

Enzyme-Linked Hyaluronectin: A Unique Reagent for Hyaluronan Assay and Tissue Location and for Hyaluronidase Activity Detection

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Several techniques for assaying and localizing hyaluronan (HA), all based on the affinity to hyaluronan of proteins isolated from cartilage, chondrosarcoma, or brain, have been proposed. We show here that a unique reagent, alkaline phosphatase-linked hyaluronectin, can be used to assay hyaluronan in biological fluids or tissue extracts (enzyme-linked sorbent assay method) and to characterize it in cells or tissue sections in two steps: reagent incubation and staining. Results of assays in biological fluids or tissue extracts showed a good correlation with results of the previously described technique using antibodies to detect hyaluronectin bound to a plastic microtest plate (B. Delpech *et al.*, 1985, *Anal. Biochem.* 149, 555–565) for both low concentrations (<1 mg/liter, $r = 0.973$, $P < 0.001$) and high concentrations (>1 mg/liter, $r = 0.953$, $P < 0.001$). The interassay variations were 8.5% when the assay was performed at 4°C and 18.5% at 37°C. The intraassay variations under those conditions were, respectively, 14.4 and 6.5%. Tissue HA could be detected easily with the reagent, as shown in fetal tissues and in tumors. Specificity of the reaction was controlled either by blocking the reagent with an excess of hyaluronan (which was not possible with other glycosaminoglycans) or by destroying tissue hyaluronan with streptomyces hyaluronidase. Alkaline phosphatase-linked hyaluronectin was also used to assay hyaluronidase activity in several biological fluids. One-hour incubation of hyaluronidase preparations on HA-coated plates made it possible to detect as low as 1 mU bovine testis hyaluronidase and 0.1 mTRU streptomyces hyaluronidase. Four-hour incubation made it possible to detect activity in a 1/12,500 dilution of human serum. © 1995

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Hyaluronan (hyaluronic acid, HA¹) is a nonsulfated glycosaminoglycan found in several organisms and tis-

¹ Abbreviations used: ABTS, 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate), diammonium salt; AHS, aminoethyl Sepharose 4B; AP, alkaline phosphatase; BSA, bovine serum albumin; CI, 95% confidence interval; ELSA, enzyme-linked sorbent assay; ELISA, enzyme-linked immunosorbent assay; HA, hyaluronan; HN, hyaluronectin; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; PO, peroxidase; TBS, Tris-buffered saline; TRU, turbidity-reducing unit.

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ues from bacteria to humans and is thought to play an important biological role particularly in growth, cell migration, and tumors (1). In clinical medicine serum hyaluronan augmentation has been observed in a few diseases related to inflammation and cancer (2–7).

The metachromatic techniques used for HA detection in tissues, and the rather complicated methods for HA assay in fluids which required differential enzyme digestion of glycosaminoglycans, have been replaced by methods based on similar affinities of proteins to HA (8–11). Among the assays, we described a method based on the use of hyaluronectin (HN), a brain-derived HA-binding glycoprotein, on HA-coated plastic microtest plates (6). The binding of HN to the plate was inhibited by HA in proportion to the HA concentration in the solution being assayed. Bound HN was then measured with enzyme-linked antibodies (ELISA method). The same principle was used to detect HA in tissues (11), with the pitfall that the use of anti-HN antibodies needed particular controls to avoid artifacts due to tissue-associated HN or the use of monoclonal species-specific antibodies (12). Biotinylated hyaluronectin also allowed very good staining in cultivated newborn rat brain (13), but as with the ELISA method, a second layer should be added before staining. Eventually, the digestion of HA adsorbed on the plastic plate by hyaluronidases could be measured by the technique (14,15).

We describe here an improvement in the different utilizations of the method for detecting and assaying HA and hyaluronidases based on the use of a unique reagent, alkaline phosphatase-linked sheep brain hyaluronectin (AP–HN). The enzyme-linked sorbent assay (ELSA) saves several hours by skipping one step (the antibody step) of the former ELISA assay and suppressing the risk of artifacts in the detection of HA in tissue. The conditions of use are slightly different from the conditions used previously, but the results are similar to those obtained with the first method.

