-kD IgE-BF preparation immunoprecipitated with anti-FcεRII mAb (lane *B*), or antiprolactin mAb (unrelated mAb of the same IgG subclass) (Lane *C*). A preparation of 37-kD IgE-BFs (0.3 nmol in 100 μl) was supplemented with 2 μg of mAbER 30 or antiprolactin mAb. After a 4-h incubation at 4°C, the mixtures were precipitated with 30 μg of affinity-purified rabbit anti-mouse IgG. The supernatants (50 μl) were incubated for 24 h at 37°C with 50 μl of ^{125}I-labeled FcεRII (5,000 cpm; ~6 pmol).

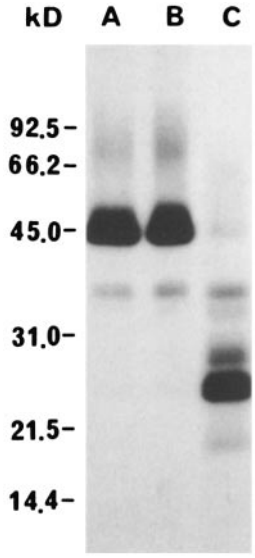

Figure 3. Effect of iodination procedure on the cleavage of FcεRII. 50 μl of ^{125}I-labeled FcεRII (5,000 cpm; ~6 pmol) were incubated for 24 h at 37°C with 50 μl of PBS (lane *A*), 50 μl of iodinated 37-kDa IgE-BFs (0.3 nmol) (lane *B*) and 50 μl of 37-kDa IgE-BFs (0.3 nmol) (lane *C*). The mixtures were analyzed by SDS-PAGE and autoradiography. SDS-PAGE molecular weight markers are indicated at the side.

by using the same lactoperoxidase method as for cell surface radioiodination and their proteolytic activity was compared to that of control 37-kDa IgE-BFs preparations treated exactly the same way except that lactoperoxidase was omitted (Fig. 3). The results show that iodinated 37-kD IgE-BFs were unable to cleave the ^{125}I-labeled FcεRII while 37-kD IgE-BFs cleaved ^{125}I-FcεRII into 25-kD IgE-BFs. The inactivation of the proteolytic activity associated with the 37-kD IgE-BFs by the iodination procedure is caused by lactoperoxidase oxidation rather than by the iodination of the molecules. Indeed, treatment of 37-kD IgE-BFs with lactoperoxidase alone abrogates the proteolytic activity (data not shown). Similar observations were also made with intact RPMI 8866 cells iodinated with cold iodine and incubated with ^{125}I-FcεRII in the presence of cycloheximide to inhibit the synthesis of new receptor molecules (data not shown).

CHO Cells Expressing FcεRII Produce IgE-BFs Identical to Those Released from RPMI 8866 Cells. Our interpretation

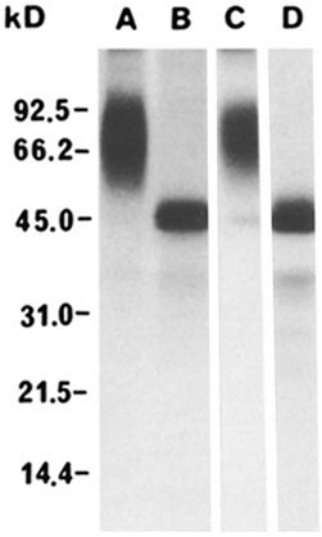

Figure 4. SDS-PAGE analysis of the affinity chromatography-purified radiolabeled FcεRII from RPMI 8866 (lanes *A* and *B*) and CHO1-7 (lanes *C* and *D*) cells. (Lanes *A* and *C*) KSCN eluates; (lanes *B* and *D*) glycine eluates. Similar amount of radioactivity was applied to each lane. SDS-PAGE molecular weight markers are indicated at the side.

