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Plasmin-Coated *Borrelia burgdorferi* Degrades Soluble and Insoluble Components of the Mammalian Extracellular Matrix

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***Borrelia burgdorferi*, the spirochetal agent of Lyme disease, binds plasminogen in vitro. Exogenously provided urokinase-type plasminogen (PLG) activator (uPA) converts surface-bound PLG to enzymatically active plasmin. In this study, we investigated the capacity of a *B. burgdorferi* human isolate, once complexed with plasmin, to degrade purified extracellular matrix (ECM) components and an interstitial ECM. In a modified enzyme-linked immunosorbent assay using immobilized, soluble ECM components, plasmin-coated *B. burgdorferi* degraded fibronectin, laminin, and vitronectin but not collagen. Incubation of plasmin-coated organisms with biosynthetically radiolabeled native ECM resulted in breakdown of insoluble glycoprotein, other noncollagenous proteins, and collagen, as measured by release of solubilized radioactivity. Radioactive release did not occur with untreated spirochetes or spirochetes treated with uPA or PLG alone. Kinetic and inhibition studies suggested that the breakdown of collagen was indirect and due to prior disruption of supportive ECM proteins. *B. burgdorferi* is an invasive bacterial pathogen that may benefit by use of the host's plasminogen activation system. The results of this study have identified mechanisms in which the spirochete can use this borrowed proteolytic activity to enhance invasiveness.**

The tick-borne spirochete *Borrelia burgdorferi*, the agent of Lyme disease (8), is introduced into the skin of the host during tick feeding and subsequently disseminates via the bloodstream to distant sites, including the heart, nervous system, and joints (19, 48). The requisite penetration of interstitium, basement membrane, and endothelium would necessitate either expression or acquisition of proteolytic enzymes for localized degradation of extracellular matrix (ECM) components. The plasminogen activation system (PAS) is a highly regulated fibrinolytic system that is widely utilized for acquisition of extracellular proteolytic activity. Its primary component is the zymogen plasminogen (PLG), a single-chain, 92-kDa glycoprotein which is abundant in plasma and tissue fluids. PLG is converted to its two-chain active form, plasmin, as a result of specific proteolytic cleavage by either urokinase-type PLG activator (uPA) or tissue-type PLG activator. Its normal physiological role as the chief fibrinolytic mediator is to dissolve fibrin clots; however, plasmin is a broad-spectrum serine protease and has been implicated in the degradation of other substrates such as the ECM components fibronectin and laminin (34, 51). In addition, plasmin can activate certain prometalloproteinases (13, 39, 52), latent elastase (9), and proplasminogen activators (42) and degrade tissue inhibitors of metalloproteinases (14).

It is now clear that bacteria utilize the PAS for a number of biological reasons (7), a phenomenon that has recently been extended to viruses (21, 24). Specifically, streptococci, staphylococci, and yersiniae have endogenous mechanisms for activation of receptor-bound PLG (7). Other gram-positive and gram-negative bacteria are able to incorporate host PLG, with subsequent activation to plasmin, utilizing host PLG activators (7). Localization of plasmin to the surface of bacteria may direct proteolytic activity to specific sites such as ECM, basement membranes, and interstitial stroma, where it is required for migration into adjacent tissues.

B. burgdorferi has also been shown to possess receptors for PLG (11, 18, 25, 32). Once bound to the surface of the spirochete, the zymogen can be converted to active plasmin by exogenously supplied uPA and is protected from inhibition by its specific plasma inhibitor, α_2-antiplasmin (11, 18, 41), and the physiological regulators of uPA, PLG activator inhibitors 1 and 2 (41). *B. burgdorferi* with complexed plasmin degrades soluble fibronectin (18) and demonstrates an enhanced capacity to penetrate endothelial monolayers (11). Finally, PLG acquisition is critical for efficient spirochete dissemination in ticks and for spirochetemia in mice (10).

To better understand the mechanism(s) by which *B. burgdorferi*, through collaboration with the host's PAS, can cause a systemic infection, we measured the degradation of purified ECM components and an insoluble, native, interstitial ECM synthesized by rat heart smooth muscle cells by plasmin-coated *B. burgdorferi*.

MATERIALS AND METHODS

Bacterial cultures. The low-passage human blood-derived (HBD) strain of *B. burgdorferi* (4) was maintained in serum-free BSK medium (5) at 34°C. The absence of rabbit serum in the culture medium allowed for the use of specific rabbit antibody for detection of substrate in the enzyme-linked immunosorbent assay (ELISA)-based degradation assay (described below) without the potential complication derived from spirochete-adsorbed rabbit immunoglobulin.

Materials. Human uPA (two-chain form) was purchased from American Diagnostica, Greenwich, Conn. Chromogenic substrate S2251 for assaying plasmin activity was purchased from Pharmacia Hepar/Chromogenix, Franklin, Ohio. Human plasma fibronectin, laminin from human placenta, human plasma vitronectin, collagen type IV from human placenta, aprotinin, and EDTA, (disodium salt) were purchased from Sigma, St. Louis, Mo. Collagen types I and III from human placenta were obtained from Biodesign International, Kennebunk, Maine. L-[6-^{3}H]fucose, L-[3,4-^{3}H]proline, and L-[^{35}S]methionine–L-[^{35}S]cysteine (Tran^{35}S-label) were purchased from ICN Biomedical Research Products, Costa Mesa, Calif. Human PLG was purified from frozen plasma by affinity chromatography as previously described (10). This preparation was used for all experiments. Briefly, 300 ml of plasma containing 100 kallikrein inhibitory units (KIU) per ml of aprotinin (Sigma) was passed through a column of lysine Sepharose 4B (Pharmacia Biotech, Piscataway, N.J.). The column was washed extensively with 0.1 M phosphate buffer (pH 7.5) containing 5 KIU of aprotinin per ml to remove

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unbound material. PLG was eluted from the column by addition of phosphate-aprotinin containing 0.05 M ε-aminocaproic acid. The ε-aminocaproic acid was removed by use of PD-10 disposable desalting columns (Pharmacia Biotech) and dialysis against phosphate buffer. Product integrity was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting.

