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IMMUNOCYTOCHEMICAL ANALYSIS OF TISSUE KALLIKREIN AND THE KININ MOIETY IN RHEUMATOID SYNOVIAL FLUID NEUTROPHILS

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SUMMARY

Polymorphonuclear leucocytes (PMNs) from the synovial fluid of patients with rheumatoid arthritis (RA) showed reduced tissue kallikrein and kinin immunoreactivity in comparison with blood PMNs from healthy individuals as judged visually using confocal microscopy. Similarly, synovial fluid PMNs exhibited reduced tissue kallikrein immunoreactivity as compared with blood PMNs from the same RA patients. Blood PMNs stimulated to degranulate *in vitro* also displayed less immunostaining for tissue kallikrein and kinin than non-stimulated PMNs. By contrast, no difference in kininogen immunostaining was detected between RA synovial fluid PMNs and blood PMNs from healthy people. It is considered that the results support the hypothesis that tissue kallikrein, released from the granules of RA synovial fluid PMNs, cleaves the kinin moiety from multifunctional kininogen protein on the surface of the PMNs.

KEY WORDS: Tissue kallikrein, Kinin moiety, Rheumatoid arthritis, Synovial fluid, Neutrophils, Degranulation.

THE pathological processes which cause acute inflammation, including joint swelling and pain in rheumatoid arthritis (RA), are debated. Classically, four major mediators, namely histamine/5-hydroxytryptamine, kinins and prostaglandins, have been recognized as dominating the early, intermediate and late phases, respectively, of the inflammatory response. In RA joints, histamine could be released from mast cells by C3a, but neither anti-histamines nor mast cell stabilizers are reported to give symptomatic relief in RA. Although 5-hydroxytryptamine is present in platelets in RA synovial fluid, its possible effects have not been pursued, probably because prostaglandin synthetase inhibitors prevent granule release. Prostaglandin synthetase inhibitors give some symptomatic relief in RA, but this is limited, and it is unclear whether the main action of these drugs is due to inhibition of prostaglandin synthetase or to some other anti-inflammatory activity.

Kinins are attractive candidates as mediators of pain and swelling in RA since they are potent stimulators of vascular permeability and pain, acting both directly and through the release of other mediators such as substance P, cytokines and prostaglandins which enhance the effects of kinins [1]. Recently, there has been renewed interest in the possible role of kinins in RA [2–4]. Kinins are formed by the action of tissue and/or plasma kallikrein on kininogen substrates which have an architecture that comprises five or six domains with the kinin moiety locked in domain 4 [5]. Tissue kallikrein is now known to be contained within the granules of polymorphonuclear leucocytes (PMNs) [6], and H- and L-kininogens are known to be displayed on the PMN surface [7, 8]. PMNs are the dominant cells found in the synovial fluid of RA patients, and because of their high numbers [9] and rapid turnover any tissue kallikrein released could make an impact on the disease process. PMNs degranulate in RA synovial fluid. In support of this assertion, PMN granule enzymes are found at high levels in RA [10, 11], but not osteoarthritis synovial fluids. Additionally, many RA synovial fluids stimulate the degranulation response of PMNs *in vitro* [11]. Since tissue kallikrein is a PMN granule enzyme and PMNs degranulate in RA synovial fluid, tissue kallikrein may be released *in vivo* and act on its substrates. Thus, it would be predicted that RA synovial fluid PMNs should contain reduced levels of tissue kallikrein. Additionally, if the enzyme acts on its substrate locally, the PMN surface kininogens should exhibit evidence of cleavage. The current work examined this hypothesis by visualizing tissue kallikrein and the kinin moiety of kininogen and comparing their levels on RA synovial fluid and blood PMNs from healthy individuals.

MATERIALS AND METHODS

Patient and healthy volunteer samples

Synovial fluid samples were obtained by therapeutic aspiration from inflamed knee joints of nine patients with RA, complying with the American College of Rheumatology (ACR) criteria [12], attending the rheumatology out-patient clinic at the Bristol Royal Infirmary (four men, median age 68 yr, range 44–72; five women, median age 54 yr, range 47–69) and eight patients (one man aged 53 yr and seven women, median age 47 yr, range 44–56) attending the rheumatology clinic at the King Edward VIII Hospital, Durban. Blood samples were obtained from nine healthy volunteers and collected in citrate (0.105 M buffered

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sodium citrate in Vacutainers) (Becton Dickinson, Cowley, Oxon.).