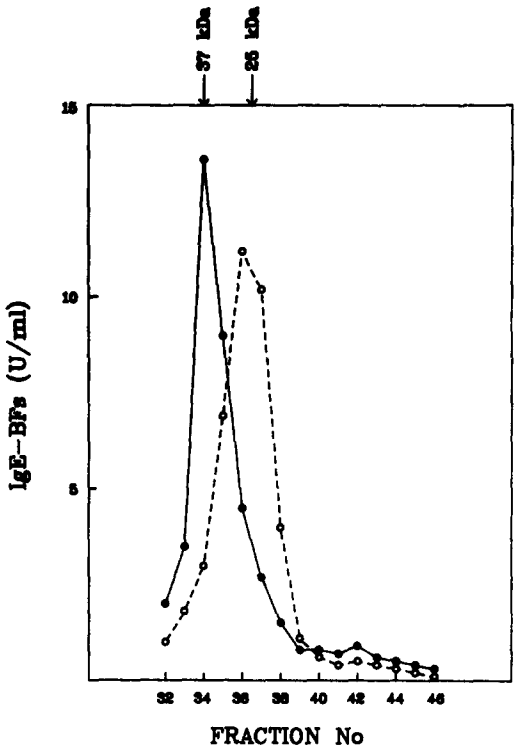

Figure 5. Fractionation by gel filtration of the supernatant from CHO1-7 cells cultured for 6 h in HB101 medium in the presence (●) or absence of 20 mM iodoacetamide (○). The supernatants were concentrated 50-fold and fractionated by HPLC on a TSK 250 column.

of the above results is that the cleavage of FcεRII and its soluble 37-kD IgE-BFs into 25-kD IgE-BFs is mediated by either an autoproteolytic mechanism or by an FcεRII-dependent ubiquitous protease. If this view is correct it may be predicted that CHO cells expressing recombinant 45-kD surface FcεRII should release IgE-BFs that are identical to those of B cell origin. In the next series of experiments we first ascertained that recombinant FcεRII on CHO cells has the same structure as on B cells (Fig. 4). We then analyzed the IgE-BFs released by CHO1-7 cells. These IgE-BFs were purified by affinity chromatography, fractionated by SDS-PAGE, and then transferred to immobilon™-P transfer membrane by electrophoretic blotting. The NH_2-terminal sequence of the first four residues was determined on the excised 25-kD stained band as described. A double sequence was obtained corresponding to Leu-Arg-Met-Glu and Met-Glu-Leu-Gln. Matching these sequences with those deduced from the FcεRII cDNA indicates that the 25-kD IgE-BFs from CHO1-7 cells starts with leucine at position 148 or methionine at position 150 of the FcεRII, which are exactly the same two proteolytic cleavage sites as for the 25-kD IgE-BFs isolated from RPMI 8866 cells culture supernatant (10–12). Moreover, in additional experiments, we observed that as in the case of RPMI 8866 cells, the 25-kD IgE-BFs are derived from soluble 37-kD IgE-BF precursors. Indeed, when CHO1-7 cells are cultured in the presence of iodoacetamide, the molecular mass of IgE-BFs

is shifted from 25 to 37-kD as determined by HPLC gel filtration (Fig. 5).

Discussion

IgE-BFs are mainly produced by the proteolytic cleavage of surface FcεRII (9, 25). The rate of this process is reduced by IgE, whereas it is increased by treatment of FcεRII-bearing cells with tunicamycin, indicating that the N-linked carbohydrate chain stabilizes the receptor (25). We recently reported that IgE-BFs are first released as 33–37-kD molecules which are subsequently transformed into 25-kD IgE-BFs (17). The latter are much more stable and they are detected in the supernatants of 2–3 wk cultures in human sera and even in urine (37).

