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Human B Lymphocytes Synthesize the 92-kDa Gelatinase, Matrix Metalloproteinase-9*

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Matrix metalloproteinases (MMPs) are involved in the remodeling of connective tissue as well as in disease states associated with acute and chronic inflammation or tumoral metastatic processes. Despite detailed and extensive studies of the mechanisms of lymphocyte extravasation, remarkably little is known about the expression and regulation of metalloproteinases involved in the migratory process. By using zymography and reverse transcription-polymerase chain reaction experiments, we have demonstrated that Epstein-Barr virus-immortalized B lymphocytes are able to secrete a 92-kDa metalloproteinase with gelatinolytic activity which has been purified and identified as being MMP-9. Moreover, the tissue inhibitor of metalloproteinase was shown to be constitutively expressed by the B cells. The expression of 92-kDa gelatinase is mediated by cytokines, growth factors, lipopolysaccharide, concanavalin A, and the tumor promotor phorbol 12-myristate 13-acetate. Time dependence activity increased rapidly up to 24 h of incubation with lipopolysaccharide or concanavalin A stimulation while it requires a delay and more time to have an optimum effect when cytokines were the stimulating agents; transforming growth factor-β abolished 92-kDa gelatinase production. Both staurosporine and wortmannin are inductive stimuli, and the level of MMP-9 secreted into the media is greater than that observed with other agents except concanavalin A. Elicitation of the chemotactic migration of B cells through a model basement membrane by lipopolysaccharide was shown to be correlated with gelatinase expression and inhibited by 7 mM captopril. Our study indicates that Epstein-Barr virus-B lymphocytes express 92-kDa gelatinase, the production of which can be modified by a variety of physiological and pharmacological signals which have been shown to differ according to the cell type.

Cells of the immune system must invade the surrounding tissue in order to reach the site of inflammation. High endothelial venules are specialized postcapillary venules that are found in lymphoïd tissues which support high levels of lymphocyte extravasation from the blood (1). The movement of lymphocytes from the circulation into the tissues requires that cells traverse the capillaries, penetrate the basement membrane, and migrate into the stroma. The basement membrane is a major barrier to leukocyte extravasation, which necessitates the proteolytic cleavage of components, including collagens (predominantly type IV collagen) and glycoproteins such as laminin (2). Since metalloproteinases (MMPs)[1] are believed to play a critical role in the degradation of the extracellular matrix (3) and to facilitate migration into the surrounding environment (4), we have reasoned that these proteinases may be involved in the movement of human lymphocytes from the circulation into the stroma (5). The matrix metalloproteinases constitute a family of zinc-dependent endopeptidases whose members have been implicated in such physiological processes as morphogenesis, angiogenesis (3), and wound repair (6), or the pathological aspects associated with inflammation (7) and tumor invasion (4). From the four subclasses of this important protease family, gelatinase A (72 kDa, type IV collagenase, MMP-2, EC 3.4.24.24) and gelatinase B (92 kDa, type IV collagenase, MMP-9, EC 3.4.24.35) have been reported as being active in the cleavage of all types of denatured collagens, type IV and type V collagens in their native forms, elastin, and other matrix proteins (3, 8–10). Gelatinases A and B are the products of distinct genes and are regulated differently (4). Their expression can be modulated by soluble mediators such as growth factors, cytokines, oncogenes, and tumor promotors (3, 11, 12). Regulation depends on coordinated increases in transcription, secretion, proteolytic activation or TIMP inactivation, and, in some instances, association of the activated forms with cell surfaces (7, 13). Neutrophils, eosinophils, macrophages, and T cells all produce and secrete MMPs with a cell-specific pattern for induction and control of MMP expression, and functional roles in the mediation of immunity and inflammation (14). Gelatinase A is the most widely distributed MMP, being produced constitutively by many cell types in culture, particularly fibroblasts (14) and endothelial cells (15). Gelatinase B can also be secreted by mesenchymal cells in culture, after induction by cytokines or other agents, but it is a major product of monocytes, macrophages, T lymphocytes, and tumor cells (14, 16). It is also found packaged in a granule fraction in polymorphonuclear neutrophils and is released upon neutrophil stimulation (17, 18). Despite detailed and extensive studies of the secretion

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[1] The abbreviations used are: APMA, *p*-aminophenylmercuric acetate; bp, base pair(s); BSA, bovine serum albumin; ConA, concanavalin A; EBV, Epstein-Barr virus; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; RT, reverse transcription; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TIMP tissue inhibitor of metalloproteinase; TGF, transforming growth factor; TNF, tumor necrosis factor; FPLC, fast protein liquid chromatography; ELISA, enzyme-linked immunosorbent assay; MOPS, 4-morpholinepropanesulfonic acid; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

of gelatinase B by infiltrating neutrophils and much speculation about the mechanisms of their extravasation (17), little is known about the involvement of MMPs in the migratory process of immune cells such as lymphocytes. It has been reported that normal T lymphocytes contain both gelatinase A and gelatinase B, which may induce basement membrane turnover by a different regulatory process (5, 19- 22). Here, for the first time, we describe the expression and regulation of gelatinase B in human B lymphocytes.

EXPERIMENTAL PROCEDURES

Materials—Reagents used in this work were obtained from the following sources: *p*-aminophenylmercuric acetate (APMA), phorbol 12-myristate 13-acetate (PMA), EDTA, gelatin agarose, recombinant (r) interleukin (IL)-10, staurosporine, wortmannin, lipopolysaccharide (LPS), concanavalin A (ConA) (Sigma); diisopropylphosphorofluoridate (Fluka, Switzerland); rIL-1β, rIL-2, rIL-4, rIL-6, rIL-8, rIL-11, rTGF-β, and rTNF-α (Boehringer Mannheim, France); Superose 12 (Amersham Pharmacia Biotech, Uppsala, Sweden); rIL-13 (RD System, Abingdon, UK); growth factor reduced Matrigel® matrix (Becton Dickinson, Bedford, MA); human TIMP-1 ELISA system and TIMP-1 (Amersham Pharmacia Biotech, Buckinghamshire, UK); Transwell inserts (Corning Costar Corporation, Cambridge, MA); Centricon 10 (Amicon, Beverly, MA); TRIzol™ reagent (Life Technologies, Inc.); captopril (D-3-mercapto-2-methylpropanoyl-L-proline) was a generous gift of Bristol Myers-Squibb Company (Paris, France).

Cell Culture—Lymphocytes from heparinized sterile venous blood were prepared by Ficoll-Hypaque density gradient centrifugation. The cells were infected with the B95-8 strain of the Epstein-Barr virus (EBV) as described previously (23). The EBV B lymphocyte cell line was maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 50 μg/ml kanamycin, and 50 μg/ml streptomycin. The culture was maintained at 37 °C under a 5% CO_2 atmosphere. The medium was changed twice weekly.

Before MMP expression and after extensive washing, the EBV B lymphocytes were grown in serum-free medium containing 0.2% (w/v) bovine serum albumin and were maintained under the same CO_2 atmosphere for 18 h until further use. Cell viability was monitored by the trypan blue exclusion method; it was of the order of 90%. All the experiments were standardized by using an equal number of cells.

