Removal of Nonspecific Antiproteinases from Serum of Rabbits Hyperimmunized Against Micrococcus and Staphylococcus Proteinases

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The naturally occurring (nonspecific) proteinase inhibitor which occurs in the alpha globulin fraction of immune rabbit serum was separated from specific staphylococcal and micrococcal antiproteinases by treatment of the antiserum with 2-ethoxy-6-9-diaminacridine. The 2-ethoxy-6-9-diaminacridine-treated serum could then be used to classify these bacterial proteinases by testing directly on casein agar without prior electrophoresis of the serum.

An immunoelectrophoretic method for the serological differentiation of extracellular bacterial proteinases has been described (O. Sandvik, Veterinary College of Norway, Oslo, 1962). Specific antiproteinases in immune rabbit sera were separated from natural proteinase inhibitors by paper electrophoresis. Their proteolytic effects were then demonstrated by inhibiting the specific enzymes from precipitating sodium caseinate in an agar medium. The method has been used to classify certain members of the family Micrococcaceae, and thus far eight groups, A through H, have been reported (1, 4).

It was considered desirable to simplify the testing of the proteinases by eliminating the step requiring electrophoresis of the immune serum. This report describes the use of Rivanol (2) for separating the nonspecific proteinase inhibitor from specific staphylococcal and micrococcal antiproteinases in immune rabbit serum.

MATERIALS AND METHODS

Microorganisms. Six proteolytic strains of Staphylococcus and two proteolytic strains of Micrococcus, classified according to their abilities to produce acid from glucose anaerobically, were used in this study (5). Five strains classified as groups A, B, C, D, and E were obtained from Olav Sandvik, Veterinary College of Norway, Oslo, Norway, and three strains classified as groups F, G, and H were isolated at the National Animal Disease Laboratory.

Enzymes. The proteinases of groups A, B, D, E, and F were produced by growing the organisms in nutrient agar with an agar content of 0.3%. The enzymes were harvested by centrifugation and then treated as described by Sandvik. After dialysis, the solutions were freeze-dried and stored at 4 C.

The proteinases of groups C, G, and H were produced by growing the organisms in a medium described by Nunokawa and McDonald (N-M medium). After centrifugation, the supernatant fluid was decanted and dialyzed against running tap water for 20 to 24 hr. The volume of the supernatant fluid was reduced by pressure filtration with a Diaflo UM-10 membrane (Amicon Corp.) at 4 C with Nz gas. The concentrate was freeze-dried and stored at 4 C. The enzyme titer of the freeze-dried material was determined by the method of Sandvik.

Immunization of rabbits. Concentrated solutions of the enzymes of groups A, B, D, E, and F, produced on 0.3% nutrient agar, were prepared in distilled water and then emulsified in mineral oil containing 3% Arlacel A (Atlas Chemical Industries) or in HP Vehicle (Hamilton Pharmacal Co.), a water-in-oil emulsion. The mixtures were injected subcutaneously into rabbits according to the protocol of Sandvik.

The freeze-dried enzymes of groups C, G, and H, produced in N-M medium, were dissolved in sterile distilled water; 1% thimerosal was added to a final concentration of 1:10,000, and the solutions were held overnight at 4 C. Before inoculation, sterile 30% Ludox HS, colloidal silica (E. I. duPont deNemours & Co.), was added as an adjuvant in the amount of 0.1 to 0.2 ml per ml of enzyme solution (H. Stone, Avian Dis., in press). The mixtures were injected subcutaneously into rabbits weekly until antiproteinases were detected. Sometimes it was necessary to hyperimmunize with intravenous injections of enzyme solutions containing no Ludox HS.

Serum treatment. Naturally occurring serum inhibitors of bacterial proteinases are associated with the alpha and beta globulins. To separate these inhibitors from specific antiproteinases, the sera from immunized rabbits were treated with 0.4% solution
of 2-ethoxy-6,9-diaminacridine lactate (Rivanol), according to the method of Horejsi and Smetana (2). The preparation of Rivanol used in this study was obtained from Winthrop Laboratories under the trade name of Ethodin. Rivanol precipitated all serum proteins except the gamma globulin and part of the beta globulin. The excess Rivanol was removed with activated charcoal (Norit A, Fisher Scientific Co.), which was separated from the solution by centrifugation and sometimes by filtration. The remaining fluid was concentrated to approximately half the volume of the original serum by pressure filtration with a Diaflo UM-10 filter membrane. The concentrated Rivanol-treated antiserum was dispensed in 1-ml volumes and was freeze-dried and stored at −65 C.

