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 immuno-electrophoretic 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 N2 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% Lodox HS, colloidal silica (E. I. duPont de Nemours & 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 Lodox 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 re-
moved 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 fil-
tration with a Diaflo UM-10 filter membrane. The
concentrated Rivanol-treated antiserum was dis-
persed in 1-ml volumes and was freeze-dried and
stored at 4 C or frozen and stored at -65 C.

Test for removal of natural inhibitors. The
Rivanol-treated antiserum were tested for removal of
the natural protease inhibitors by use of a Micro-
zone electrophoresis system (Beckman Instruments,
Inc.). Untreated serum (1 ml)ter and Rivanol-treated
antiserum (2 mlters) were applied to the Microzone
cellulose acetate membrane in slots 1 and 6 (un-
treated serum) and slots 3 and 8 (Rivanol-treated
antiserum). Electrophoresis was performed in Ve-
ronal 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 contained 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 antiprotein-
ases, 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 anti-
serum to be absorbed by the agar, the strip was re-
moved, 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 Ri-
avol-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 protease 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 cellu-
lose acetate membrane. Figure 1B shows the
electrophoretic patterns for the same un-
treated 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 anti-
serum. The specific protease 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 con-
taining Rivanol-treated antiserum and enzyme
on casein agar is illustrated in Fig. 2A and 2B.
a positive test in which the homologous anti-
serum neutralizes the enzyme is shown in Fig.
2C. A negative test, in which no inhibition
occurs in the zone of precipitation when a pro-
teinase is tested with a heterologous antiser-
um, 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 anti-
serum, or one enzyme can be tested against
eight different Rivanol-treated antisera.
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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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