Purification of MMP-9 from Human Neutrophils and Production of Antisera—Secreted gelatinase B was purified from 0.8 nM PMA-stimulated human neutrophils as described previously (24). Briefly, released proteins were fractionated by DEAE-Sephacel anion exchange chromatography and affinity chromatography on gelatin agarose. Fractions containing gelatinase activity were pooled and processed using preparative SDS-polyacrylamide gel electrophoresis (PAGE) (5–15% gel). A 92-kDa band corresponding to the native neutrophil gelatinase was electroeluted and injected into rabbits for antibody production. Purified gelatinase (50 μg/0.5 ml of phosphate-buffered saline) was mixed with 0.5 ml of Freund's complete adjuvant and injected into rabbits. Booster injections using Freund's incomplete adjuvant were started on day 15 and repeated every 2nd week for 2 months. Blood was collected 2 weeks after the last booster and allowed to clot. Antisera were separated by centrifugation and stored at −20 °C. Serum immunoglobulins were pelleted down twice with 33–40% (w/v) $(NH_4)_2SO_4$, and filtered through a DEAE-cellulose matrix to eliminate plasma α_2-macroglobulin. Immunoglobulins G (IgG) were further isolated onto protein A-Sepharose. The specificity of antisera and immunoglobulins with respect to binding and inhibition has been reported previously (24).

Purification of a Metalloproteinase with Gelatinolytic Activity from EBV B Lymphocytes in Culture—A metalloproteinase with gelatinolytic activity, from crude EBV B lymphocyte culture medium supernatant, was purified using substrate affinity chromatography on gelatin agarose, and gel filtration on FPLC Superose 12. Approximately 40 ml of serum-free, conditioned medium (10^8 EBV B lymphocytes) was dialyzed against 0.05 M Tris-HCl, pH 7.6, buffer containing 0.005 M $CaCl_2$, 0.02% (w/v) NaN_3, 0.5 M NaCl, 0.05% (v/v) Brij 35. The dialyzed medium was applied to a gelatin agarose column that had been equilibrated in the same buffer. After extensive washing with equilibration buffer containing 1 M NaCl, the bound enzyme was eluted with 10% (v/v) dimethyl sulfoxide, which was added to the latter buffer. Fractions with zymographically determined gelatinolytic activity were pooled, concentrated by ultrafiltration through Centricon 10, and applied to a Superose 12 column prepared in 0.05 M Tris-HCl, pH 8, buffer containing 0.3 M NaCl and 0.05% (v/v) Brij 35. The fractions were collected by FPLC, at a flow rate of 0.25 ml/min at a pressure of 1 MPa. Eluates showing gelatinolytic activity were pooled and frozen until further use.

Migration Assay—Cell migration was quantified using Transwell inserts as described previously (5). 10^6 EBV B lymphocytes in 0.5 ml of serum-free culture medium containing 0.2% (w/v) BSA were added to the 12-mm diameter inserts of Transwell chambers over 12-μm pore polycarbonate filters with a continuous even coating of 100 μl of growth factor reduced Matrigel® matrix, which separated the cells from 1.5 ml of the same medium in the lower compartment. In this experiment, EBV B cells were preincubated with 10 ng/ml LPS at 37 °C for 18 h in the 0.2% BSA serum-free medium and then added to the upper compartment of the insert. The Transwell chambers were incubated in a 5% CO_2 environment at 37 °C for 24 h. In some experiments, different concentrations of captopril were added to the two compartments. Cells in the lower compartment were detached by shaking and collected for counting.

SDS-PAGE and Immunoblotting—Proteins were separated in parallel with appropriate controls and molecular weight markers using SDS-PAGE (25) in 10% (w/v) acrylamide gel with a 5% (w/v) stacking gel, and stained with Coomassie Brilliant Blue R-250. Nitrocellulose transfer of proteins separated by SDS-PAGE was performed according to the method described by Towbin *et al.* (26). After this, the blotting membranes were incubated with specific antiserum raised against neutrophil-purified gelatinase (1:200 dilution) in 0.05 M Tris-HCl, 0.2 M NaCl, 0.05% (v/v) Tween 20, pH 7.5, followed by goat anti-rabbit IgG alkaline phosphatase conjugate (1:1000 dilution in the same buffer), and stained with nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate reagent, according to the manufacturer's instructions.

Gelatin Zymography—Zymographic analysis was carried out in 10% (w/v) SDS-polyacrylamide gels containing gelatin (0.5 mg/ml), as described previously (15, 16). The proteins collected from conditioned medium or chromatography eluates were concentrated using Centricon 10. They were applied to the gel in a sample buffer containing 2.3% (w/v) SDS but lacking β-mercaptoethanol and were not boiled prior to loading. The gels were washed twice for 15 min in 0.05 M Tris-HCl, pH 7.6, containing 0.005 M $CaCl_2$, 0.001 mM $ZnCl_2$, and 2.5% (w/v) Triton X-100, in order to remove SDS, followed by 5-min washes in buffer devoid of Triton X-100. After 3.5 h of incubation at 37 °C with 0.001 M APMA in the same buffer, containing 1% (w/v) Triton X-100, the gels were stained with Coomassie Brilliant Blue R-250 and destained as described previously (27). Zones of enzymatic activity were shown by negative staining and quantitated by scanning densitometry at 600 nm (CD 60, Desaga, Sarstedt Gruppe). Enzyme activity was expressed in arbitrary units from a standard curve corresponding to the gelatin zymography of increasing concentrations of latent purified gelatinase B (27).

Protein content was estimated using the micro BCA method (28). TIMP-1 was measured by using a two-site ELISA "sandwich" format (ELISA system, code RPN 2611, Amersham Life Science, Inc.). Standards (purified TIMP-1, Amersham Life Science, Inc.) and samples (flow-through from gelatin agarose matrix and culture medium) were incubated with anti-TIMP-1 antibody. The TIMP-1 was detected by a conjugate (peroxidase-labeled antibody to TIMP-1); the reaction was then quantitated as indicated by the manufacturer's instruction. The concentration of TIMP-1 in a sample was determined by interpolation from a standard curve.