Antibodies. Goat anti-human fibronectin and rabbit anti-human laminin were purchased from Sigma. Rabbit anti-human vitronectin and rabbit antibodies to human type I, III, and IV collagens were purchased from Biodesign International. Rabbit anti-goat immunoglobulin G (IgG) and goat anti-rabbit IgG, conjugated to alkaline phosphatase, were purchased from Kirkegaard & Perry Laboratories, Gaithersburg, Md.

Degradation assay for soluble ECM proteins. We designed a modified ELISA to measure degradation of immobilized ECM components by using polyclonal antibodies for substrate detection. The absence of serum in the spirochete culture medium ensured that recognition of spirochete-adsorbed serum IgG by antibodies did not contribute to the ELISA signal. Purified, soluble fibronectin, laminin, vitronectin, and collagen types I, III, and IV were diluted in pH 9.6 carbonate buffer (1 μg/ml) and adhered to 96-well polystyrene plates (Becton Dickinson, Franklin Lakes, N.J.) (30 μl per well) by incubation at 4°C for 16 h. Plate wells were washed four times with phosphate-buffered saline (PBS, pH 7.2) (200 μl per well), and the plates were stored at −20°C until needed. On the day of the assay, spirochetes were enumerated, centrifuged, and resuspended in Hanks' balanced salt solution (HBSS) containing 100 μg of human PLG and 60 U of human uPA per ml. The PLG concentration chosen for these experiments (100 μg/ml) is within the physiological range of PLG in human plasma. Control spirochete preparations received HBSS alone, uPA in HBSS, or PLG in HBSS. A sham preparation tube received PLG and uPA but no spirochetes and was treated identically to the spirochete preparations. Sterile 1.5-ml microcentrifuge tubes were used for all incubations. All preparations were incubated for 1.5 h at 34°C, with gentle end-over-end agitation, washed four times with HBSS, and resuspended in HBSS. For this and all subsequent experiments, a synthetic chromogenic substrate (S2251) assay (11) was used to verify that the spirochetes had acquired plasmin activity. Prior to addition of spirochetes, the coated wells were incubated with HBSS containing 2% bovine serum albumin (100 μl per well, 1 h, 34°C) to minimize spirochete adherence to the polystyrene. The preparations (six replicates per experimental group, 100 μl per well) were added, and the plate was centrifuged for 15 min at 180 × *g* to ensure contact between spirochetes and substrate. Six wells received HBSS alone and served as the positive control in the subsequent ELISA step. Following incubation (6 h at 34°C), the ELISA plate wells were washed five times with PBS containing 0.02% Tween 20 (200 μl per well) to remove the spirochetes. The relative amount of undigested substrate was determined by using a polyclonal antibody specific for each substrate and a species-specific secondary antibody conjugated to alkaline phosphatase, followed by the phosphatase substrate *p*-nitrophenyl phosphate (2 mg/ml in 0.05 M carbonate buffer [pH 9.8] containing 0.001 M $MgCl_2$). Absorbance was measured with a microplate reader fitted with a 410-nm filter (MR 700; Dynatech Laboratories Inc., Chantilly, Va). Percent degradation was determined as $[(A - B)/A](100)$, where A equaled the mean HBSS-positive control absorbance and B equaled the mean absorbance for the experimental group.

Preparation of radiolabeled ECM. Cell culture and production of ECM were carried out as described previously (29, 44, 45), with some modifications. R22 rat heart smooth muscle cells (the generous gift of Sanford Simon, Department of Pathology, School of Medicine, State University of New York at Stony Brook) were cultured in Dulbecco's modified Eagle medium (Gibco, Grand Island, N.Y.) containing 10% fetal bovine serum (HyClone Laboratories, Logan, Utah), 2% tryptose phosphate, and penicillin (100 U/ml) plus streptomycin (100 μg/ml) (Gibco). For preparation of ECM, trypsinized cells were passed into growth medium and seeded at $5 \times 10^3/cm^2$ in 96-well tissue culture plates (Becton Dickinson). Cells reached confluency after 4 days of growth at 37°C and 5% CO_2, at which time fresh medium supplemented with 50 μg of ascorbic acid (Wako Chemicals, Waco, Tex.) per ml and either [^{3}H]fucose (1.85×10^4 Bq/ml [0.5 μCi/ml]) alone or [^{3}H]proline (1.85×10^4 Bq/ml [0.5 μCi/ml]) and [^{35}S]methionine-cysteine (Tran^{35}S-label) ($7.4\ 10^4$ Bq/ml [2.0 μCi/ml]) together was added. Cells received fresh medium with radioactive label again at 8 days. At day 12, the cells were lysed with 25 mM NH_4OH and the resultant ECMs were examined microscopically to verify that there was complete cell lysis. The ECMs were carefully rinsed three times with sterile distilled water and once with PBS containing 0.02% sodium azide, dried, and stored at 4°C until needed.

Degradation assay for radiolabeled ECM. Plasmin-coated *B. burgdorferi* and controls were prepared as described above. Previously prepared ECMs in 96-well plates (described above) were rinsed six times with HBSS (100 μl per well) to remove the sodium azide. Plasmin-coated *B. burgdorferi* (three to six replicates of 10^8 per well in 100 μl) and controls were added to the wells, and the plates were centrifuged at 180 × *g* to ensure contact between the spirochetes and substrate. This was followed by an incubation period for 6 h at 34°C. Kinetic and dose-response experiments were carried out by varying incubation times and spirochete densities, respectively. For inhibition experiments, plasmin-coated *B. burgdorferi* was resuspended in HBSS containing aprotinin (100 KIU/ml) or EDTA (1 and 10 mM) prior to incubation with the ECM. Inhibitors were present for the entire incubation. After incubation, the supernatants containing released radioactive counts were carefully removed from each well and placed in 3.25-ml scintillation fluid (EcoLume; ICN Pharmaceuticals, Costa Mesa, Calif.) each. Each well then received 100 μl of 2 N NaOH, and the plates were incubated for an additional 2 h at 25°C. The contents of each well were then neutralized by addition of 100 μl of 2 N HCl, and the entire sample containing digested residual radioactive counts was placed in 3.25 ml of EcoLume. The total released radioactivity in each sample was determined in a liquid scintillation counter (LKB Wallac, Gaithersburg, Md.). Percent radioactivity release was determined as $[A/(A + B)] \times 100$, where A and B equaled mean supernatant and mean NaOH digest counts per minute, respectively. For ECM labeled with ^{3}H and ^{35}S, a program designed for counting dual labels was used.