MATERIALS

Sheep brain was obtained at slaughter and could be stored at -30°C for up to 6 months without any detect-

able alteration of the protein extracted. Ultra-Turrax was from Bioblock (Illkirch, France). Aminoethyl Sepharose 4B was from Pharmacia (St. Quentin en Yvelines, France). HPLC was performed with a Superose 12 column (Pharmacia) driven by a Beckman device (Gagny, France) with a continuous-flow recorder. For preparatory purposes, chromatography was performed on an AcA 34 (Sepracor, Villeneuve la Garenne, France).

Hyaluronan from human umbilical cord (H 1504), diethanolamine, bovine serum albumin, pronase E from *Streptomyces griseus* (4.4 units/mg), Tween 20, bovine testis hyaluronidase (290 units/mg), guanidinium chloride, Fast Red, naphthol As-Mx phosphate, and levamisole were from Sigma (Coger, Paris, France). Alkaline phosphatase from calf intestine (3000 U/mg protein), *p*-nitrophenyl phosphate, peroxidase from horseradish, and ABTS were purchased from Boehringer (Meylan, France). Chondroitinase was purchased from Seikagaku Corp. (Tokyo, Japan).

Glutaraldehyde was from TAAB Laboratories (Saint Germain en Laye, France). Streptomyces hyaluronidase (200 TRU/mg) was purchased from Calbiochem (Meudon, France). Other chemical reagents were from ProLabo (Paris, France) and were of analytical quality.

Microtest plates were purchased from Nunc (Poly Labo, Strasbourg, France). Absorbances were recorded on a Titertek Multiskan instrument (Flow Laboratories, Les Ulis, France). Online calculations were made with a Victor computer. Buffers used were PBS (phosphate-buffered saline, 0.14 M NaCl in 0.01 M sodium phosphate, pH 7.4) and TBS (Tris-buffered saline, 0.14 M NaCl in 0.1 M Tris-HCl, pH 7.4) for HA assay and tissue detection; 0.1 M acetate with 0.15 M NaCl at pH 5 for bovine testicular hyaluronidase and pH 6 for streptomyces hyaluronidase; and 0.1 M citrate with 0.05 M NaCl at pH 3.8 for human serum and hepatoma cell culture hyaluronidase.

METHODS

Preparation of HA-linked aminoethyl Sepharose, of HA-coated plastic microtest plates, and of standard HA solutions and HA assay in sera and biological fluids with the enzymeimmunological assay were described previously and used without any modification (6). The HA used for plate coating was not as critical in the ELSA method as it has been in the ELISA method used previously, since it was not necessary to get rid of any cross-reactivity with anti-HN antibodies. However, as with the ELISA method, we selected HA batches which did not cross-react with anti-HN antibodies.

HA used as a standard solution in both methods was prepared as described (6). Briefly, HA was digested with chondroitinase ABC for 1 h at 37°C and then digested with Pronase E from *S. griseus* for 18 h, and protease was denatured by 20-min heating at 100°C. The solution was dialyzed against 1000 vol of 0.2 M

glycine-HCl buffer, pH 2.2, and dialyzed twice against 1000 vol of PBS. HA standard solution was estimated with the carbazole method.