**Test for removal of natural inhibitors.** The Rivanol-treated antisera were tested for removal of the natural proteinase inhibitors by use of a Microzone electrophoresis system (Beckman Instruments, Inc.). Untreated serum (1 μl) and Rivanol-treated antiserum (2 μl) were applied to the Microzone cellulose acetate membrane in slots 1 and 6 (untreated serum) and slots 3 and 8 (Rivanol-treated antiserum). Electrophoresis was performed in Vero nal buffer (Spinco B-1), pH 8.6, 0.05 M, at 200 v for 1 hr. The membrane was cut in half longitudinally. One half was stained in Ponceau-S dye fixative to show the proteins. The other half of the membrane was placed on casein agar containing in 12 by 18 cm plates (4). After 3 to 5 hr of incubation at 37 C, the membrane was removed and discarded. Filter-paper strips (Whatman no. 1 or 2), 2 to 4 mm wide and 7 to 9 cm long, were laid over the area of absorbed serum components, and a homologous enzyme was pipetted onto the strip. After development at 37 C overnight, the strips were removed. A zone of casein precipitation appeared along the enzyme-containing strips except for the areas containing antiproteinases, which appeared as clear spaces or marked indentations in the zone of precipitation.

**Routine test for antiproteinase activity.** Freeze-dried Rivanol-treated antiserum, dissolved in sterile distilled water, or thawed Rivanol-treated antiserum was pipetted onto a filter-paper strip which had been previously placed on the surface of caseinate agar in a petri dish (Fig. 2A). After incubation at 37 C for 2 to 4 hr to allow the Rivanol-treated antiserum to be absorbed by the agar, the strip was removed, and another filter paper strip, 2 to 4 mm wide by 3 to 3.5 cm long, containing a proteinase solution (liquified gelatin, etc.) was placed on a perpendicular line across the previously applied Rivanol-treated antiserum, shown by the dotted lines in Fig. 2B. The agar plate was developed overnight at 37 C.

**RESULTS**

The removal of the natural proteinase inhibitor from rabbit antiserum by treatment with Rivanol is shown in Fig. 1. Figure 1A shows the electrophoretic patterns of untreated and Rivanol-treated antiserum on a stained cellulose acetate membrane. Figure 1B shows the electrophoretic patterns for the same untreated and Rivanol-treated antiserum when the other half of the membrane was transferred to casein agar and developed with a casein precipitation enzyme homologous to the antiserum. The specific proteinase is present in the gamma globulin fraction, and the naturally occurring inhibitor is located in the alpha-1 fraction of the serum.

The positioning of the filter-paper strip containing Rivanol-treated antiserum and enzyme on casein agar is illustrated in Fig. 2A and 2B. A positive test in which the homologous antiserum neutralizes the enzyme is shown in Fig. 2C. A negative test, in which no inhibition occurs in the zone of precipitation when a proteinase is tested with a heterologous antisem, is shown in Fig. 2D. By arranging the paper strips containing enzymes as spokes on a wheel, up to eight different enzyme solutions can be tested against one Rivanol-treated antiserum, or one enzyme can be tested against eight different Rivanol-treated antisera.

![Fig. 1. Electrophoretic patterns for an untreated and a Rivanol-treated rabbit antiserum run in Vero nal buffer (Spinco B-1) at pH 8.6, 0.05 M, for 1 hr at 200 v in a Microzone apparatus. (A) Stained cellulose acetate membrane: 1, untreated rabbit antiserum; 2, Rivanol-treated rabbit antiserum. (B) Development of casein agar with an enzyme homolo- gous to the antiserum after removal of the unstained cellulose acetate membrane: 3, untreated rabbit anti- serum; 4, Rivanol-treated rabbit antiserum. AP = line of application; n = natural inhibitor; s = spe- cific antienzyme.](image-url)
FIG. 2. (A) Filter-paper strip for Rivanol-treated antiserum. (B) Filter-paper strip for enzyme (with outline showing where Rivanol-treated antiserum had been placed). (C) Positive test result. (D) Negative test result.

DISCUSSION

Elimination of the electrophoretic step in the serological classification of extracellular bacterial proteinases is desirable. This has been achieved by removing the natural inhibitor from the serum of rabbits immunized against staphylococcal and micrococcal proteinases, by treatment with Rivanol. Thus far, the only natural inhibitor we have found in rabbit serum was in the alpha-1 globulin fraction. Sandvik found up to three zones of inhibition against bacterial casein precipitation enzymes caused by natural inhibitors when rabbit serum was separated by paper electrophoresis, but usually only one was present. The beta globulin fraction was active against the proteinases of certain organisms; however, these were not specified in his report. The difference in results might be attributed to the fact that we studied only proteinases produced by staphylococci and micrococci, whereas Sandvik’s study included organisms from other genera.

Since our final Rivanol-treated immune serum fraction contained some beta globulin, as well as gamma globulin, it would be necessary to determine the presence or absence of natural inhibitors before it could be used to test proteinases of organisms other than staphylococci and micrococci.

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