Statistics. The data were tested for statistical significance by Student's *t* test as part of the InStat 2.01 statistical software package (GraphPad, San Diego, Calif.), where $P < 0.05$ was chosen as the alpha value for statistical significance. In some instances, analysis of variance and the Mann-Whitney test were used.

RESULTS

Plasmin-coated *B. burgdorferi* degrades immobilized ECM components. The purified human ECM components fibronectin, laminin, vitronectin, and type I, III, and IV collagens were adhered to 96-well polystyrene plates. Degradation of these substances by *B. burgdorferi* pretreated with HBSS alone, uPA in HBSS, PLG in HBSS, or PLG and uPA together in HBSS to form spirochete surface-associated plasmin is shown in Fig. 1. Plasmin-coated *B. burgdorferi* consistently degraded immobilized fibronectin (~8-fold, $P < 0.0001$), vitronectin (~7-fold, $P < 0.0001$), and laminin (~3-fold, $P < 0.01$) compared to control preparations (Fig. 1A to C). In time course assays, degradation was maximized after 6 h of incubation (not shown). This incubation time was then chosen for all assays. Laminin was consistently the most resistant substrate (Fig. 1B). Plasmin-coated *B. burgdorferi* did not significantly degrade any of the collagen types tested (Fig. 1D to F). Control preparations had no significant effect on any of the substrates tested, indicating that loss of signal is associated with substrate degradation, rather than solubilization over the course of the assay. Degradation of fibronectin, laminin, and vitronectin occurred in a concentration-dependent manner and was statistically significant, with 10^7 plasmin-coated spirochetes for fibronectin (Fig. 2A), 5×10^7 plasmin-coated spirochetes for laminin (Fig. 2B), and 10^6 plasmin-coated spirochetes for vitronectin (Fig. 2C).

Plasmin-coated *B. burgdorferi* degrades insoluble mammalian ECM. The capacity for plasmin-coated *B. burgdorferi* to degrade soluble fibronectin, laminin, and vitronectin in vitro led to a series of experiments designed to assess whether receptor-bound plasmin on *B. burgdorferi* could degrade native mammalian ECM in vitro. R22 rat heart smooth muscle cells, which produce an insoluble ECM consistent with mammalian interstitium (1), were cultured in the presence of the radiolabeled precursors [^{35}S]methionine-cysteine, [^{3}H]fucose, and [^{3}H]proline to label preferentially noncollagenous protein, glycoprotein, and collagenous protein respectively, in the resultant synthesized ECM. After lysis of the cells with 25 mM NH_4OH and washing with HBSS, the immobilized ECM was overlaid with either HBSS or *B. burgdorferi* pretreated with HBSS, uPA in HBSS, PLG in HBSS, or PLG and uPA together in HBSS to form spirochete surface-associated plasmin. A representative radiolabel release experiment is shown in Fig. 3. Spontaneous release of radioactivity was minimal as shown by the controls with HBSS alone. All other controls also showed minimal radioactivity release. Significant release of radioactivity occurred in wells that received plasmin-coated *B. burgdorferi*. The release of ^{35}S from ECM labeled with [^{35}S]methionine-cysteine (Fig. 3A), which labels preferentially noncollagenous ECM protein, was fivefold greater than control levels ($P < 0.01$). The levels of release of ^{3}H from ECM labeled with [^{3}H]fucose (Fig. 3B), which labels preferentially glycoprotein, and [^{3}H]proline (Fig. 3C), which labels preferen-