Purification of Sheep Brain Hyaluronectin

A sheep brain (about 100 g) was thawed and ground in 300 ml of 0.2 M glycine-HCl buffer, pH 2.2. The final pH of the extract was 2.8. The extract was centrifuged for 20 min at 32,000g at 4°C. The supernatant pH was brought to 5 with sodium hydroxide, the extract was spun again as above, and the precipitate was discarded. The supernatant pH was adjusted to 7 with sodium hydroxide and passed through a 15-ml gel of HA-linked aminoethyl Sepharose, overnight at 4°C. The flow rate was about 15 ml/h. The column was washed with 0.5 liter of PBS and 1 liter of 1 M NaCl in 0.01 M sodium phosphate at pH 7.4, and the protein was eluted with 0.2 M glycine-HCl buffer, pH 2.8. The tubes with absorbance above 0.1 at 280 nm were pooled and the pH was adjusted to 6–7 with 2 M sodium hydroxide. The pool was concentrated under pressure at 4°C to about one-tenth the initial volume and stored at –30°C or dialyzed against 1 liter of PBS twice. The amount of HN obtained for one brain was 3–5 mg on the basis of the absorbance at 280 nm (0.8 for 1 mg/ml under standard conditions). The purity of the preparation was studied by HPLC through Superose 12 in PBS. During the two first cycles of the column, heavy HA–HN complexes may be found in the void volume (under 20 min) due to the release of some micrograms of HA by the column. Only the protein found in the 25- to 27-min tubes was kept for further work; it was extensively dialyzed against distilled water and freeze-dried. Its affinity to HA was verified with the same method: the addition of HA to HN amplified the void volume protein peak and reduced the peak at 25–27 min by more than 95%.

The total cycling procedure could be performed at least 10 times with the same column.

Preparation of Enzyme-Linked HN

The glutaraldehyde technique was used and the optimal technical conditions selected were as follows: One milligram of HN was mixed with 0.5 mg of alkaline phosphatase in a final volume of 1 ml of PBS and dialyzed against 1 liter of PBS. The mixture was supplemented with 40 μ l of glutaraldehyde at 4.2% and rocked gently for 2 h at room temperature. The excess of glutaraldehyde was inhibited by dialysis against 1 liter of TBS. The AP–HN complexes were isolated by AcA 34 chromatography in that buffer. The tubes with AP–HN were detected by incubation of 1/20 dilutions on HA-coated plastic microtest plates, for 2 h at 4°C. Only the tubes with good HA-binding activity (that is, those that gave a final substrate absorbance over 0.5 after 1 h of incubation of the substrate) were kept. Tubes with free alkaline phosphatase activity were dis-

carded (they contained also a small amount—less than 5%—of HN). The preparations were supplemented with 1 g/liter bovine serum albumin and 0.5 g/liter sodium azide and titrated on an HA-coated plate, which was stored at 4°C. The activity had not significantly decayed after 18 months. The final dilution used in the HA assay was that which gave a final absorbance of the substrate of 1 at 405 nm within 1 h incubation of the substrate.

The technique was used under the same conditions to prepare peroxidase-linked HN, with the only differences that azide was not used in solutions and that PO–HN was stored under sterile conditions after filtration through 0.22- μ m Millipore filters.

Estimation of HN in the AP–HN Fraction

AP–HN complexes were isolated by affinity chromatography on HA-linked aminohexyl Sepharose and elution with 2 M guanidinium chloride, since we found that 2 M guanidinium chloride did not alter the phosphatase activity of the AP–HN fraction, and dialyzed against PBS.

HN activity in the AP–HN fraction was measured by its capacity to compete with peroxidase-linked sheep brain HN in the binding to the HA-coated plate. The standard was made with pure sheep brain 45–70K HN, which had been purified on HPLC and whose reactivity with HA was verified. The incubation was for 3 h at 4°C. PO–HN activity was measured with ABTS (7.5 mg in 10 ml of 0.1 M acetoacetic buffer at pH 5.6 with 1 μ l of hydrogen peroxide) at 405 nm.

Estimation of Alkaline Phosphatase in the AP–HN Fraction

A 0.1-ml alkaline phosphatase sample was added to 0.9 ml of paranitrophenyl phosphate, 1 g/liter in 1 M diethanolamine, pH 9.8, with 0.2 M $MgCl_2$ and incubated in a water bath at 37°C for 30 min. The absorbance was read at 405 nm.

The Enzyme-Linked Sorbent Assay

The principle of the reaction is based on the affinity of the AP–HN complex to HA adsorbed to the plastic microtest plate. The binding of AP–HN to the HA-coated plate decreases when AP–HN is solubilized in a solution containing HA. Plate wells were coated with 100 μ l of HA as described and could be stored for 1 month at 4°C. The samples tested were protease digested in Eppendorf tubes overnight at 37°C by dilution of 0.05 ml of the sample in 0.45 ml of TBS with 0.5 mg of protease. A control was made with BSA in TBS. The protease was destroyed at 100°C for 20 min and the samples were centrifuged when necessary (tissue extracts, undiluted sera). Two blank wells were made in which HA-coated wells were filled with buffer alone.