Analysis of the Isolated 92-kDa Gelatinase and TIMP-1 mRNAs by Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)—Total RNA was isolated (29) from about 10^8 EBV B lymphocytes, using a modification of the single-step method described by Chomczynski and Sacchi (29), involving 5-min incubation of the total cell pellets with 1 ml of TRIzol™ reagent. The cell pellets were disrupted by repetitive push-pull through a 1-ml Pipetman tip. Cell lysates were transferred to RNase-free sterile Eppendorf tubes, and RNA was extracted over 0.2 ml of chloroform by centrifugation (10,000 × *g*, 15 min, 4 °C). RNA was precipitated from the supernatant phase with 0.5 ml of isopropyl alcohol and washed in 70% (v/v) ethanol. The optical density of RNA resuspended in sterile water was recorded ($A_{260\ nm/280\ nm}$ ratio >1.8–2.0). The yield from this procedure varied between 50 and 200 μg of total RNA. Aliquots of 5 μg of total RNA were reverse-transcribed in 20 μl of RT buffer, using oligo(dT) primers and a cDNA synthesis kit used according to the manufacturer's instructions. cDNA (2.5 μl per test) was immediately amplified by PCR, using 2.5 units of *Taq* polymerase in 100 μl (final volume) of *Taq* buffer containing 0.2 mM dNTP and 0.25 μM sense and antisense oligonucleotides. The oligonucleotides used as primers were synthesized from the *timp-1, mmp-9* gene sequences of the EMBL cDNA library as follows. MMP-9 (92-kDa gelatinase) primers, sense 5′-116–136 bp/antisense 5′-392–372 bp were designed to

TABLE I
Composition of oligonucleotide primers

Positions of the 5′-ends of the primers are numbered from the ATG inhibition codon of the MMP-9 or TIMP-1 gene. The MMP-9 gelatinase primers and TIMP-1 primers correspond to cDNA fragments of 277 and 769 bases, respectively.

Oligoprimers	Sequence	Position	T_m
			°C
MMP-9			
Oligo sense	5′-GGAGACCTGAGAACCAATCTC-3′	+116	64
Oligo antisense	5′-TCCAATAGGTGATGTTGTCGT-3′	+392	60
TIMP-1			
Oligo sense	5′-GGCCATCGCCGCAGATCC-3′	+1	62
Oligo antisense	5′-GCTGGGTGGTAACTCTTTATTTCA-3′	+769	68

amplify a 277-bp cDNA fragment; TIMP-1 primers: sense 5′-1–18 bp/antisense 5′-769–746 bp (769-bp complete cDNA) (Table I). Thirty-five cycles (denaturation, 1 min at 94 °C; annealing, 1 min at 57 °C for MMP-9, at 60 °C for TIMP-1; extension, 2 min at 72 °C) followed by a 7-min elongation period were performed with a M J Research PTC 150 thermocycler with Peltier effect. Commercial actin primers were run in parallel PCR tests as a control for PCR and RNA extraction efficiency. Aliquots of 10 μl of PCR products in bromphenol blue solution were run together with a scale of DNA ladders (markers VI, Boehringer Mannheim, France) on 1.5% (w/v) agarose gels containing 1 μg/ml ethidium bromide. The bands were photographed using Polaroid film and UV transillumination.

Sequencing of the PCR Products—PCR products were gel-purified and automatically sequenced by Genome Express Ltd., Grenoble, France, with forward and backward primers for TIMP-1 and MMP-9.

Northern Blot Analysis—24 h after addition of 0.5 nM PMA, 1 μg/ml concanavalin A, or 10 ng/ml LPS, total RNA was extracted from EBV B lymphocytes by the TRIzol™ method as described previously. Poly(A)$^+$ RNA was isolated onto an oligo(dT) cellulose matrix by rocking total RNA with oligo(dT) cellulose in a high salt buffer (400 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.2% (w/v) SDS, pH 7.4) for 2–3 h at room temperature and elution of mRNA with a buffer with no salt (5 mM Tris, 1 mM EDTA, 0.2% (w/v) SDS, pH 7.4). After denaturation at 65 °C for 15 min, poly(A)$^+$ RNA from control or test samples was size-fractionated on a 1% (w/v) agarose-formaldehyde gel in 1× MOPS buffer, blotted onto a positive nylon membrane in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7) by capillary action and immobilized by UV cross-linking using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The 277-bp *mmp-9* PCR product was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, France) by PCR with the same oligonucleotide primers as those used before and hybridized to the filter overnight at 42 °C in a high SDS buffer (7% (w/v) SDS, 50% (v/v) formamide, 5× SSC, 2% (w/v) blocking reagent, 50 mM sodium phosphate, pH 7, 0.1% (w/v) *N*-lauroylsarcosine). The membrane was washed twice in 2× SSC with 0.1% (w/v) SDS at room temperature for 5 min and twice in 0.1× SSC with 0.1% (w/v) SDS at 50 °C for 15 min. The detection was performed using anti-digoxigenin (Fab) fragments conjugated to alkaline phosphatase followed by a chemiluminescent reaction using the CDP-Star system (Boehringer Mannheim, France), according to the manufacturer's instructions. Chemiluminescent signals were detected by exposing the blot onto Hyperfilm MP (Amersham Life Science, Inc.) for 10 min. The integrity and equal gel loading of mRNA were assessed by visualizing the remaining 28 and 18 S ribosomal RNA bands under UV light after staining with ethidium bromide and by a second hybridization of the membrane with a digoxigenin-labeled probe of the housekeeping *g3pdh* gene. Quantitation (ratio mRNA for gelatinase B/mRNA for G3PDH) was performed by scanning densitometry at 400 nm of the bands of MMP-9 and G3PDH seen on x-ray films (CD 60, Desaga, Sarstedt Gruppe).

Statistical Methods—The variations are expressed as mean ± S.E.; p values were calculated by Student's paired t test.

RESULTS

Production of a Metalloproteinase with Gelatinolytic Activity by Epstein-Barr Virus-immortalized B Lymphocytes—Human B cells were isolated from peripheral blood, transformed by Epstein-Barr virus, and cultured *in vitro* as described under "Experimental Procedures." We analyzed EBV B lymphocytes for metalloproteinase activity secreted into the culture-conditioned medium. Because of low levels of expression, the isolation of a metalloproteinase with gelatinolytic activity was carried out onto a gelatin agarose matrix (Fig. 1*A*). Analysis of the eluates by gelatin zymography (Fig. 1*A*, *inset*) revealed that proenzyme was present in eluates 11–15, which were recovered from the gelatin affinity chromatography once dimethyl sulfoxide was added to the NaCl washing buffer; maximum gelatinolytic activity occurred in fractions 12 and 13 in both the control experiment and after treatment of the cells with 0.5 nM PMA. Treatment with PMA induced a high level of secretion of the gelatinolytic enzyme compared with the control. Similar results were observed with untransformed B lymphocytes (not shown). Metalloproteinases are known to be secreted as latent precursors of higher molecular weight than the mature enzyme. 1.8 mM APMA induced the conversion of the proenzyme to active forms (Fig. 1B, *lane 1* (latent) and *lane 2* (active)), yielding gelatinolytically active products at 78 and 71 kDa molecular masses. 10 mM EDTA completely inhibited the activity of all the gelatinases (Fig. 1*B*, *lane 3* (latent) and *lane 4* (active)). The inhibition of gelatinolytic activity by EDTA indicated that the enzyme displays the characteristics of a metalloproteinase. Gelatinase-free TIMP-1 was recovered in the flow-through of the gelatin agarose chromatography during the isolation of 92-kDa gelatinase, as shown in Fig. 1*A*. TIMP-1 was quantitated using ELISA, giving a 94% recovery of TIMP-1 (flow-through *versus* culture medium). The 92-kDa gelatinase isolated on gelatin agarose was purified by FPLC gel filtration chromatography (not shown). The eluates which show gelatinolytic activity were pooled and processed using SDS-PAGE and Western blotting (Fig. 1, *C* and *D*). About 10 ng of purified gelatinase were isolated from 196 ml of serum-free conditioned medium of 8×10^8 EBV B lymphocyte culture in a purification process which yielded a 471-fold purified protein. The final material ran as a major 90-kDa band on reducing SDS-polyacrylamide gel electrophoresis (Fig. 1*C*, *lane 2*). Western blot analysis of the purified protein with a polyclonal antibody specific for neutrophil MMP-9 labeled a protein of molecular mass in the range of 90 to 94 kDa (Fig. 1*D*). This corresponds to the 92-kDa protein with gelatinolytic activity detected by zymography (Fig. 1*A*, *inset*). Additional confirmation that the enzyme was indeed gelatinase B (MMP-9) was obtained by detection of specific mRNA by PCR. Total RNA was extracted from EBV B lymphocytes (10^8 cells) and 5 μg of total RNA were subjected to RT and amplification by PCR, as described under "Experimental Procedures." As shown in Fig. 2, MMP-9 and TIMP-1 PCR products were clearly observed. RT-PCR yielded low but significant levels of MMP-9 products (Fig. 2, *lane 1*) while the amount of TIMP-1 transcript seemed to be higher (Fig. 2, *lane 2*); the amplification products have the expected size and 99.3 and 99.5% homology, respectively, with TIMP-1 and MMP-9 cDNA published sequences.