Isolation of PMNs

PMNs were isolated according to Dularay *et al*. [11] using the method of Dooley *et al*. [13]. Citrated blood, diluted 1:3:1 (v/v) with phosphate-buffered saline citrate [PBS citrate: 0.14 M NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 14.7 mM KH_2PO_4 and 13.6 mM sodium citrate (pH 7.2)] and dextran (Gentran 70, Baxter, UK) in a glass conical flask was incubated for 30–45 min at room temperature and the erythrocyte-depleted supernatant harvested. The cells were resuspended in 50% percoll (Sigma Chemical Co., Poole, Dorset) and layered onto a discontinuous gradient of 82.5 and 65% percoll, and centrifuged at 270 *g* for 20 min. The PMNs were removed from the 65%–82.5% interface and washed twice with PBS citrate. These cells were diluted in trypan blue (0.25% w/v in PBS) (Sigma) and counted using a haemocytometer. Their viability was >98% as assessed by their ability to exclude trypan blue.

PMNs from synovial fluid were sedimented by centrifugation (170 $g \times 10$ min) and isolated in a similar manner, except that the dextran sedimentation step was omitted.

Immunocytochemistry

PMNs were pipetted onto glass coverslips and fixed with freshly prepared 4% paraformaldehyde (w/v in PBS) for 10 min and permeabilized (2 min) with 0.2% Triton X-100 (Sigma) (v/v in PBS). To block Fc receptors, PMNs were incubated (three cycles of 10 min) with 1% human IgG (Blood Transfusion Service, Bristol) in PBS. The PBS/IgG was also used as an antibody diluent and for washes. PMNs were separately incubated with primary antibodies directed against tissue kallikrein [polyclonal antibodies to human urinary kallikrein from rabbit [6] or from goat (Protogen AG, Switzerland)], kininogen (AS218 polyclonal antisera to domain 5 of L-kininogen from rabbit [8] and I108 affinity-purified polyclonal antibodies to the heavy domain of kininogen from sheep [14]) or bradykinin (SBK1 mouse monoclonal antibody to bradykinin [15]). Following incubation with the primary antibody, cells were washed and incubated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of anti-species IgG (Sigma). Cells were imaged with a confocal scanning fluorescence microscope after a final wash. For controls, the primary antibody was replaced with buffer or, where appropriate, with rabbit IgG (Sigma). No or at most minimal immunostaining was observed with these negative controls.

Cells were imaged blind to minimize observer bias. Comparisons were made only between PMNs stained and imaged in the same session, at the same laser settings and gain controls. For the purpose of these experiments, the relative fluorescence intensities of the samples were judged by the observer to be higher than, lower than or the same as those with which they were being compared.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

Electrophoresis was performed according to Laemmli [16]. Recombinant tissue kallikrein (a gift from Dr M. Kemme, Germany) produced in baculovirus-infected insect cells [17], pure human urinary tissue kallikrein (Protogen), PMNs, human pooled saliva and biotinylated molecular weight markers (BioRad, Hemel Hempstead) were diluted in a reducing gel sample buffer [20% v/v glycerol, 4% w/v SDS, 25% v/v 0.35 M Tris (pH 6.8), 4.5% saturated bromophenol blue, 10% mercaptoethanol] (BioRad) and boiled for 3 min before electrophoresis on a 10% polyacrylamide gel in the presence of SDS under reducing conditions.

Protein was transferred from gels to nitrocellulose paper using a wet blotting system (Biorad) at 100 V for 1 h. To block non-specific antibody binding, blots were incubated (30 min) with PBS/Tween-20 (Sigma)/5% Marvel (w/v) (skimmed milk powder). This buffer was also used as an antibody diluent. Blots were incubated with rabbit anti-tissue kallikrein for 1 h and then washed (three cycles of 5 min) in PBS/Tween-20 (25 drops/l). Blots were further incubated for 1 h with anti-rabbit horseradish peroxidase and avidin horseradish peroxidase (both BioRad, diluted 1:3000) and washed as previously. Immunoreactivity was detected with 3,3′-diaminobenzidine (0.06% w/v in PBS with 0.06% v/v hydrogen peroxide).