An assay at 4°C was performed for low concentra-

tions (below 1 mg/liter in the sample), which was generally the case for sera. We found the affinity of HN for soluble HA to be much higher at 4°C than at 37°C. This was also seen for AP–HN. The standard HA solutions were made in TBS at concentrations of 2, 4, 8, 16, 32, 64, and 128 μ g/liter. They were mixed (v/v) with AP–HN (corresponding to HN equivalent activity of 2 mg/liter) diluted in TBS with 1 g/liter Tween 20, 1 M NaCl, and 1 g/liter BSA for 1 h at 4°C. The plate was rinsed with 0.1 M acetic acid and with PBS and kept at 4°C for 1 h before use. The plates were set on ice, and the wells were filled with 100 μ l of the solutions. The standards were incubated in the two series of wells of the middle of the plate, in duplicate. To optimize the results, the solutions were assayed in duplicate on each side of the standard wells, which minimized a 20% absorbance shift that was often seen from the left to the right of the plates. Two wells were filled with a known sample to verify the reproducibility of the assay. The plate was incubated at 4°C for 3 h in a water bath, rinsed with PBS, and incubated with 250 μ l substrate per well.

An assay at 37°C was performed for samples with HA concentrations above 1 mg/liter (pleural fluid, tumour extracts). The standard solutions were 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mg/liter. The same AP–HN dilution as that at 4°C was used and the assay was run as above, with all reagents and incubations at 37°C. In all cases the plates were washed three times with PBS and stained with paranitrophenyl phosphate, 1 g/liter in 1 M diethanolamine, pH 9.8, with 0.2 M $MgCl_2$. Plates were incubated in water for 1 h at 37°C in a heated cabinet. Plates were read at 405 nm on a Titertek Multiskan (Flow) and calculations were made by an online computer.

Specificity tests included digestions of samples with streptomyces hyaluronidase for 48 h at 37°C. The tests showed complete abolition of the inhibition of AP–HN binding to HA-coated plates by the samples tested.

The recovery test was performed as follows: A known amount of 10 μ g/liter HA was added to a solution containing less than 10 μ g HA/liter. The sample was digested and assayed as above.

Reproducibility tests were performed with one sample assayed in 40 wells of the same plate (intraassay variation) and on two wells of 15 plates (interassay variation).

Detection of HA on Cultivated Cells and on Tissue Sections

Cultivated human glioma-derived cells CB74, CB191, and CB193, isolated in our laboratory, were grown on coverslips and fixed in 1% acetic acid in ethanol (1/99 v/v) for 10 min.

Mouse grafted tumors derived from cultivated CB109 glioma cells (13) and HT29 colonic carcinoma cells, rat

kidney and cerebellum, and human tumors, spleen, and bone marrow biopsies were fixed in either liquid nitrogen or acetic alcohol for 24 h, paraffin-embedded, and cut into 5- μ m sections. Paraffin was extracted, and sections and cells were rehydrated and washed for 10 min in TBS and incubated with AP–HN preparation (equivalent to 20 mg HN/liter) for 30–40 min at 37°C. Alkaline phosphatase was detected with Fast Red staining for 30 min at room temperature (18). Sections were counterstained with hematoxylin when necessary.

The specific controls were made by digesting tissue–HA with 20 TRU/ml streptomyces hyaluronidase twice for 2 h at 37°C or by adding soluble hyaluronan to the AP–HN reagent (50/100, w/w) 12 h before incubation on the tissue section.