Taken together, these results conclusively indicate that EBV B lymphocytes secrete a 92-kDa gelatinolytic enzyme which can be identified as MMP-9 according to the following criteria: molecular weight, specific inhibition by EDTA, the pattern of activation products generated by APMA, binding to gelatin agarose matrix and cleavage of gelatin, identification of specific mRNA, and immunoreactivity.

Regulation of Expression of the Metalloproteinase with Gelatinolytic Activity Secreted from Epstein-Barr Virus-immortalized B Lymphocytes—Under the conditions used for short term cultures, EBV B lymphocytes secreted a slight constitutive gelatinolytic proteinase of approximately 92-kDa molecular mass, which has been identified as being MMP-9. Treatment of EBV B lymphocytes with PMA resulted in an enhanced production of the 92-kDa gelatinase (Fig. 1*A*, *inset*). To determine the optimal PMA concentration for enzyme induction, the cells

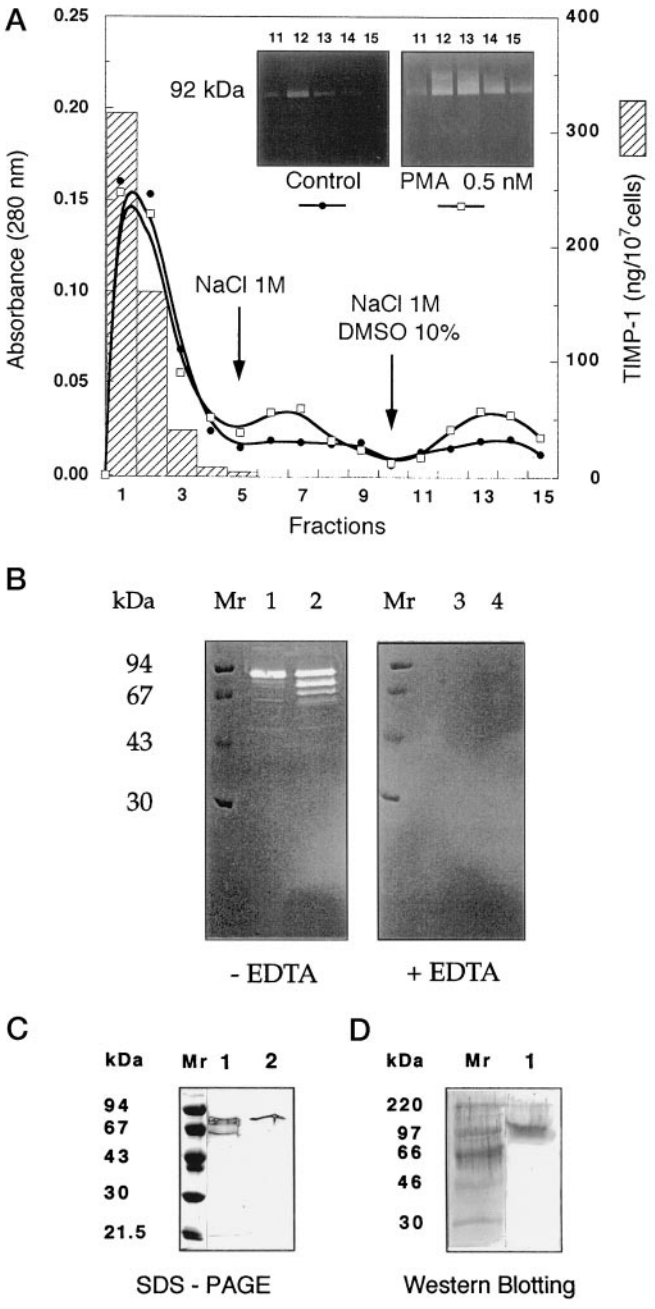

FIG. 1. **Isolation of a metalloproteinase with gelatinolytic activity from serum- free conditioned medium of cultured EBV B lymphocytes.** *A*, serum-free conditioned medium of resting (control) or 0.5 nM PMA-stimulated 10^8 EBV B lymphocytes cultured at 37 °C for 72 h was filtered through a gelatin agarose matrix as described under "Experimental Procedures." A 92-kDa metalloproteinase with gelatinolytic activity was eluted from the matrix by using 10% Me_2SO and 1 M NaCl added to the equilibrium buffer. *Fractions 11–15* of the control ( ) or PMA assay (□) were concentrated onto Centricon 10 (Amicon) and submitted to gelatin zymography (*inset*). Following Coomassie staining, *white bands* represent zones of lysis of the gelatin substrate. TIMP-1 (▨) was measured using ELISA, as reported under "Experimental Procedures" and expressed as nanograms/10^7 cells. Results are representative of 20 experiments. *B*, fractions of the previous gelatin agarose chromatography performed with the conditioned medium from PMA-stimulated EBV B lymphocytes were pooled and subjected to gelatin zymography, after activation of the proteinase using 1.8 mM APMA *(lanes 2* and *4)* at 37 °C for 45 min in the absence *(lanes 1* and *2)*, or the presence *(lanes 3* and *4)* of 10 mM EDTA; *lanes 1* and *3*, control experiment without APMA activation. *C*, the pooled fraction from *B* was subjected to FPLC Superose 12 gel filtration for purification as described under "Experimental Procedures." The purified gelatinase-enriched pooled fraction was submitted to SDS-PAGE. *Lane 1*, gelatin agarose, 35 μg; *lane 2*, FPLC Superose, 20 μg. Results are representative of three experiments. *D*, Western blotting of the purified protein. Specific antibodies were polyclonal antibody raised against neutrophil MMP-9. Staining was performed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. *kDa* represents the molecular mass standards. Results are representative of three experiments.