In the course of our initial studies on the cloning of FcεRII cDNA, we noticed that after purification to homogeneity, 45-kD FcεRII molecules were unstable and degraded into 37-, 33-, 29-, and 25-kD fragments. In contrast, most preparations of radiolabeled receptors isolated from surface radioiodinated cells were rather stable (in the absence of inhibitors), even when incubated for 24 h or more at 37°C. Such preparations were therefore used as a substrate for the identification of the proteolytic enzymes that cleave FcεRII. In this study, we first observed that the ability of a given cell line to cleave ^{125}I-labeld 45-kD FcεRII or 37-kD IgE-BFs is dependent upon the expression of FcεRII. Indeed, ^{125}I-labeled 45-kD FcεRII and its 37-kD fragments are transformed into 25-kD IgE-BFs when incubated with several FcεRII-bearing cells (including the transfected CHO1-7 cells), while the same preparations remain stable when incubated with several FcεRII$^-$ cells (including CHO control). There are two possible explanations for these observations: (*a*) FcεRII is degraded by a receptor-associated proteolytic mechanism (where one FcεRII molecule could cleave adjacent identical molecules), or (*b*) it is degraded by a protease that is both ubiquitous and activated by FcεRII. These two possibilities are also supported by the observation that CHO1-7 cells, expressing functional recombinant IgE receptor, release IgE-BFs identical to those released by B cells (i.e., they have the same molecular weight and the same NH_2-terminal end cleavage sites). To examine these alternatives it was necessary to test the ability of purified unlabeled FcεRII to cleave radioiodinated FcεRII. Because it is difficult to obtain convincingly pure preparations of 45-kD FcεRII, the proteolytic activity of soluble IgE-BFs was examined. The instability of 37-kD IgE-BFs suggested that it might be endowed with such activity. Indeed, highly purified 37-kD IgE-BFs proved to be capable of cleaving radiolabeled 45-kD FcεRII, as well as radiolabeled 37-kD IgE-BFs, into 25-kD IgE-BFs. Moreover, recombinant and stable 29-kD IgE-BFs but not native or recombinant 25-kD IgE-BFs displayed the same activity. Three observations militate against the possibility that the proteolytic activity of the purified 37- and 29-kD molecules was due to a co-purified protease. First, the 24-h culture supernatant of FcεRII-bearing B cells (containing 25-kD IgE-BFs) has no activity (28). Second, immunoprecipitation of the 37-kD IgE-BFs specifically removed the proteolytic activity of the preparation. Third, when 37 and 25 kD were isolated from the culture supernatants of the same cell line using identical purification procedures, only the 37-kD molecules were capable of cleaving the labeled 45-kD FcεRII. Taken collectively, the present data suggest, but do not demonstrate, that IgE-BFs are produced by an autoproteolytic mechanism. This possibility is currently examined more directly by (*a*) using synthetic peptides as substrate, and (*b*) comparing the proteolytic activity of several FcεRII mutants, aiming to localize the putative active site(s).

Autoproteolysis was recently described as a mechanism to activate or inactivate an increasing number of biologically active proteins in bacteria (38, 39), virus (40–42), and eucaryotes (43–47). For example, in polymorphonuclear leukocytes, calpain (a protease) is derived from its inactive precursor by autoproteolysis (43–44). Other examples include cathepsin D (45) and human collagenase (46). The current observations of an FcεRII-associated proteolytic activity raises the intriguing possibility that this receptor or its 37-kD fragment may be involved in the cleavage of other biologically relevant molecules. In this regard, it is worth noting that some differentiation antigens were recently shown to display protease activity: examples include CD10 (or CALLA, endopeptidase −24.11) (47, 48), CD13 (aminopeptidase N) (49), and CD26 (dipeptidyl peptidase IV) (50).

We thank Dr. Michel Van Der Rest (McGill University, Montreal, Quebec) for the NH_2-terminal sequencing of IgE-BFs. The advice and the constructive criticism of Dr. M. Sarfati (University of Montreal) are fully appreciated. The excellent secretarial assistance of Mrs. Norma Del Bosco is gratefully acknowledged.

This work was supported by a grant from the Medical Research Council of Canada. G. Delespesse is a Research Associate from the Medical Research Council of Canada. M. Letellier is a postdoctoral fellow from the Fonds de la Recherche en Santé du Québec.

Address correspondence to Dr. G. Delespesse, Notre-Dame Hospital, Research Center, 1560 Sherbrooke Street East, Montreal, Quebec, Canada H2L 4M1.

Received for publication 13 November 1989 and in revised form 12 April 1990.