Hyaluronidase Activity Detection

The presence of hyaluronidase in a sample results in the loss of the HN-binding capacity of an HA-coated plate. Samples were diluted in the appropriate buffer. All buffers were supplemented with 0.1% BSA. One hundred-microliter samples were incubated in each well at 37°C for 1 or 4 h. The plate was rinsed with 4 M guanidinium chloride and then with water and incubated for 30 min with TBS containing 1 g/liter BSA to saturate plastic sites made free by the removal of hyaluronan. Wells were incubated with AP–HN diluted as for the HA assay, for 3 to 4 h at 4°C. The substrate was as described above. The sample activities were calculated by reference to a standard hyaluronidase preparation incubated on the plate under the same conditions. The activities of commercial testicular hyaluronidase and streptomyces hyaluronidase were expressed in units per milligram (one unit being equivalent to one National Formulary Unit (17)) and in TRU, respectively. Because no standard human seric hyaluronidase was available, the sensitivity of the assay was expressed as the highest dilution of a pool of sera that gave a decrease in the maximum absorbance of 10%.

Statistical Methods

Since the distribution of serum HA values was not normal, statistical calculations for regression analysis were performed using log values.

RESULTS

Composition of the AP–HN Complex

The linking of HN and AP led to loss of 90% of activity for both. This could be due to denaturation of HA-binding sites on HN or of enzyme sites on AP, to their involvement in the linking, or to steric encumbrance. A comparison of the ability of the preparation to compete with peroxidase-linked HN with that of pure HN revealed an activity equivalent to 10 to 12.5% of the initial activity. The phosphatase activity of the prepa-

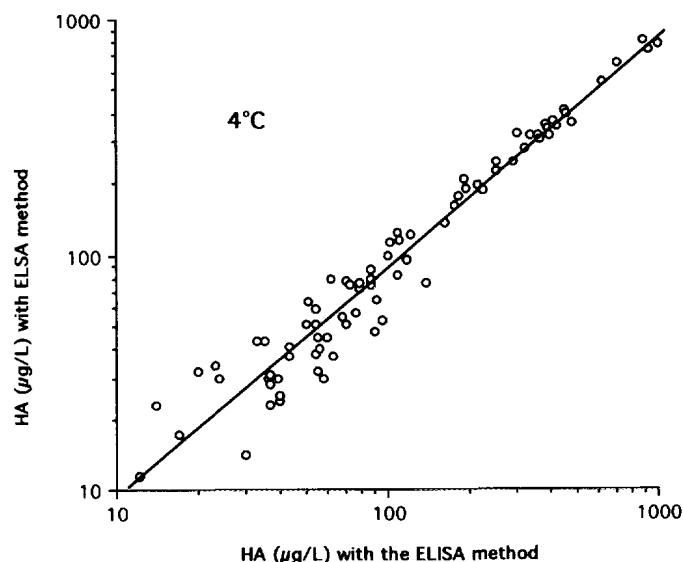


FIG. 1. Linear regression between ELISA and ELSA assays performed at +4°C. $r = 0.973$ ($P < 0.001$); slope = 0.978 (CI = 0.926 to 1.030); intercept = -0.026 (CI = -0.273 to $+0.221$).

ration was equivalent to 8 to 10% of the initial activity. We can estimate from the values that the mean complex composition was one to two molecules HN per molecule of enzyme. Since the enzyme denaturation rate is about 90%, the results fit with the requirement of an HN equivalent concentration about 10-fold higher in the ELSA method than in the ELISA test.

The best activity was obtained under the described linking conditions. The glutaraldehyde concentration was the most critical factor. A 20- μ l volume of 4.2% glutaraldehyde provided a rather poor reagent. With 80 μ l glutaraldehyde, the reagent, although excellent for the microtest plate assay, was not as suitable for histochemistry as the standard preparation made with 40 μ l.