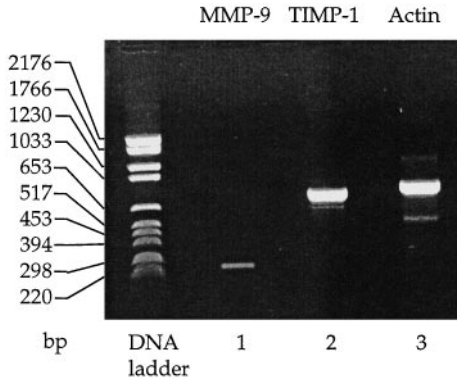

FIG. 2. **RT-PCR of actin, MMP-9 and TIMP-1 of RNA from unstimulated EBV B lymphocytes.** The experiment was performed with 5 μg of RNA for each specimen and 35 cycles for the PCR as described under "Experimental Procedures." Samples without RT showed no PCR products. Results presented illustrate 1 of 10 experiments.

were incubated in medium containing increasing concentrations of PMA, for 18 h. Maximum induction of gelatinase activity was observed in the range of 0.1 to 0.5 nM and was correlated with good cell viability. The kinetics of enzyme induction were then examined. The cells were grown in medium containing 0.5 nM PMA, and at defined time periods, medium samples were withdrawn. Gelatinase was then isolated onto a gelatin agarose matrix and analyzed for gelatinolytic activity. Increased levels of secreted proteinase activity were visible 10 h post-treatment (Fig. 3). As PMA is an inducer of the membrane-associated protein kinase C and in order to determine whether it was inducing gelatinase activity via activation of protein kinase C in EBV B lymphocytes, the effect of staurosporine, a protein kinase C inhibitor, was investigated. For these studies, EBV B lymphocytes were cultivated in the absence or the presence of increasing concentrations of staurosporine up to 20 nM (Fig. 4*A*). Staurosporine increased gelatinase expression and at the concentrations used, had no effect on cell survival as determined by trypan blue exclusion; in all treatment groups, the viability of the cells was 90% (data not shown). A similar stimulation was observed after incubation of EBV B lymphocytes for 18 h with wortmannin, suggesting the involvement of phosphatidyl inositiol 3-kinase in signal transduction pathways. Basal expression of gelatinase was optimum with 20 nM staurosporine and 200 nM wortmannin (Fig. 4, *A* and *B*), and four times more than that measured when PMA was the stimulating agent. EBV B lymphocytes were then exposed to a variety of physiological and pharmacologic modulators. In order to determine the best conditions for enzyme induction, the cells were incubated in medium containing increasing concentrations of stimulating agents. The metalloproteinase from the medium was isolated onto a gelatin agarose matrix and analyzed by zymography as reported previously for PMA. As shown in Table II and Figs. 3 and 5, basal expression of gelatinase activity was affected by exposure of the cells to different cytokines, TNF-α, TGF-β, LPS, ConA, or PMA. Increased levels of secreted gelatinolytic enzyme were visible at 10 and 8 h post-treatment of the cells with PMA (Fig. 3), and LPS or ConA (Fig. 5*B*), respectively, but only at 24 h when IL-13 was the stimulating agent (Fig. 5*A*). Stimulation of EBV B lymphocytes showed variations in gelatinase secretion ac-

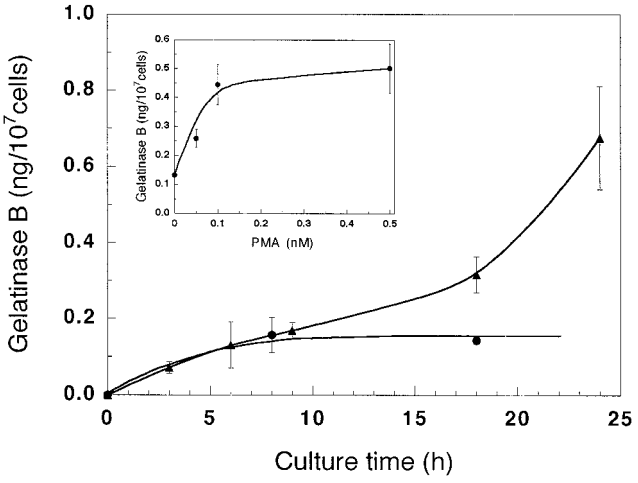

FIG. 3. **Time course of gelatinase B (92 kDa) induction in EBV B lymphocytes stimulated by 0.5 nM PMA.** 2–5 × 10^7 EBV B lymphocytes were stimulated by 0.5 nM PMA (▲) or not (control,) and cultivated at 37 °C (+5% CO_2) at different incubation times. The supernatant was next collected and assayed for gelatinase B production using gelatin zymography. Gelatinase activity was quantitated by scanning densitometry and expressed from arbitrary units extrapolated to a concentration of MMP-9 obtained from a standard curve drawn up after gelatin zymography of latent purified gelatinase B (24, 27). Results are mean ± S.E. and representative of four experiments (each in triplicate). *Inset*, dose-response effect of PMA on gelatinase B production. 2–5 × 10^7 EBV B lymphocytes were stimulated by increasing PMA concentrations up to 0.5 nM and cultivated for 18 h at 37 °C. Gelatinase activity was quantitated as described previously. The results are mean ± S.E. and representative of four experiments (each in triplicate).

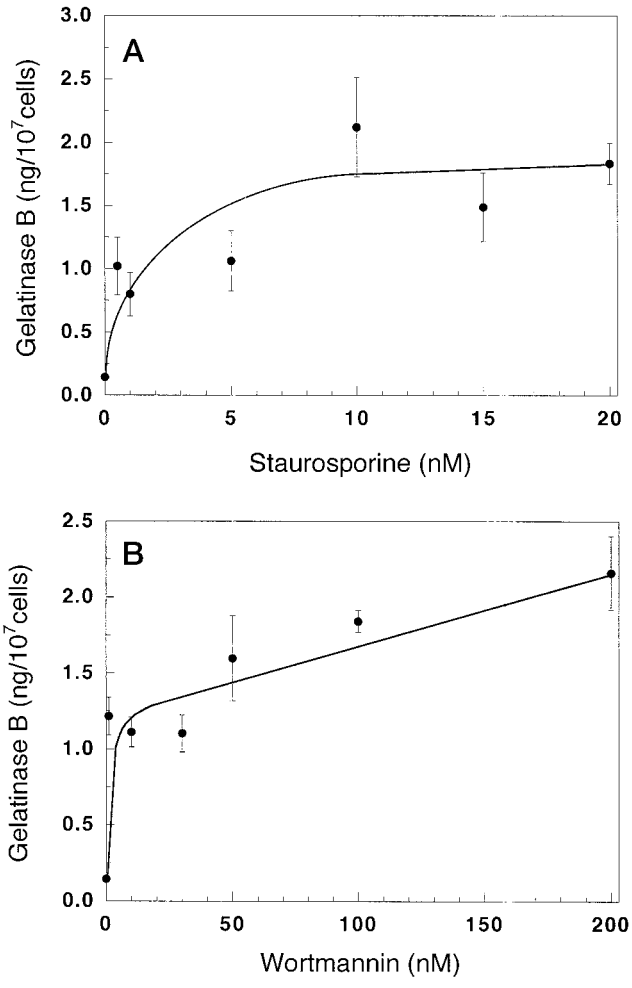

FIG. 4. **Dose-response effect of staurosporine (*A*) and wortmannin (*B*) on constitutive gelatinase B and TIMP-1 production.** 2–5 × 10^7 EBV B lymphocytes were treated with increasing concentrations of staurosporine (up to 20 nM) (*A*) and wortmannin (up to 200 nM) (*B*) and cultivated for 18 h at 37 °C (+5% CO_2). Gelatinase production was measured using gelatin zymography and densitometry, as described under "Experimental Procedures." Results are the mean ± S.E. from three experiments (each in triplicate).