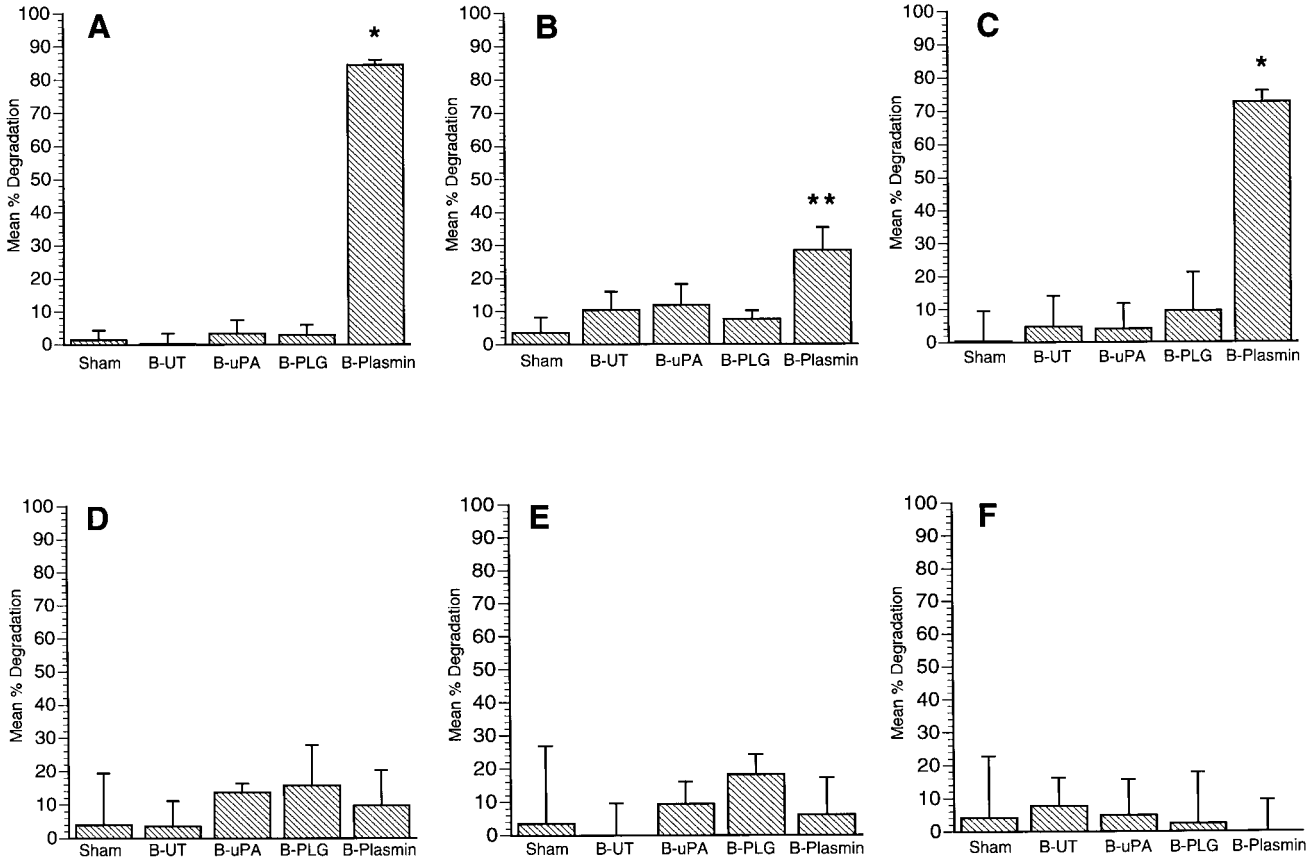

FIG. 1. Degradation of soluble ECM components by plasmin-coated *B. burgdorferi*. The substrates tested were human fibronectin (A), laminin (B), vitronectin (C), collagen type I (D), collagen type III (E), and collagen type IV (F). Spirochetes were incubated in HBSS with no additions (B-UT) and with addition of uPA alone (B-uPA), PLG alone (B-PLG), and PLG and uPA together to form spirochete surface-associated plasmin (B-Plasmin). A sham preparation to control for free plasmin carryover in the latter group consisted of PLG and uPA in HBSS but no *B. burgdorferi*. ELISA plate wells (six replicates) coated with substrate were incubated for 6 h with 10^8 spirochetes from each experimental group. Undegraded substrate was detected, and percent degradation (a reduction in absorbance value was interpreted as substrate degradation) was calculated as described in Materials and Methods. Bars represent mean percent substrate degradation relative to the positive control (0% degradation) ± the standard deviation of six replicate wells for each experimental group. *, statistically significant ($P < 0.0001$); **, statistically significant ($P < 0.01$) compared to the positive control. The experiment was performed three times with similar results.

tially collagen, by plasmin-coated *B. burgdorferi*, were approximately three- and fourfold, respectively, over control levels ($P < 0.01$). Radioactivity release occurred in a consistent, concentration-dependent manner, with statistical significance being achieved at concentrations of 2×10^6 plasmin-coated spirochetes per well (Fig. 4).

The amount of bound, active plasmin per organism was calculated for each of the labeling conditions through free plasmin standard curves and radioactivity release data (Fig. 5). These amounts were 2.0×10^{-5}, 1.5×10^{-5}, and 2.5×10^{-5} fmol for plasmin-coated spirochete degradation of ECM labeled with [^{35}S]methionine-cysteine, [^{3}H]fucose, and [^{3}H]proline, respectively.

Components of the ECM labeled with [^{35}S]methionine-cysteine are degraded first by plasmin-coated *B. burgdorferi*. The release of radioactivity from ECM labeled with [^{3}H]proline by plasmin-coated *B. burgdorferi* could reflect either direct action on collagen by plasmin, the activation of a prometalloproteinase residing in the ECM by plasmin, which, in turn, could act on collagen, or destabilization of collagen through degradation of ECM glycoproteins and proteoglycans. We attempted to distinguish these possibilities through experiments to determine the kinetics of label release and by use of inhibition assays. A degradation assay was carried out as described above except that released radioactivity was sampled at time points of 0.5, 1, 2, 4, and 6 h in order to determine the kinetics of ECM component release. The first label to be released in detectable quantities was ^{35}S, in ECM that had been labeled with [^{35}S]methionine-cysteine. This was followed by ^{3}H from matrix that had been labeled with [^{3}H]proline and finally ^{3}H from matrix that had been labeled with [^{3}H]fucose (Fig. 6).

Serine protease inhibition prevents degradation of ECM. *B. burgdorferi* pretreated with HBSS alone or PLG and uPA in HBSS to form surface-associated plasmin was incubated for 6 h with radiolabeled ECM in the presence and absence of the serine protease inhibitor aprotinin (Fig. 7). The release of radiolabel from the ECM in wells that contained no inhibitor was consistent with that obtained in previous experiments. The release of radiolabel in wells that contained aprotinin, however, was reduced to levels similar to the background level obtained with HBSS alone. In another experiment, inhibition of release of ^{35}S from ECM labeled with [^{35}S]methionine-cysteine and ^{3}H from ECM labeled with [^{3}H]proline by plasmin-coated *B. burgdorferi* in the presence of the chelating agent EDTA was measured (Fig. 8). EDTA did not inhibit the release of ^{35}S from noncollagenous protein. In addition, there was no significant inhibition of release of ^{3}H from ECM-labeled with [^{3}H]proline.