HA assays were performed in TBS with 1 g/liter Tween and 1 M NaCl, to avoid nonspecific interactions that may lead to loss of accuracy and overestimation of values lower than 100 μ g/liter. Despite the high concentrations of hyaluronidase used to digest serum or tissue extract HA, positive results (that is, a decrease in the alkaline phosphatase activity in the tests compared to that in controls with hyaluronidase-digested pure HA) were still observed when incubations of sera with AP–HN were performed in PBS, whereas they were abolished when incubations were performed in TBS/Tween/NaCl. This is the main difference from the ELISA method, suggesting that the AP–HN complexes might exhibit some nonspecific weak affinities to other serum components or that AP was slightly inhibited by those components. Under the conditions described, the results of the ELSA method were in good correlation with and gave results close or identical to those obtained with the ELISA method at 4 and 37°C (Figs. 1

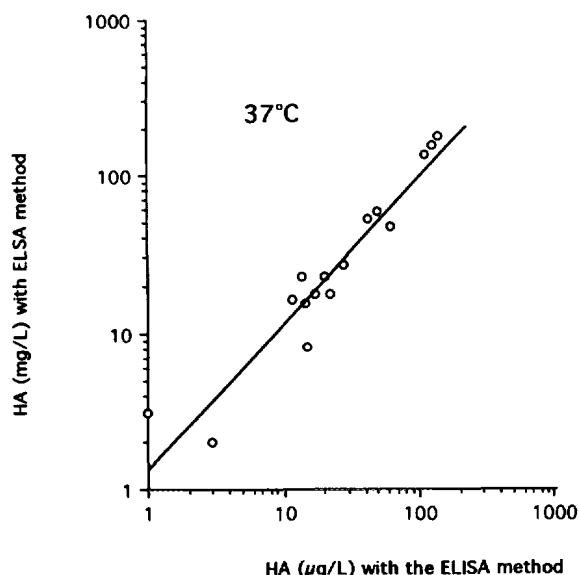


FIG. 2. Linear regression between ELISA and ELSA assays performed at +37°C. $r = 0.953$ ($P < 0.001$); slope = 0.927 (CI = 0.759 to 1.095); intercept 0.344 (CI = -0.225 to +0.913).

and 2). The regression slopes were not different from 1 and intercepts were not different from 0.

Tissue Location

The reagent allowed specific histological and cytological characterization of HA. In tumors, HA has already been described as a constant marker of desmoplasia, and AP-HN gave the same pattern as those previously described with other probes (19,20). Superimposition of HA with the reticulin network was seen in both myeloproliferative spleen and bone marrow (Fig. 3A) where fibrosis was increased, and the reticulin fibers were underlined by HA. HA was also constantly seen around the follicular and trabecular arteries (Fig. 3C), which were the only positive areas of normal spleens. With the AP-HN method, tissue HA characterization on frozen sections was obtained within 2 h after tissue removal.

The specificity of the reaction was verified with two types of controls. One was the abolition of the enzyme staining when the tissue section had been incubated with streptomyces hyaluronidase, an enzyme specific for HA (Fig. 3B). The second was the blocking of the AP-HN reactivity by the addition of hyaluronan (50/100, HA/HN (w/w)) to the reagent (Fig. 3D).

Hyaluronidase Assay

With 1 h incubation at 37°C, the method could detect 1 mU bovine testis hyaluronidase or 0.1 mTRU streptomyces hyaluronidase. The significant threshold was a 10% decrease in the absorbance (Fig. 4). After a 4-h incubation, hyaluronidase could be still detected in a

1/12,500 dilution of human serum, whereas it was not detectable in undiluted rabbit serum.

DISCUSSION

We have shown that a unique reagent can be used to assay HA in solution, to detect HA on tissue sections, or to detect hyaluronidase activity in biological fluids or tissue extracts. The reagent is relatively easy to prepare and can be stored without decay for months in a cold room. From 1 mg sheep brain HN, a reagent that allows several thousand assays or several hundred tissue detections can be prepared within a few hours.

Compared with methods previously described for the HA assay and detection with HN and antibodies, the methods described here are faster because they omit the antibody steps which were needed to detect and measure HN bound to HA. The preparation leads to the loss of about 90% of the original HN activity, which is not truly a drawback since the source of HN is common and inexpensive.