References

1. Capron, A., J.P. Dessaint, M. Capron, J.H. Ouma, and A.E. Butterworth. 1987. Immunity to schistosomes: progress toward vaccine. *Science (Wash. DC).* 238:1065.
2. Capron, A., J.P. Dessaint, M. Capron, M. Joseph, J.C. Ameisen, and A.B. Tonnel. 1986. From parasites to allergy: a second receptor for IgE. *Immunol. Today.* 7:15.
3. Bonnefoy, J.Y., J.P. Aubry, C. Peronne, J. Wijdenes, and J. Banchereau. 1987. Production and characterization of a monoclonal antibody specific for human lymphocyte low affinity receptor for IgE: CD23 is a low affinity receptor for IgE. *J. Immunol.* 138:2970.
4. Yukawa, K., H. Kikutani, H. Owaki, K. Yamasaki, A. Yokota, H. Nakumura, E.L. Barsumian, R.R. Hardy, M. Suemura, and T. Kishimoto. 1987. A B cell-specific differentiation antigen, CD23, is a receptor for IgE (FcεR) on lymphocytes. *J. Immunol.* 138:2576.
5. Kehry, M.R., and L. Yamashita. 1989. Low-affinity IgE receptor (CD23) function on mouse B cells: Role in IgE-dependent antigen focusing. *Proc. Natl. Acad. Sci. USA.* 86:7556.
6. Meinke, G.C., A.M. Magro, D.A. Lawrence, and H.L. Speigelberg. 1978. Characterization of an IgE receptor isolated from cultured B-lymphoblastoid cells. *J. Immunol.* 121:1321.
7. Peterson, L.H., and D.H. Conrad. 1985. Fine specificity, structure, and proteolytic susceptibility of the human lymphocyte receptor for IgE. *J. Immunol.* 135:2654.
8. Nakajima, T., and G. Delespesse. 1986. IgE receptors on human lymphocytes. I. Identification of the molecules binding to monoclonal anti-Fcε receptor antibodies. *Eur. J. Immunol.* 16:809.
9. Letellier, M., T. Nakajima, and G. Delespesse. 1988. IgE Receptor on Human Lymphocytes. IV. Further analysis of its structure on the role of N-linked carbohydrates. *J. Immunol.* 141:2374.
10. Kikutani, H., S. Inui, R. Sato, E.L. Barsumian, H. Owaki, K. Yamasaki, T. Kaisho, N. Uchibayashi, R.R. Hardy, T. Hirano, S. Tsunasawa, F. Sakiyama, M. Suemura, and T. Kishimoto. 1986. Molecular structure of human lymphocyte receptor for immunoglobulin E. *Cell.* 47:657.
11. Ludin, C., H. Hofstetter, M. Sarfati, C.A. Levy, U. Suter, D. Alaimo, E. Kilchherr, H. Frost, and G. Delespesse. 1987. Cloning and expression of the cDNA coding for a human lymphocyte IgE receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:109.
12. Ikuta, K., M. Takami, C. Won Kim, T. Honjo, T. Miyoshi, Y. Tagaya, T. Kawabe, and J. Yodoi. 1987. Human lymphocyte Fc receptor for IgE: sequence homology of its cloned cDNA with animal lectins. *Proc. Natl. Acad. Sci. USA.* 84:819.
13. Bevilacqua, M.P., S. Stengelin, M.A. Gimbrone, and B. Seed. 1989. Endothelial leukocyte adhesion molecule 1: An inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science (Wash. DC).* 243:1160.
14. Sarfati, M., T. Nutman, C. Fonteyn, and G. Delespesse. 1986. Presence of antigenic determinants common to Fc IgE receptors on human macrophages, T and B lymphocytes and IgE-binding factors. *Immunology.* 59:569.
15. Sarfati, M., and G. Delespesse. 1988. Possible role of human lymphocyte receptor for IgE (CD23) or its soluble fragments in the in vitro synthesis of Human IgE. *J. Immunol.* 141:2195.
16. Pène, J., F. Rousset, F. Brière, I. Chrétien, J. Wideman, J.Y. Bonnefoy, and J.E. De Vries. 1988. Interleukin 5 enhances interleukin 4-induced IgE production by normal human B cells. The role of soluble CD23 antigen. *Eur. J. Immunol.* 18:929.