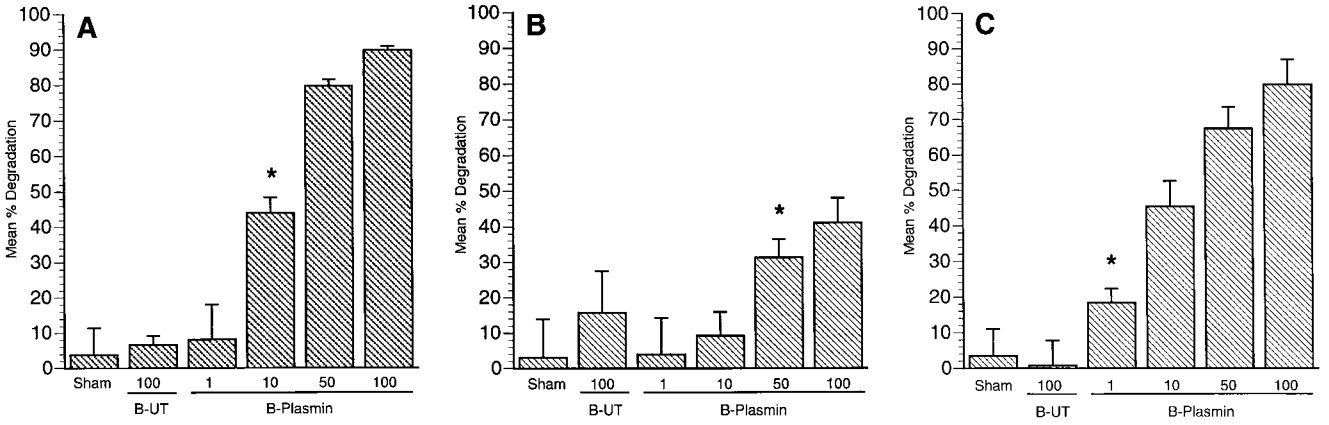

FIG. 2. Degradation of soluble ECM components by graded concentrations of plasmin-coated *B. burgdorferi*. The substrates tested were fibronectin (A), laminin (B), and vitronectin (C). Spirochetes were incubated in HBSS with no additions (B-UT) and with addition of PLG and uPA together to form spirochete surface-associated plasmin (B-Plasmin). A sham preparation to control for free plasmin carryover in the latter group consisted of PLG and uPA in HBSS but no *B. burgdorferi*. ELISA plate wells (six replicates) coated with substrate were incubated for 6 h with a range of plasmin-coated *B. burgdorferi* concentrations (10^6, 10×10^6, 50×10^6, and 100×10^6 per well) as well as 100×10^6 untreated spirochetes. Undegraded substrate was detected, and percent degradation (a reduction in absorbance value was interpreted as substrate degradation) was calculated as described in Materials and Methods. Bars represent mean substrate degradation relative to the positive control (0% degradation) ± the standard deviation of six replicate wells for each experimental group. *, statistically significant ($P < 0.0001$) compared to ELISA positive control. The experiment was performed twice with similar results.

DISCUSSION

In this study, we demonstrated that *B. burgdorferi*, once complexed with the host's plasmin, could degrade the soluble forms of the ECM glycoproteins fibronectin, laminin, and vitronectin but not collagen. Furthermore, *B. burgdorferi* with associated plasmin degraded insoluble components from a biosynthetically radiolabeled native interstitial ECM. Degradation was not observed in any of the untreated control spirochete preparations or those treated with uPA or PLG alone. Breakdown of the insoluble matrix was fully inhibited by the serine protease inhibitor aprotinin but not by the chelating agent EDTA.

The genomic sequence of *B. burgdorferi* has recently been published (17). It is remarkable for the relatively small size of the chromosome (<1 Mb), lack of recognizable genes associated with virulence, and small number of proteolytic enzymes. The ability of the spirochete to disseminate from the skin and cause organ-specific disease (19, 48) despite a limited armamentarium (17) would require mechanisms for adhesion to, and degradation of, basement membrane and interstitial ECM. Previous studies have shown that *B. burgdorferi* has a strong affinity for ECM (20, 33, 49) and ECM components (23, 26). In addition, the organism binds PLG in vitro, with conversion to active plasmin being achieved by addition of exogenous PLG activator (11, 18, 25, 32), and penetrates endothelial cell monolayers in vitro (12, 49), a process that is enhanced by spirochete plasmin acquisition (11). In experimental *B. burgdorferi* infections, the acquisition of PLG by the spirochete occurs in the feeding tick. The concomitant release of PLG activator, due to localized cellular injury at the site of the tick bite (uPA is detectable in the tick bloodmeal contents) (10), leads to active plasmin formation on the spirochete surface. Subsequently, plasmin-coated *B. burgdorferi* could enter and reside in tissues, where there is significant microscopic evidence both in patients (16, 31, 35, 43, 50) and in animal models (2, 3, 15, 46, 53) to indicate that the preferred niche for the spirochete is in the interstitial ECM of the colonized tissues. With this specificity in mind, we therefore sought to determine the ability of *B. burgdorferi* with surface-localized plasmin to degrade an in vitro model of an insoluble interstitial ECM.

The insoluble ECM synthesized by the R22 rat heart smooth muscle cells is a model of an native, interstitial ECM and is composed of fibronectin, elastin, collagen types I and III, and proteoglycans (1, 29, 44, 51). The percent composition of this ECM is 51% glycoproteins and proteoglycans, 37% collagen, and 12% elastin (44). ECM from R22 cells has been used to demonstrate protease production by activated phagocytic cells (28) and human tumor cell lines (27), degradation and chemotaxis in studies using neutrophils (45), and binding of human α_1-proteinase inhibitor (44). Incubation of R22 cells with radiolabeled precursors leads to ECM components that are labeled selectively (29, 30, 44). We used radiolabeled methionine-cysteine, fucose, and proline to label preferentially noncollagenous protein, glycoprotein, and collagen, respectively, and measured the release of radioactivity after incubation of the ECM with plasmin-coated *B. burgdorferi*. Solubilization of counts in ECM labeled with [^{35}S]methionine-cysteine after incubation with plasmin-coated *B. burgdorferi* is reflective of attack on noncollagenous protein and is associated with the degradation of fibronectin and/or the protein core of proteoglycan (51). Released counts in supernatants of ECM labeled with radiolabeled fucose have been previously shown to be indicative of fibronectin release alone (29). In addition, fucose is present in fibronectin but generally lacking in other ECM glycoproteins (38, 47). Further support is provided by the ratio of released counts after incubation with plasmin-coated *B. burgdorferi* in ECMs labeled with [^{35}S]methionine-cysteine and [^{3}H]fucose—the percent release of radioactivity from [^{3}H]fucose, representing fibronectin alone, was always quantifiably less than that of [^{35}S]methionine-cysteine, which is preferentially incorporated into other ECM components in addition to fibronectin. The degradation of soluble and insoluble fibronectin by plasmin-coated *B. burgdorferi* in our study confirms and extends the results obtained previously with soluble fibronectin alone (18).