cording to the stimulus (Table II). The results obtained at 18 + 72 h of culture showed a significant 2-fold increase in IL-1β-induced gelatinolytic activity, compared with the control. Interleukins, IL-2, IL-4, IL-6, IL-10, IL-11, and TNF-α failed to up- or down-regulate significantly gelatinase biosynthesis, while IL-13 required more time to have an optimum effect (Fig. 5*A*); 1 ng/ml TGF-β completely abolished gelatinase synthesis and secretion. The production of gelatinase was strongly stimulated by LPS in a dose-dependent manner, up to 20 ng/ml (not shown). It was optimum with 10 ng/ml LPS and 1 μg/ml ConA at 24-h incubation.

92-kDa Gelatinase mRNA expression by EBV B lymphocytes was evidenced by using a reverse transcription-polymerase chain reaction and Northern blot. We were successful in performing RT-PCR for 92-kDa gelatinase and TIMP-1 from resting EBV B lymphocytes (Fig. 2). When EBV B lymphocytes were cultured in the presence of cytokines or growth factors, there was no increase in the levels of the enzyme and TIMP-1 inhibitor mRNAs (not shown); these findings differ from the slight but significant increase in both proteins secreted after stimulation of the cells by IL-1β, IL-8, and IL-13. On the contrary when PMA, LPS, and ConA were the stimulating agents the MMP-9 mRNA message was enhanced while there was no significant differences *versus* control for TIMP-1 (not shown). Northern blot analysis of poly(A)$^+$ RNA prepared from EBV B lymphocytes was carried out with a cDNA probe specific for human MMP-9 to determine if the changes in secreted gelatinolytic activity were reflective of significantly increasing amounts of mRNA present (Fig. 5*C*). Poly(A)$^+$ mRNA from resting cells hybridized as a single band which corresponded to the MMP-9 transcript (30). Treatment of EBV B lymphocytes with PMA, ConA, or LPS increased significantly the expression of MMP-9 mRNA of 1.7-, 2.7-, and 3.5-fold, respectively, as shown by densitometric analysis (ratio of mRNA for gelatinase B/mRNA for G3PDH).

EBV B Lymphocyte Migration through a Basement Membrane Equivalent—The recruitment of blood B cells to tissue sites of immune responses and chronic inflammation involves their adhesion to and movement between endothelial cells, and migration through the vascular basement membrane and into tissues. The spontaneous and LPS- or ConA-enhanced migration of human EBV-transformed B cells across a layer of growth factor-reduced Matrigel® which consists of basement membrane matrix constituents was carried out in order to assess the role of secreted 92-kDa gelatinase in the transmigration process.

10^6 EBV B cells were layered onto a polycarbonate micropore filter coated with Matrigel® (Fig. 6). Migration assays were performed using unstimulated and LPS-prestimulated B lymphocytes. The specificity of gelatinase-mediated migration was demonstrated by using captopril, a recently reported inhibitor of zinc MMPs and angiogenesis (31, 32). As expected, prestimulation of the EBV B lymphocytes with 10 ng/ml LPS induced a large migration increase (Fig. 6) which was inhibited by 10 mM EDTA (not shown). Captopril was able to inhibit LPS-induced migration at concentrations ranging from 1 to 15 mM. The dose-response curve illustrated first a sharp rise in inhibition of migration up to a 7 mM captopril concentration followed by a plateau in inhibitory activity at captopril concentrations from 7 to 15 mM (Fig. 6, *inset*). There was no toxic effect, as measured

TABLE II
Induction of gelatinolytic activity in EBV B lymphocytes stimulated with cytokines, and growth factor, TGF-β

Conditioned medium from stimulated EBV B lymphocytes was withdrawn after 18 h and 18 h + 72 h of culture and filtered through a gelatin agarose matrix. The eluted fractions were analyzed for gelatinase activity by zymography. Zones of enzymatic activity were shown by negative staining and quantitated by scanning densitometry at 600 nm (CD60, Desaga, Sarstedt Gruppe). The arbitrary units that were obtained were extrapolated to a concentration of MMP-9 from a range of increasing concentrations of purified gelatinase B of neutrophils (27).

Stimulating agent	Concentration	Conditioned medium[a]	
		Time in culture after addition of stimulating agent	
		18 h	18 h + 72 h
	ng/ml	*ng gelatinase per 10^7 cells*	
Control		0.15 ± 0.009 (10)	0.18 ± 0.029 (5)
IL-1β	1	0.13 ± 0.014 (3)	0.43 ± 0.084* (5)
IL-2	25	0.04 ± 0.020 (3)	0.10 ± 0.016 (5)
IL-4	20	0.13 ± 0.026 (5)	0.15 ± 0.056 (4)
IL-6	10	0.07 ± 0.035* (3)	0.09 ± 0.021 (4)
IL-8	100	0.21 ± 0.011* (6)	0.32 ± 0.066* (5)
IL-10	20	0.10 ± 0.019* (4)	0.18 ± 0.039 (4)
IL-11	100	0.09 ± 0.031* (4)	0.20 ± 0.018 (3)
IL-13	50	0.00 ± 0.000* (3)	0.27 ± 0.017* (4)
TNF-α	20	0.04 ± 0.021 (3)	0.04 ± 0.013* (5)
TGF-β	1	0.00 ± 0.000* (3)	0.00 ± 0.000* (3)

[a] Results are mean ± S.E. (n) number of experiments. The asterisk (*) indicates $p < 0.05$ *versus* control.

by trypan blue exclusion, of captopril to the cell in culture up to 12 mM. In the absence of stimulus, 10 mM captopril had no effect on basal migration corroborating that at this concentration it was not toxic to the B lymphocytes.