PMN degranulation

PMNs were suspended in Hank's balanced salt solution (HBSS) [136 mM NaCl, 5.4 mM KCl, 1 mM

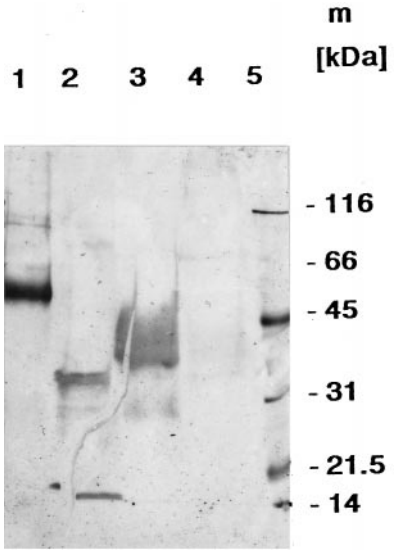


FIG. 1.—Western blot of tissue kallikrein immunoreactivity. Lane 1, recombinant tissue kallikrein (100 ng); lane 2, PMNs (2.5×10^5 cells); lane 3, human urinary tissue kallikrein (125 ng); lane 4, pooled human saliva (25 μl of 1:4 dilution); lane 5, biotinylated markers (2.5 μl of 1:4 dilution). Samples were subjected to electrophoresis on a 10% polyacrylamide gel in the presence of SDS under reducing conditions. The gel was then subjected to Western blot analysis and probed with rabbit anti-tissue kallikrein.

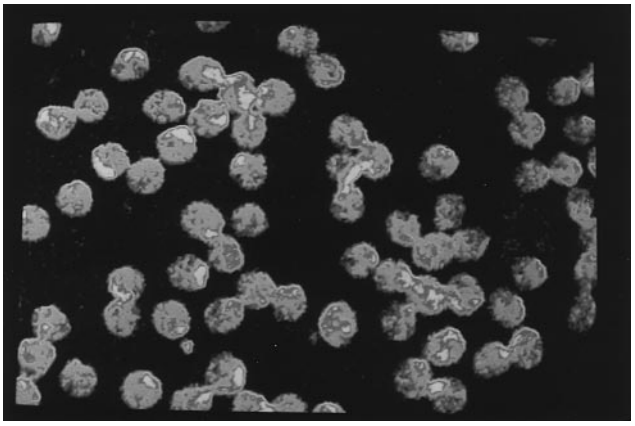
glucose, 1 mM $MgCl_2$, 5.5 mM HEPES (Sigma), 10 mM $CaCl_2$ (pH 7.2)] containing gelatin (Sigma, 1 mg/ml) at 10^7 cells/ml. The cells were pre-incubated with cytochalasin b (Sigma) [CTC-B; 1 mg/ml in dimethyl sulphoxide (DMSO; Sigma), final concentration of 20 μg/ml] for 10 min at room temperature. A total of 10^6 cells/ml were stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (Sigma) (FMLP; 100 mM in DMSO, final concentration of 20 μM) or HBSS–gelatin buffer for 30 min at 37°C. The PMNs were centrifuged (170 $g \times 10$ min), and the supernatants assayed for myeloperoxidase activity by the method of Suzuki *et al*. [18]. The cells were resuspended in PBS for immunocytochemistry.

RESULTS

Tissue kallikrein

Experiments were set up to determine the identity of immunoreactive tissue kallikrein detected in PMNs. Recombinant tissue kallikrein, PMNs, pure human urinary tissue kallikrein and human saliva were subjected to SDS–PAGE and Western blot analysis (Fig. 1). Note that anti-tissue kallikrein detected specific bands in all four samples. For PMNs, a major immunoreactive band was seen at 32–35 kDa and another band at 16 kDa, while for recombinant tissue kallikrein a band of 54–62 kDa was obtained. Diffuse bands were detected about 27 kDa and from 36 to 54 kDa for human urinary tissue kallikrein and at 35–49 kDa for saliva.

(a)



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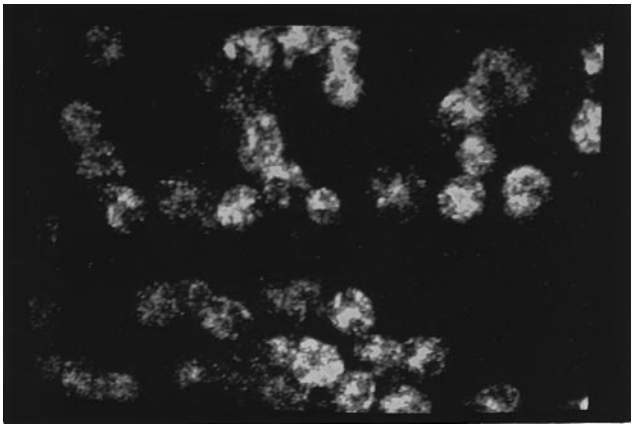
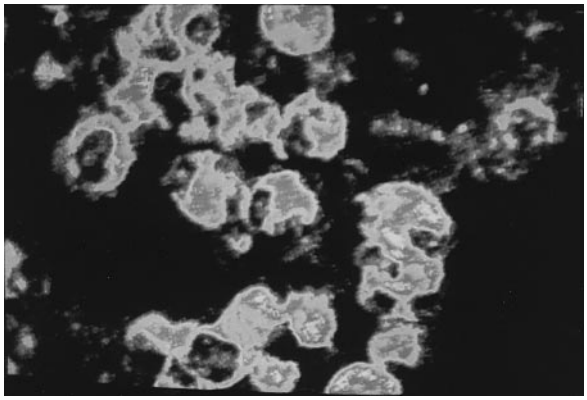