A previous report has described a collagenolytic activity in detergent extracts of *B. burgdorferi* (22). Furthermore, two putative zinc proteases have been identified by homology in the genome of *B. burgdorferi* (17), but they have not been characterized biologically. Therefore, we sought to determine

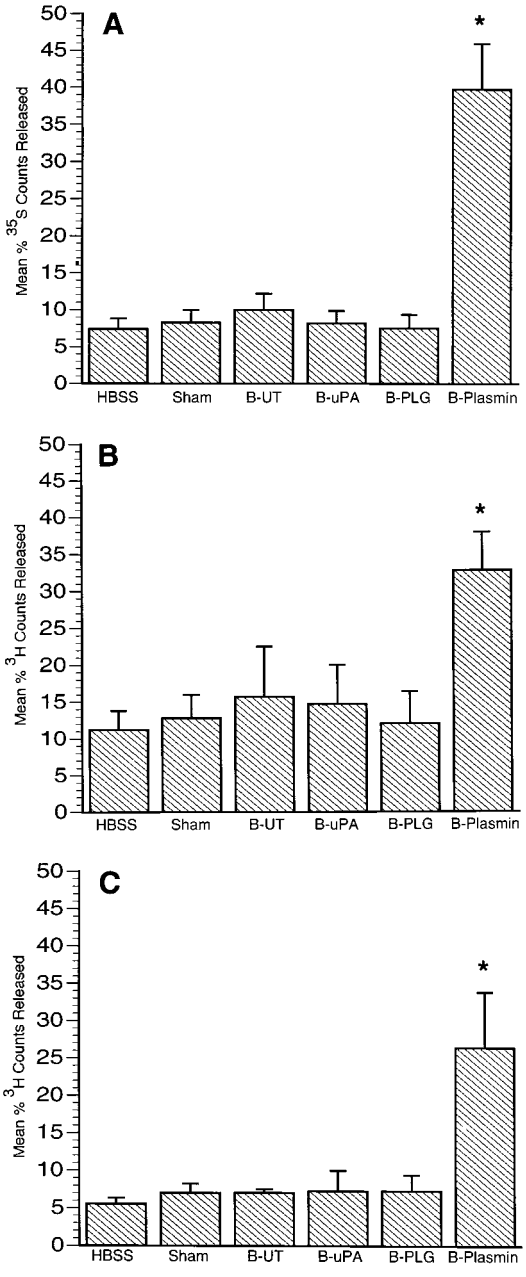

FIG. 3. Degradation of radiolabeled, insoluble R22 ECM by plasmin-coated *B. burgdorferi*. ECM components were labeled preferentially with [^{35}S]methionine-cysteine (noncollagenous protein) (A), [^{3}H]fucose (glycoprotein) (B), and [^{3}H]proline (collagen) (C). Spirochetes were incubated in HBSS with no additions (B-UT) and with addition of uPA alone (B-uPA), PLG alone (B-PLG), and PLG and uPA together to form spirochete surface-associated plasmin (B-Plasmin). A sham preparation to control for free plasmin carryover in the latter group consisted of PLG and uPA in HBSS but no *B. burgdorferi*. ECMs were incubated for 6 h with 10^8 spirochetes from each experimental group. Released (supernatant) and unreleased (2 N NaOH digest of undegraded ECM) radioactivity was counted for each well. Percent release of the total radioactive counts present in each well was calculated as described in Materials and Methods. Bars represent mean percent radioactivity release ± standard deviation of five replicate wells per experimental group. *, statistically significant ($P < 0.01$) compared to HBSS control. The experiment was performed twice with similar results.