DISCUSSION

One of the effector functions of activated MMPs expressed in immune cells is to promote transbasement membrane migration of lymphocytes. Here, we have shown that EBV-immortalized B lymphocytes constitutively express gelatinase B and that expression can be modulated by cytokines, growth factors, and tumor promotors; there was no evidence of phenotypic expression of gelatinase A. The characteristics of the 92-kDa gelatinase isolated from EBV-immortalized B lymphocytes and demonstrated in this study are similar to those reported for neutrophils, monocytes, and tumor cells (19, 33–35). It is a metalloproteinase that is dependent on zinc and calcium ions, with degradative activity against gelatin. Our data indicate that the isolated 92-kDa form is a proenzyme that can be activated *in vitro* by organomercurials and *in vivo* after secretion as all metalloproteinases except membrane-type MMPs and MMP-11. Disruption of the endothelial basal lamina is a prerequisite for migration of immunoactive cells through postcapillary venules. It has been reported that normal human T cells express two matrix metalloproteinases, gelatinase A and B, both being detectable in their inactive proenzyme forms as well as in their active forms (5). However, the enzymatic events that underlie this capability in B cells are unknown. Here, we demonstrate the selective and constitutive expression in EBV B lymphocytes of progelatinase B, which can be modulated by different mediators. Among the proinflammatory cytokines, only IL-1β and IL-8 enhanced gelatinase synthesis, while IL-6 and TNF-α had no significant effect. The expression of MMP with gelatinolytic activity was suppressed by TGF-β, providing an interesting contrast with the MMP-enhancing expression observed in T lymphocytes, which suggests the existence of a differential regulation of the MMP gene in the two lymphocyte cell types (19, 22). IL-4, another anti-inflammatory cytokine, has no effect, and in contrast, IL-13 induces significant gelatinase expression but as IL-1β and IL-8 requires a delay for the response. There is no possible contamination of the medium by other sources of gelatinase; this was ensured by the use of serum-free media and a highly purified EBV-immortalized B cell population.

As observed with lymphocytes and with various cell lines, metalloproteinase expression and regulation is cell type-specific. In uterine cervical fibroblasts, the synthesis of pro-MMP-9 was recently shown to be regulated by PMA, IL-1, and TNF-α, but the combined effect of PMA or TNF-α and then of IL-1 resulted in distinct responses suggesting a different regulation process of production (36). Differential signaling pathways mediated by TGF-β were also evidenced in dermal fibroblasts and epidermal keratinocytes (37). Finally the expression and suppression of matrix metalloproteinase biosynthesis was differently reported. While IL-4 and IL-10 inhibited 92-kDa gelatinase, matrilysin, and collagenase in mononuclear phagocytes (38–40), they had little or no effect upon fibroblast (40) or EBV B lymphocyte (this work) MMP expression. In contrast, TGF-β suppressed gelatinase production in B cells, while it had no effect upon the mononuclear phagocyte production of matrilysin, interstitial collagenase, or 92-kDa gelatinase (38). TGF-β is known to achieve its anti-inflammatory properties by the inhibition of IL-8 gene expression (41). These results argue in favor of a great complexity of regulation mechanisms of MMPs produced by the inflammatory cells and a strict dependence on cell specificity.

T and B lymphocytes, neutrophils, and macrophages attracted to damage sites by various chemotactic factors are often in close spatial proximity and may interact with each other or with resident tissue cells. The consequences of the interaction have been demonstrated recently by the induction of MMP expression when neutrophils adhere to the endothelium (15) or by a direct contact between T cells and monocytes (42, 43). The present results suggest that gelatinase B expression in B lymphocytes might depend on an imbalance between pro- and anti-inflammatory stimuli, through which the cytokines could play a central role. Our results therefore have shown that inflammation will depend not only on the presence of proinflammatory mediators such as IL-1, IL-8, and IL-13 but also on the absence of negative regulatory factors such as TGF-β (41). Like other MMPs, gelatinase B is secreted from cells as an inactive zymogen (pro-MMP-9) and the recent finding that pro-MMP-9 forms a specific complex with the MMP inhibitor TIMP-1 (16, 44) has introduced another level of complexity into the regulatory mechanisms of MMP activity. In the present work, during the 92-kDa gelatinase purification process, we did not collect any gelatinase-TIMP-1-associated complex, while the ratio of TIMP-1 *versus* progelatinase B in the culture medium recovered after 72 h of culture time was in favor of the inhibitor (TIMP-1/MMP-9 = 1000). The local concentration of these molecules (mediators and inhibitors) will thus be critical in infiltration of inflammatory cells and regulation.

An interesting finding of our study is that the constitutive expression of latent 92-kDa progelatinase by EBV B lymphocytes is greatly enhanced when they are stimulated by LPS, ConA, and PMA. In many inflammatory cell types, including macrophages, MMP genes encoding gelatinase B but not gelatinase A respond in a similar fashion to LPS and PMA stimulation (45) and contain cis-acting elements such as AP-1 and NF-κB-binding sites in their promoter region (45). The gelatinase content of EBV B lymphocytes was investigated after homogenization of the cells and found to be in the same range as that measured in the culture medium at resting states (0.17 ng/10^7 cells inside, *versus* 0.11–0.18 ng/10^7 cells outside, respectively). Secretion of gelatinase in the medium increased upon PMA stimulation without any modification of its content