FIG. 2.—Comparison of confocal scanning laser micrographs of PMNs from (a) normal blood and (b) RA synovial fluid stained with FITC-linked anti-rabbit IgG following incubation with rabbit anti-tissue kallikrein.

(a)



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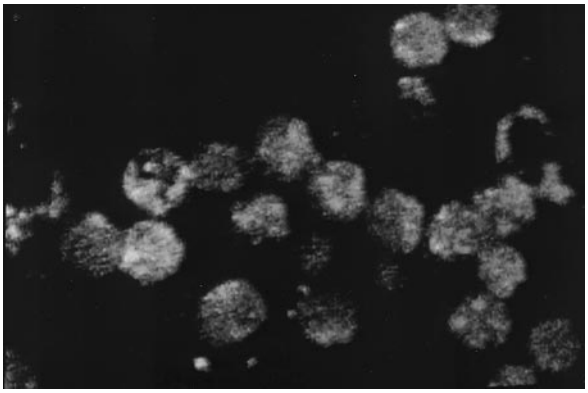

FIG. 3.—Comparison of confocal scanning laser micrographs of PMNs from (a) normal blood and (b) RA synovial fluid stained with FITC-linked anti-mouse IgG following incubation with anti-bradykinin.

PMNs, from five RA synovial fluid and five blood samples from healthy volunteers, were stained with anti-tissue kallikrein and examined by confocal microscopy. A typical result is illustrated in Fig. 2. It can be seen that the RA synovial fluid PMNs were much less stained for tissue kallikrein than the blood PMNs, and similar results were observed for the other four paired samples. In separate experiments, blood PMNs from eight RA patients were stained with anti-tissue kallikrein and, although there was some variation, all were stained. By contrast, little or no staining was exhibited by paired synovial fluid PMNs from three of these patients.

Kinin moiety of kininogen

To determine whether or not the kinin moiety was present within kininogen molecules on the external surface of RA synovial fluid PMNs, samples from five patients and five blood samples from healthy

volunteers were stained with anti-bradykinin and examined by confocal microscopy. From Fig. 3, it can be seen that the RA synovial fluid PMNs were much less stained for bradykinin than the blood PMNs, and similar results were observed for the other four paired samples.

Since the kinin moiety is an integral part of the kininogen molecule, it could be argued from the above results that the entire protein was lost from the RA synovial fluid PMNs. Therefore, two of the paired samples were probed for kininogen immunoreactivity. The antibodies used were specific to the light and heavy chain domains of kininogen which lack the kinin moiety. Figure 4 shows that PMNs both from RA synovial fluids and from the blood of healthy individuals stained similarly.

Effect of degranulation on PMN tissue kallikrein and kinin moiety

As already mentioned, RA synovial fluid PMNs degranulate in the inflamed joint space. Thus, the observed pattern of staining for tissue kallikrein and the kinin moiety may result from PMN degranulation. If so, PMNs stimulated to degranulate *in vitro* should exhibit a similar pattern. To examine this possibility, PMNs were stimulated with or without FMLP, in the presence of CTC-B. These cells were stained with anti-tissue kallikrein or anti-bradykinin. A typical result is depicted in Fig. 5. It is clear that stimulated PMNs exhibited decreased staining with both anti-tissue and anti-bradykinin. To confirm that degranulation had occurred in the FMLP-stimulated PMNs, the cell supernatants were collected and assayed for the PMN granule enzyme, myeloperoxidase. Myeloperoxidase activity in supernatants from FMLP-stimulated PMNs was 1.250 ± 0.16 absorbance units (mean $\pm$ S.E.M.) and 0.087 ± 0.027 absorbance units for PMNs incubated with CTC-B alone ($n = 8$, $P = 0.0006$, Mann–Whitney test).