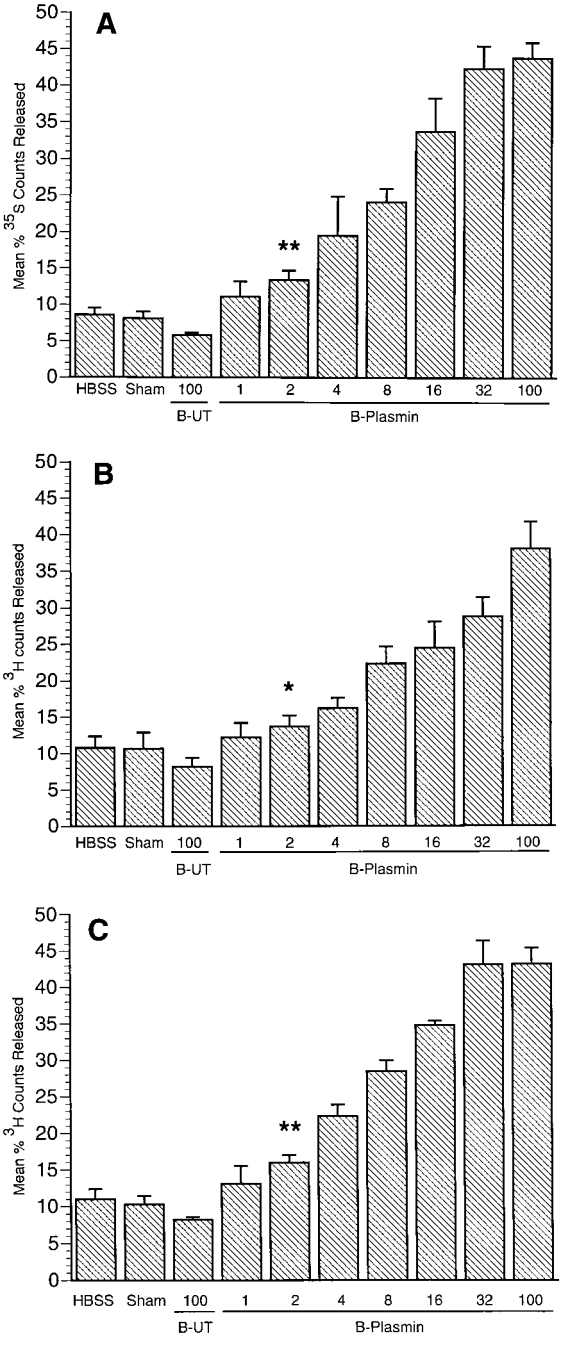

FIG. 4. Degradation of radiolabeled, insoluble R22 ECM by graded concentrations of plasmin-coated *B. burgdorferi*. ECM components were labeled preferentially with [^{35}S]methionine-cysteine (noncollagenous protein) (A), [^{3}H] fucose (glycoprotein) (B), and [^{3}H]proline (collagen) (C). Spirochetes were incubated in HBSS with no additions (B-UT) and with addition of PLG and uPA together in HBSS to form spirochete surface-associated plasmin (B-Plasmin). A sham preparation intended to control for free plasmin carryover in the latter group consisted of PLG and uPA in HBSS but no *B. burgdorferi*. Tissue culture plate wells containing labeled ECM were incubated for 6 h with a range of plasmin-coated *B. burgdorferi* concentrations (10^6, 2×10^6, 4×10^6, 8×10^6, 16×10^6, 32×10^6, and 100×10^6 per well) as well as 100×10^6 untreated spirochetes. Released (supernatant), and unreleased (2 N NaOH digest of undegraded ECM) radioactivity was counted for each well. Percent release of the total radioactive counts present in each well was calculated as described in Materials and Methods. Bars represent mean percent radioactivity release ± standard deviation of five replicate wells per experimental group. *, statistically significant ($P < 0.05$) compared to HBSS control; **, statistically significant ($P < 0.001$) compared to HBSS control. The experiment was performed twice with similar results.

if *B. burgdorferi*, either untreated or plasmin coated, exhibited collagenolytic activity in our assays. In our study, which included only intact organisms, *B. burgdorferi*, either alone or treated with uPA, PLG, or PLG, and uPA together to form

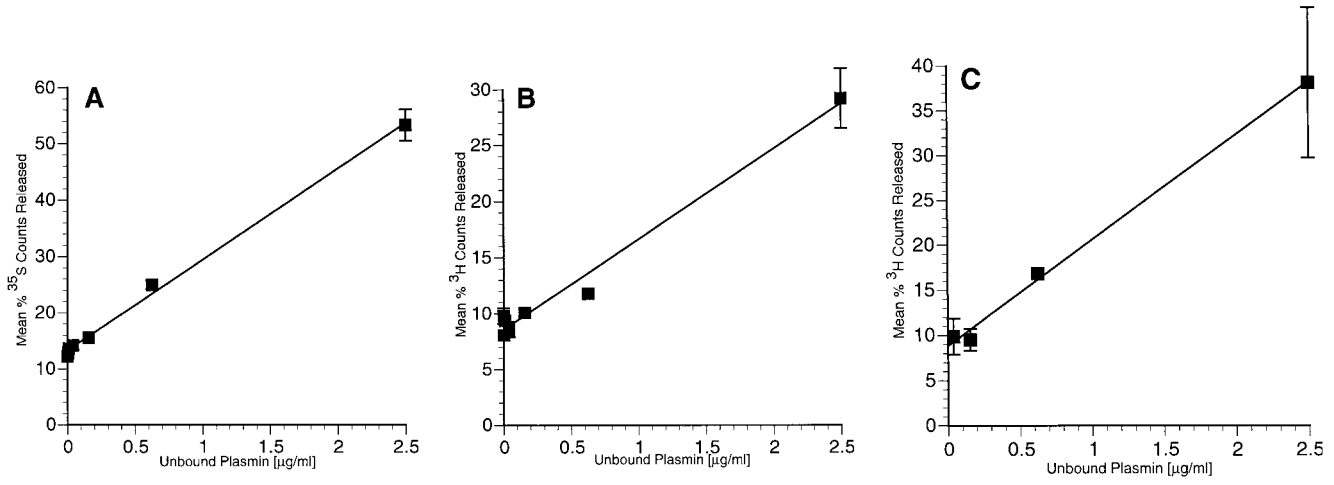

FIG. 5. Measurement of active plasmin bound to *B. burgdorferi* as determined by release of radioactivity from radiolabeled R22 ECM. Standard curves generated for unbound plasmin degradation of ECM labeled with [^{35}S]methionine-cysteine (A), [^{3}H]fucose (B), and [^{3}H]proline (C) were used to calculate the amount of plasmin bound per organism. Tissue culture plate wells containing labeled ECM were incubated for 6 h with 2.5×10^7, 5×10^7, and 10^8 plasmin-coated spirochetes and graded concentrations of unbound plasmin per well. Released (supernatant) and unreleased (2 N NaOH digest of undegraded ECM minus supernatant) radioactivity was counted for each of three replicate wells. Percent release of the total radioactive counts present in each well was calculated as described in Materials and Methods. Linear curve fits were calculated by the least squares method, using the DeltaGraph 4.0 software package. The experiment was performed twice with similar results.

plasmin, failed to degrade soluble, immobilized collagen; however, release of radioactivity from insoluble ECM labeled with [^{3}H]proline after incubation with plasmin-coated *B. burgdorferi* did occur. [^{3}H]proline is overwhelmingly incorporated into the collagens of the ECM, which are rich in proline and hydroxyproline (6), but can also be found in fibronectin, which has substantially fewer proline residues (40). Some of the release observed in ECM labeled with [^{3}H]proline could also be due to degradation of fibronectin by spirochete-bound plasmin. Since native collagen is highly resistant to direct plasmin action (36), for collagen damage to occur in vivo, the participation of a complex battery of specific proteolytic enzymes is required. In our studies, release of collagen was probably the result of the destabilization of the ECM by prior degradation of other supportive components. In support of this possibility, ^{35}S-labeled proteins (noncollagenous) were released first as a result of the action of the plasmin-bound spirochetes. Further-