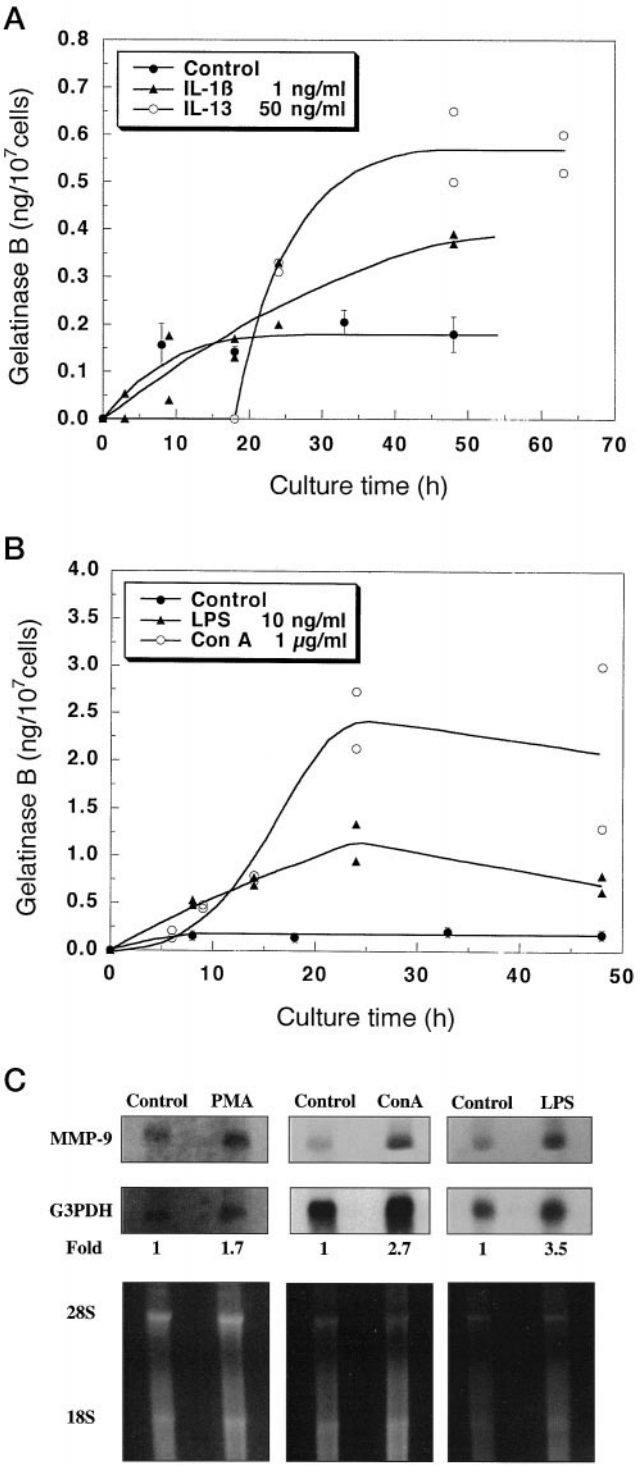

FIG. 5. **Time course of gelatinase induction by cytokines, LPS, or ConA in EBV B lymphocytes.** *A*, 2–5 × 10^7 EBV B lymphocytes were either stimulated or not stimulated (*control*) ( ) by 1 ng/ml IL-1β (▲), or 50 ng/ml IL-13 (○) at different incubation times. Serum-free conditioned medium containing 0.2% (w/v) BSA was withdrawn, and 92-kDa metalloproteinase with gelatinolytic activity was isolated onto a gelatin agarose affinity matrix and quantitated by zymography as described under "Experimental Procedures." *B*, same experiment as in *A*, using 10 ng/ml LPS (▲), 1 μg/ml ConA (○) as the stimulating agent. The results in *A* and in *B* are representative of two experiments, except for the control, the results of which are mean ± S.E. (three experiments). *C*, Northern blot analysis of *mmp-9* transcription of stimulated EBV B lymphocytes. 3 × 10^8 EBV B lymphocytes were cultivated with 0.5 nM PMA, 1 μg/ml ConA, or 10 ng/ml LPS for 24 h at 37 °C (+5% CO_2). Total RNA was extracted and oligo(dT) cellulose purified poly(A)$^+$ RNA was analyzed by Northern blot using a digoxigenin-labeling MMP-9 DNA probe as described under "Experimental Procedures." The filter was reprobed with a specific G3PDH DNA probe; mRNA for gelatinase was quantitated by normalizing to mRNA for G3PDH. Ribosomal RNA bands (28 and 18 S) visualized under ultraviolet light after staining with ethidium bromide show the amounts and quality of the RNA (*bottom panel*). The results are representative of two experiments.

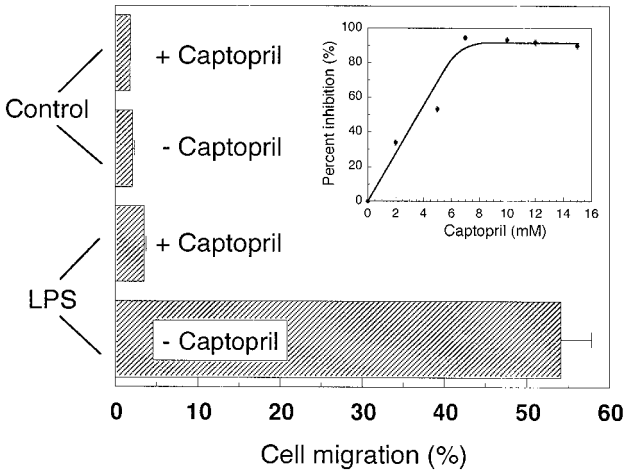

FIG. 6. **Transwell migration.** Resting or 10 ng/ml LPS-prestimulated EBV B lymphocytes (10^6 cells) were added to the upper chamber of a Transwell over a continuous coating of growth factor reduced Matrigel® matrix and incubated in 0.5 ml of 0.2% (w/v) BSA serum-free culture medium supplemented (or not, control) with 10 mM captopril added to the two chambers. After 24-h incubation at 37 °C in a 5% CO_2 atmosphere, the B cells in the lower compartment were collected by shaking and numbered by trypan blue exclusion. LPS preincubation of the B cells was performed at 37 °C for 18 h. The results are representative of four experiments, each in quadriplicate. *Inset*, dose-response effect of captopril on cell migration. 10^6 resting EBV B lymphocytes in 0.2% (w/v) BSA serum-free culture medium were layered onto the upper Transwell chamber and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. Increasing concentrations of captopril (up to 15 mM) were added to the two chambers. At the end of incubation, the B cells which migrated in the lower chamber were detached and numbered. The results are representative of three experiments each in duplicate.

inside the cells (not shown). The results suggest that, contrary to neutrophils which release outside of the cells their storage granule content upon stimulation (18, 46), there was no accumulation of gelatinase inside the B lymphocytes before secretion. Moreover, a 20-fold increase of 92-kDa gelatinase production was recently demonstrated in bronchial epithelial cells (47) upon LPS stimulation with a minimal change of mRNA level, suggesting post-transcriptional modifications. RT-PCR and Northern blot experiments carried out with EBV B lymphocytes indicated that message was increased upon PMA, LPS, and ConA stimulation, while it was unchanged when cytokines or growth factors were the ligand. These latter aspects are now under work, particularly referring to the half-life of 92-kDa gelatinase mRNA according to the stimulation conditions.

Protein kinase C signaling pathways have been involved in the expression of metalloproteinase genes; surprisingly staurosporine, a broad spectrum protein kinase inhibitor, not only failed to block response but itself stimulated expression of gelatinase B (this work) or of collagenase (48). This observation is of particular interest in view of the finding that staurosporine possesses tumor-promoting activity in mouse skin keratinocytes (49). The discordant effects of protein kinase C inhibitors on gelatinase expression raise questions with respect to their therapeutic use in the treatment of cancer since recent reports suggest that gelatinase B may play a role in metastasis development (45).

Lymphocytes continuously circulate from the blood through lymphoid and other tissues, and back through the lymphatics

to the blood (1), the first critical step in lymphocyte migration being adhesion to the vascular endothelium. Gelatinases A and B were recently shown to mediate the invasion of the basement membrane by cytotrophoblasts and tumor cells *in vitro* (4); inhibition of T cell homing by interference with gelatinase function was proposed to represent a useful approach to the treatment of T cell-mediated autoimmune disease (5, 50). Degradation of the basement membrane by MMP-9 was reported to play an important role in transmigration of human eosinophils (51) as of lymphocytes (5, 22) or neutrophils (52). The data presented above or previously reported (32) clearly identify captopril as being a general inhibitor of the migratory behavior of cells. The demonstrated ability of captopril to inhibit *in vitro* gelatinase B (31) may partly explain its antiangiogenic activity. The study presented here argues in favor of the expression of gelatinase B by B lymphocytes as tools for migration through high endothelial venules, and for induction of the proteolysis process at the cell surface. The latter aspect has yet to be demonstrated and is currently under study.

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