17. Sarfati, M., E. Rector, K. Wong, M. Rubio-Trujillo, A.H. Sehon, and G. Delespesse. 1984. In vitro synthesis of IgE by human lymphocytes. II. Enhancement of the spontaneous IgE synthesis by IgE-binding factors secreted by RPMI 8866 lymphoblastoid B cells. *Immunology.* 53:197.
18. Sarfati, M., E. Rector, M. Rubio-Trujillo, K. Wong, A.H. Sehon, and G. Delespesse. 1984. In vitro synthesis of IgE by human lymphocytes. III. IgE-potentiating activity of culture supernatants from Epstein-Barr Virus (EBV) transformed B cells. *Immunology.* 53:207.
19. Swendeman, S., and D.A. Thorley-Lawson. 1987. The activation antigen BLAST-2, when shed, is an autocrine BCGF for normal and transformed B cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1637.
20. Gordon, J., J.A. Cairns, M.J. Millsum, S. Gillis, and G.R. Guy. 1988. Interleukin 4 and soluble CD23 as progression factors for human B lymphocytes: analysis of their interactions with agonists of the phosphoinositide "dual pathway" of signalling. *Eur. J. Immunol.* 18:1561.
21. Armitage, R.J., and L.K. Goff. 1988. Functional interaction between B cell subpopulations defined by CD23 expression. *Eur. J. Immunol.* 18:1753.
22. Armitage, R.J., L.K. Goff, and P.C.L. Beverley. 1989. Expression and functional role of CD23 on T cells. *Eur. J. Immunol.* 19:31.
23. Mossalayi, M.D., J.-C. Lecrom, A.H. Dalloul, M. Sarfati, J.-M. Bertho, H. Hofstetter, G. Delespesse, and P. Debré. 1990. Soluble CD23 (FcεRII) and interleukin synergistically induce early human thymocyte maturation. *J. Exp. Med.* 171:959.
24. Flores-Romo, L., J.A. Cairns, M.J. Millsum, and J. Gordon. 1989. Soluble fragments of the low-affinity IgE receptor (CD23) inhibit the spontaneous migration of U937 monocytic cells: maturation of MIF-activity by a CD23 antibody. *Immunology.* 67:547.
25. Nakajima, T., M. Sarfati, and G. Delespesse. 1987. Relationship between human IgE-binding factors (IgE-BF) and lymphocyte receptors for IgE. *J. Immunol.* 139:848.
26. Lee, B.W., C.F. Simmons, T. Wileman, and R.S. Geha. 1989. Intracellular cleavage of newly synthesized low affinity Fcε receptor ($Fc\varepsilon R_2$) provides a second pathway for the generation of the 28-kDa soluble $Fc\varepsilon R_2$ fragment. *J. Immunol.* 142:1614.
27. Sarfati, M., T. Nakajima, H. Frost, E. Kilccherr, and G. Delespesse. 1987. Purification and partial biochemical characterization of IgE-binding factors secreted by a human B lymphoblastoid cell line. *Immunology.* 60:539.
28. Letellier, M., M. Sarfati, and G. Delespesse. 1989. Mechanisms of formation of IgE-BFs (soluble CD 23). I. FcεRII bearing B cells generate IgE-binding factors of different molecular weights. *Mol. Immunol.* 26:1105.
29. Guy, G.R., and J Gordon. 1987. Coordinated action of IgE and a B-cell-stimulatory factor on the CD23 receptor molecule up-regulates B-lymphocyte growth. *Proc. Natl. Acad. Sci. USA.* 84,6239–6243.
30. Thorley-Lawson, D.A., S.L. Swendeman, and C.M. Edson. 1986. Biochemical analysis suggests distinct functional roles for the BLAST-1 and BLAST-2 antigens. *J. Immunol.* 136:1745.
31. Rector, E., T. Nakajima, C. Rocha, D. Duncan, D. Lestourgeon, R.S. Mitchell, J. Fisher, A.H. Sehon, and G. Delespesse. 1985. Detection and characterization of monoclonal antibodies specific to IgE receptors on human lymphocytes by flow cytom-