The use of AP-HN was optimized in TBS by the addition of 1 g/liter Tween and 1 M NaCl to the Tris Buffer. In PBS we observed amplification of low results (compared to that in the ELISA HA assay) in part resistant to hyaluronidase, suggesting that, as opposed to pure HN, AP-HN complexes could establish low-affinity binding with irrelevant components of the serum or extracts or that AP was slightly inhibited even after protease digestion. TBS/Tween/NaCl abolished the weak unspecific interactions and gave good correlation of the results obtained with ELISA and ELSA methods.

Detection of tissue HA in rat cerebellum with an antigen-antibody complex method specific for rat species tissues was previously described (11). More recently, with the biotinylated HN probe, detection of HA in human tumors as well as in grafted tumors was described (16). It was claimed that biotin can bind, in some cases, to avidin-like molecules in endogenous tissue (21,22). Other work showed that tissues can exhibit an avidin-binding activity (23). The need for other controls with biotinylated reagents or a streptavidin layer does not affect AP-HN, which is easier to use. The AP-HN probe used here is a more convenient tool for one-step detection of HA in tissue sections and cultivated cells whatever their species of origin. Tissue labeling was specific for HA, since all staining was abolished by tissue digestion with hyaluronidase or by incubation of the probe with soluble HA. Other glycosaminoglycans had no effect on the reactivity of the probe with HA.

The method was extended to the detection and measurement of hyaluronidase activity. Usual biochemical techniques are based on the determination of the reducing groups released by HA during its digestion with hyaluronidase (24,25). Compared to those techniques, the method described here is much more sensitive and