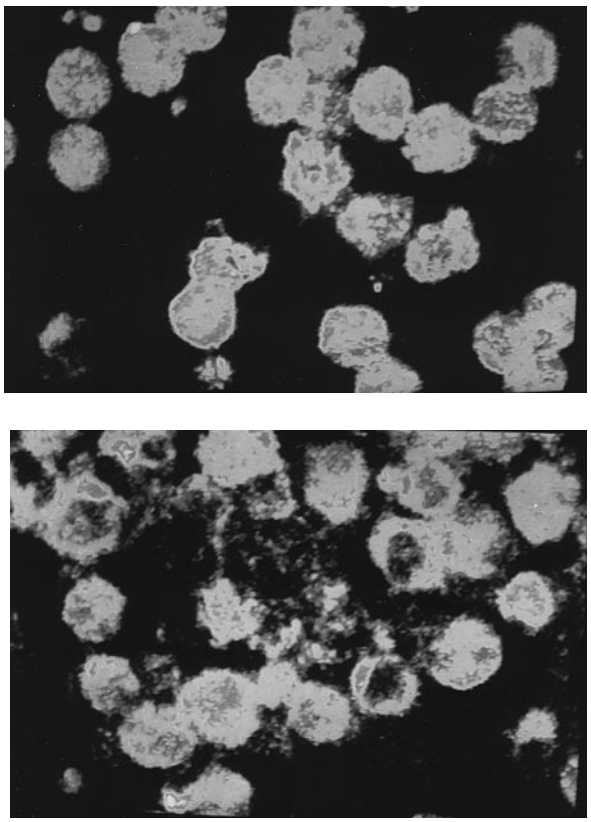

FIG. 4.—Comparison of confocal scanning laser micrographs of PMNs from (a) normal blood and (b) RA synovial fluid stained with FITC-linked anti-sheep IgG following incubation with sheep anti-kininogen.

DISCUSSION

Anti-tissue kallikrein was used to visualize tissue kallikrein in human PMNs. It could be argued that the antibody reacted with other PMN proteins. However, Western blot analysis revealed not only that the antibodies recognized one major band with recombinant tissue kallikrein, but also discrete immunoreactive bands with PMNs. Similar discrete bands were obtained with human urinary tissue kallikrein which were of a molecular weight previously reported by others [19]. The fact that broad bands were obtained with both PMNs and human urinary tissue kallikrein is consistent with the known differential glycosylation of tissue kallikrein [1] and may account for the observed variation in the molecular weight of tissue kallikrein from different sources.

One important result reported here is that tissue kallikrein immunoreactivity was lower in RA synovial fluid PMNs than in those from the blood of healthy individuals or RA patients. As tissue kallikrein is a PMN granule enzyme [6], this result supports the hypothesis that tissue kallikrein is released *in vivo* from RA synovial fluid PMNs by degranulation. If so, it would be expected that the levels of tissue kallikrein immunostaining exhibited by blood PMNs stimulated to degranulate *in vitro* should be similar to those of the RA synovial fluid PMNs. This expectation was borne out by the results. PMNs stimulated to degranulate *in vitro* displayed less tissue kallikrein immunoreactivity than their non-stimulated counterparts.

PMN-bound kininogen may be cleaved in RA synovial fluid by tissue kallikrein released from PMN granules. In support of this view, comparisons of immunoreactivity to the kinin moiety of kininogen in PMNs from RA synovial fluid and from the blood of healthy individuals showed lower levels of immunoreactive enzyme in the RA synovial fluid PMNs. The results also showed that the kinin moiety was cleaved from the PMN surface kininogen as opposed to the whole molecule merely being released from the cell surface. PMNs from RA synovial fluid and from the blood of healthy individuals exhibited similar levels of kininogen staining. In other words, the lower level of kinin immunoreactivity on RA synovial fluid PMNs was not due to loss of the entire kininogen molecule from the PMN surface. It is known that PMN kinin immunoreactivity can be reduced *in vitro* by incubation with plasma kallikrein or tissue kallikrein [14]. Thus,