etry. *Immunology.* 55:481.
32. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
33. Lazure, C., N.G. Seidah, M. Chrétien, R. Lallier, and S. St-Pierre. 1983. Primary structure determination of Escherichia coli heat-stable enterotoxin of porcine origin. *Can. J. Biochem. Cell Biol.* 61:287.
34. Asselbergs, F.A., H. Will, P. Wingfield, and M. Hirschi. 1986. A recombinant Chinese hamster ovary cell line containing a 300-fold amplified tetramer of the hepatitis B genome together with a double selection marker expresses high levels of viral protein. *J. Mol. Biol.* 189:401.
35. Dorsch-Hasler, K., G.M. Keil, F. Weber, M. Jasin, W. Schaffner, and U.H. Koszinowski. 1985. A long and complex enhancer activates transcription of the gene coding for the highly abundant immediate early mRNA in murine cytomegalovirus. *Proc. Natl. Acad. Sci. USA.* 82:8325.
36. Van Heinje, G. 1983. Patterns of amino acids near signal-sequence cleavage sites. *Eur. J. Biochem.* 133:17.
37. Sarfati, M., D. Bron, L. Lagneaux, C. Fonteyn, H. Frost, and G. Delespesse. 1988. Elevation of IgE-Binding Factors in serum of patients with B cell-derived chronic lymphocytic leukemia. *Blood.* 71:94.
38. Slilaty, S.N., J.A. Rupley, and J.W. Little. 1986. Intramolecular cleavage of Lex A and phage Lambda repressors: dependence of kinetics on repressor concentration, pH, temperature, and solvent. *Biochemistry.* 25:6866.
39. Burckhardt, S.E., R. Woodgate, R.H. Scheuermann, and H. Echols. 1988. UmuD mutagenesis protein of *Escherichia coli*: overproduction, purification, and cleavage by Rec A. *Proc. Natl. Acad. Sci. USA.* 85:1811.
40. Giam, C.Z., and I. Boros. 1988. In vivo and In vitro autoprocessing of human immunodeficiency virus protease expressed in *Escherichia coli*. *J. Biol. Chem.* 263:14617.
41. Hahn, C.S., E.G. Strauss, and J.H. Strauss. 1985. Sequence analysis of three sindbis virus mutants temperature-sensitive in the capsid protein autoprotease. *Proc. Natl. Acad. Sci. USA.* 82:4648.
42. Richards, O.C., L.A. Ivanoff, K. Bienkowska-Szewczyk, B. Butt, S.R. Petteway, M.A. Rothstein, and E. Ehrenfeld. 1987. Formation of poliovirus RNA polymerase 3D in *Escherichia coli* by cleavage of fusion proteins expressed from cloned viral cDNA. *Virology.* 161:348.
43. Legendre, J.L., and H.P. Jones. 1988. Purification and characterization of calpain from human polymorphonuclear leukocytes. *Inflammation.* 12:51.
44. Morelli, A., M. Grasso, T. Meloni, G. Forteleoni, E. Zocchi, and A. De Flora. 1987. Favism: impairment of proteolytic systems in red blood cells. *Blood.* 69:1753.
45. Nishimura, Y., M. Higaki, and K. Kato. 1987. Identification of a precursor form of cathepsin D in microsomal lumen: Characterization of enzymatic activation and proteolytic processing in vitro. *Biochem. Biophys. Res. Commun.* 148:335.
46. Grant, G.A., A.Z. Eisen, B.L. Marmer, W.T. Roswit, and G.I. Goldberg. 1987. The activation of human skin fibroblast procollagenase. Sequence identification of the major conversion products. *J. Biol. Chem.* 262:5886.
47. Letarte, M., S. Vera, R. Tran, J.B.L. Addis, R.J. Onizuka, E.J. Quackenbush, C.V. Jongeneel, and R.R. McInnes. 1988. Common acute lymphocytic leukemia antigen is identical to neutral endopeptidase. *J. Exp. Med.* 168:124.
48. Shipp, M.A., J. Vijayaraghavan, E.V. Schmidt, E.L. Masteller, L. D'Adamio, L.B. Hersh, and E.L. Reinherz. 1989. Common acute lymphoblastic leukemia antigen (CALLA) is active neutral endopeptidase 24.11 ("enkephalinase"): direct evidence by cDNA transfection analysis. *Proc. Natl. Acad. Sci. USA.* 86:297.
49. Amascato, A.A., J.W. Alexander, and G.F. Babcock. 1989. Surface amino peptidase activity of human lymphocytes. I. Biochemical and biologic properties of intact cells. *J. Immunol.* 142:1245.
50. Mentlein, R., E. Heymann, W. Scholz, A.C. Feller, and H.D. Flad. 1984. Dipeptidyl peptidase IV as a new surface marker for subpopulation of human T lymphocytes. *Cell. Immunol.* 89:11.