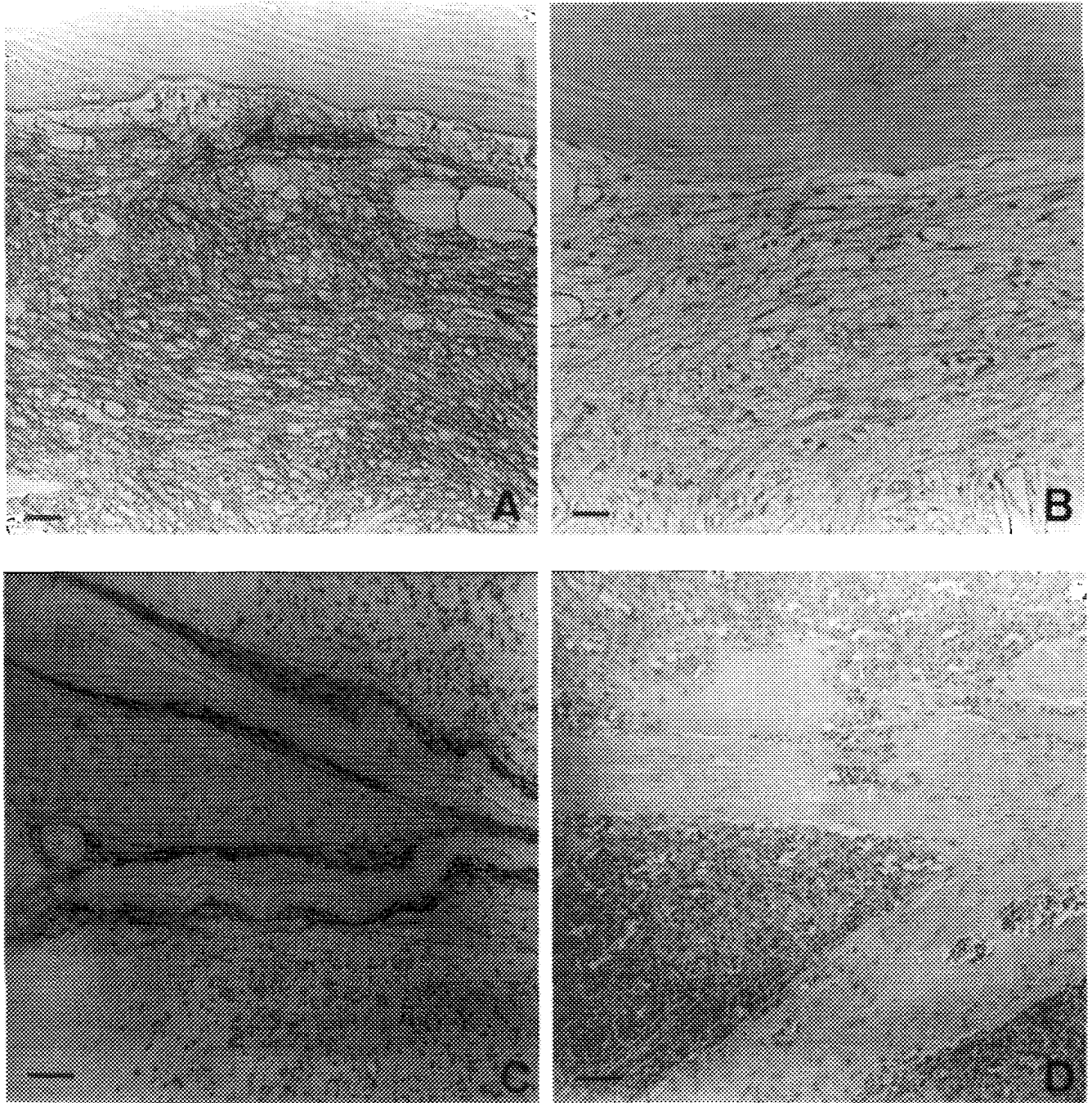


FIG. 3. HA detection with AP-HN in tissue sections. (A) Pathological bone marrow with dense myelofibrosis. Reticular pattern of HA. (B) Control of A. The staining was suppressed by hyaluronidase digestion of the section. (C) Normal spleen. Specific staining of an artery adventitia. There is no counterstaining. (D) Control of C. The specific staining was suppressed when the reagent was supplemented with HA. Nuclear counterstaining was necessary to see the tissue structure on the control section. Bar, 20 μm .

is easier to implement. A few precautions should be taken to avoid misinterpretation. One is to wash out unspecific molecules bound to HA after enzymatic digestion, with a dissociative reagent such as guanidinium or acid. Another is to saturate the plastic plate with albumin after the digestion step, to avoid coating plastic sites with AP-HN. Eventually, too long an incu-

bation with AP-HN (e.g., overnight) can lead to high background noise and loss of sensitivity, indicating that exchanges can still occur with the plastic plate after HA digestion, which is not the case when the plate is completely coated with HA (see Results). For that reason, incubation with AP-HN must not exceed 4 h. The method made it possible to detect and determine

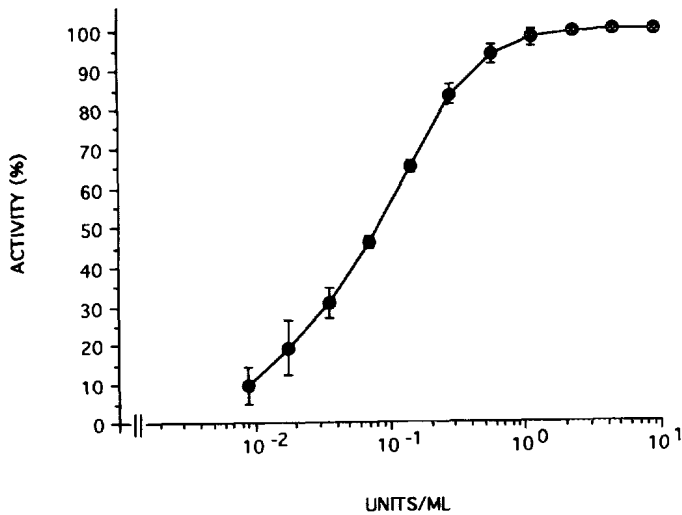


FIG. 4. Sensitivity of the hyaluronidase assay. Sensitivity was measured with bovine testicular hyaluronidase incubated for 1 h at 37°C. The decrease in the maximum absorbance (A_m) measured without enzyme is a function of the enzyme activity, which was calculated as follows: activity (%) = $(1 - A/A_m) \cdot 100$, where A is the absorbance measured for samples and A_m the maximum absorbance read in the absence of hyaluronidase.

the enzyme parameters for several hyaluronidases from human sera and tumors, bee venom, bovine testis, and bacteria.

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