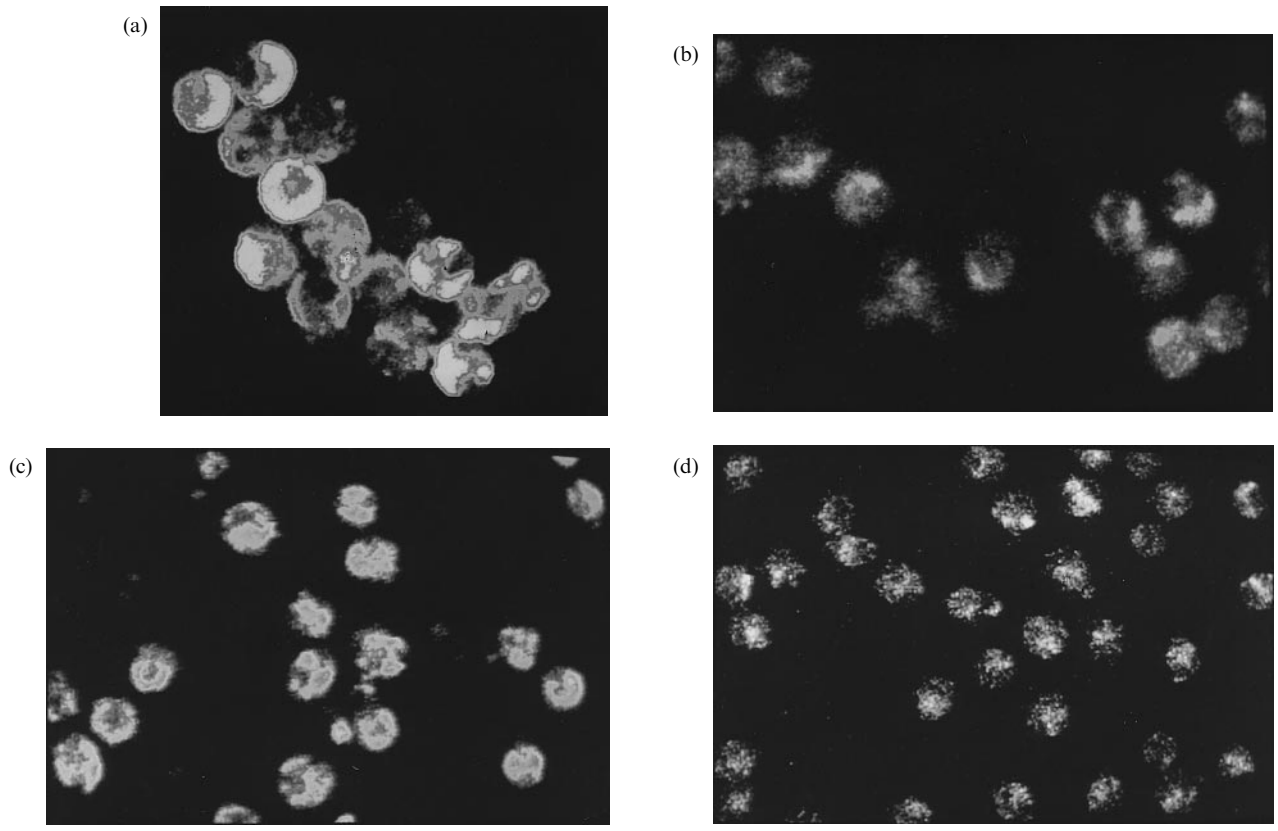

FIG. 5.—Comparison of confocal scanning laser micrographs of PMNs from a degranulation experiment stained with FITC-linked anti-species IgG following incubation with anti-tissue kallikrein or anti-bradykinin. (a) PMNs stimulated with CTC-B alone and then stained with anti-tissue kallikrein; (b) PMNs stimulated with CTC-B and FMLP (20 μM), and then stained with anti-tissue kallikrein; (c) PMNs stimulated with CTC-B alone and then stained with anti-bradykinin; (d) PMNs stimulated with CTC-B and FMLP (20 μM), and then stained with anti-bradykinin.

in addition to PMN-derived tissue kallikrein, PMN-bound kininogen could be cleaved by the action of activated surface-bound plasma prekallikrein [14], or by kininogenases in synovial fluid [3, 4, 20]. However, if tissue kallikrein released from PMN granules does cleave the kinin moiety from the surface of RA synovial fluid PMNs, then PMN from healthy individuals stimulated to degranulate *in vitro* should also display reduced kinin levels. Again, the results agree with this postulate. Kinin immunoreactivity was lower on stimulated than non-stimulated PMNs. Additionally, although tissue kallikrein has been detected in RA synovial fluids, it is mainly bound to inhibitors [3, 4]. One might therefore envisage that tissue kallikrein released from PMN granules could act locally on cell surface kininogen before it is inactivated by inhibitors.

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