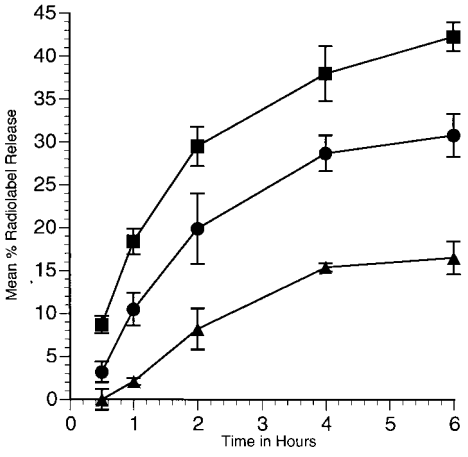

FIG. 6. Kinetics of release of radiolabeled, insoluble R22 ECM components by plasmin-coated *B. burgdorferi*. ECM components were labeled preferentially with [^{35}S]methionine-cysteine (noncollagenous protein), [^{3}H]fucose (glycoprotein), and [^{3}H]proline (collagen). Spirochetes were incubated with PLG and uPA together, in HBSS, to form spirochete surface-associated plasmin. Tissue culture plate wells containing ECM labeled with [^{35}S]methionine-cysteine (■), [^{3}H]fucose (▲), and [^{3}H]proline (●) were incubated with 10^8 plasmin-coated spirochetes per well. At fixed intervals (0.5, 1, 2, 4, and 6 h), released (supernatant) and unreleased (2 N NaOH digest of undegraded ECM) radioactivity were counted for each of five replicate wells. Percent release of the total radioactive counts present in each well was calculated as described in Materials and Methods. Chart points represent the mean percent radioactivity release (minus the experimental background [mean value for wells that received HBSS alone]) ± standard deviation of five replicate wells per experimental group per time point. The experiment was performed twice with similar results.

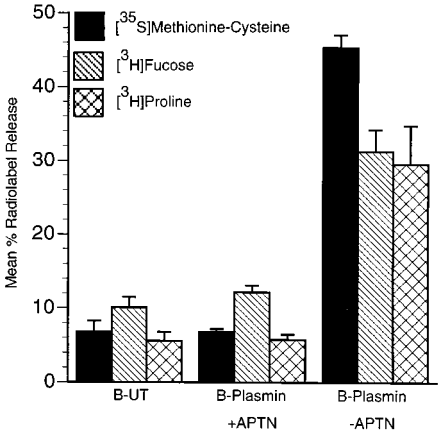

FIG. 7. Effect of the serine protease inhibitor aprotinin on the degradation of insoluble R22 ECM labeled with [^{35}S]methionine-cysteine, [^{3}H]fucose, and [^{3}H]proline by plasmin-coated *B. burgdorferi*. ECM components were labeled preferentially with [^{35}S]methionine-cysteine (noncollagenous protein), [^{3}H]fucose (glycoprotein), and [^{3}H]proline (collagen). Spirochetes were incubated in HBSS with no additions (B-UT) and with addition of PLG and uPA together in HBSS to form spirochete surface-associated plasmin (B-Plasmin). Spirochetes were added to tissue culture plate wells containing ECM in both the presence and the absence of aprotinin (APTN) and incubated for 6 h. Released (supernatant) and unreleased (2 N NaOH digest of undegraded ECM) radioactivity was counted for each well. Percent release of the total radioactive counts present in each well were calculated as described in Materials and Methods. Bars represent mean percent radioactivity release ± standard deviation of three replicate wells per experimental group. The experiment was performed twice with similar results.

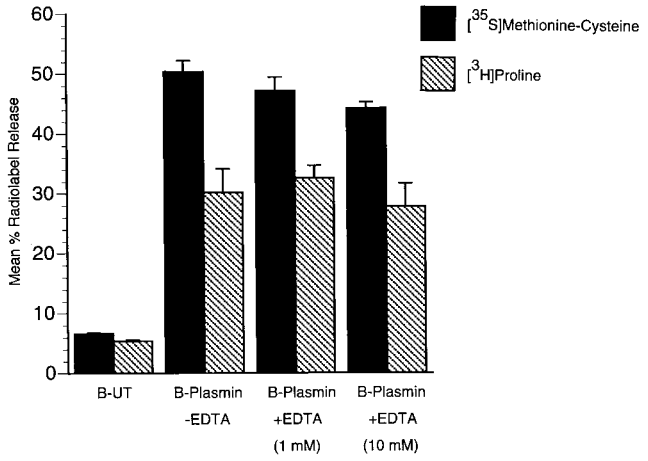

FIG. 8. Effect of EDTA on the degradation of insoluble R22 ECM labeled with [^{35}S]methionine-cysteine and [^{3}H]proline by plasmin-coated *B. burgdorferi*. ECM components were labeled preferentially with [^{35}S]methionine-cysteine (noncollagenous protein) and [^{3}H]proline (collagen). Spirochetes were incubated in HBSS with no additions (B-UT) and with addition of PLG and uPA together in HBSS to form spirochete surface-associated plasmin (B-Plasmin). Spirochetes were added to tissue culture plate wells containing ECM in the absence and in the presence of EDTA and then incubated for 6 h. Released (supernatant) and unreleased (2 N NaOH digest of undegraded ECM) radioactivity was counted for each well. Percent release of the total radioactive counts present in each well was calculated as described in Materials and Methods. Bars represent mean percent radioactivity release ± standard deviation of three replicate wells per experimental group. The experiment was performed twice with similar results.

more, EDTA, which is a potent collagenase inhibitor (39), did not inhibit degradation of ECM labeled with either [^{35}S]methionine-cysteine or [^{3}H]proline. Earlier studies have shown that once the structural architecture of the R22 ECM is disrupted by proteolysis (37, 51), the remaining collagen becomes susceptible to collagenolytic attack.

B. burgdorferi is found primarily in connective tissue of the affected organs in Lyme disease. Colonization of this niche is likely to require localized degradation of the insoluble matrix, which as we have shown cannot be accomplished by the organisms themselves but can be achieved by the organisms with surface-bound plasmin. The resulting proinflammatory degradation products are also likely to contribute to the focal inflammation characteristic of the affected tissues in Lyme disease.

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