Immunosuppressive Activity of the 3a-1 Fraction of Papain

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The maximal nonlethal dose of the 3a-1 fraction of papain, determined by use of 9-week-old white albino rabbits, was 10 mg per injection, administered intravenously. The immunosuppressive activity of the 3a-1 fraction of papain was studied by its inhibition of sheep red blood cell hemolysin, bacterial agglutinin, and horse serum precipitin. Immune suppression was observed when papain injections preceded antigen injections by 12 to 18 hr. The enzyme preparation was analyzed by use of cellulose acetate and starch-gel electrophoresis and by the micro-Kjeldahl method. Starch-gel electrophoresis of serum samples revealed qualitative alterations in the α 1 region 7 hr after papain administration. Changes were also observed in the urinary aminopolysaccharide content.

Halpern et al. (4) found that the injection of crystalline papain produced a collagen-like disease. His group observed glycoprotein thesaurosisis in the liver, lesions in the epiphyseal plate of the long bones, and marked osteoporosis in young rabbits after 23 injections of papain during a 70-day period. Further, Morard et al. (8) reported autoantibodies against cartilage, renal papilla, and liver extracts. These autoantibodies caused lesions in the pleura, pericardium, and peritoneum. J. E. Fidler (Ph.D. Thesis, Univ. of Nebraska, Lincoln, 1966) reported that young rabbits given 500 μg of the 3a-1 fraction of papain failed to produce detectable streptococcal anti-M antibodies after exposure to viable streptococci 7 hr later.

Thomas (11) reported that solutions of papain (3a-1) caused ear collapse of rabbits weighing 1 kg. The ear collapse was associated with the depletion of the ear cartilage and disappearance of basophilia from the matrix. The effects of papain (3a-1) were not reproduced by separate injections of crystalline papain protease or crystalline plant lysozyme. The third constituent, chymopapain, of the 3a-1 fraction may be the causative agent.

This paper describes the immunosuppressive activity of the 3a-1 fraction of papain and concomitant findings in the urine and serum of the rabbit.

MATERIALS AND METHODS

Experimental animals. White albino rabbits, 9 weeks old and weighing 1 kg each, were used. They were maintained on a diet of antibiotic-free Purina Rabbit Chow and water ad libitum.

Preparation of 3a-1 fraction of papain. This fraction was prepared by the method of Kimmel and Smith (5). A 180-g amount of dried papaya latex (Wallerstein Co., Staten Island, N.Y.), 100 g of Celite, and 150 g of washed sand were mixed in a Waring Blender with 200 to 300 ml of 0.04 M cysteine (6.3 g of cysteine hydrochloride in 1,000 ml of 0.054 M NaOH).

With the use of the supernatant fluid, the extraction and grinding procedures were repeated with the cysteine solution until a total volume of 1 liter had been used. The resulting suspension was mixed and filtered by use of a 0.5-cm layer of Hyflo Super-Cel (Johns-Manville, New York, N.Y.) on Whatman no. 1 filter paper. The filtrate (fraction 1) was opalescent and greenish-yellow, with a pH of approximately 5.5.

Fraction 1 was adjusted to pH 9.0 with 1 N NaOH. The resulting gray precipitate was removed by centrifugation at 2,600 rev/min for 1 hr. The supernatant fluid (fraction 2) was clear.

Fraction 2 was brought to 0.4 saturation with solid ammonium sulfate (250 g per liter). After 1 to 2 hr at 4 C, the white precipitate (fraction 3) was again centrifuged and the supernatant solution (fraction 3a) was saved. Fraction 3a was brought to 0.8 saturation with ammonium sulfate and allowed to stand overnight at 4 C. The resultant precipitate (fraction 3a-1) was again collected by centrifugation. The material was dialyzed against deionized water until the dialysate was free from NH₄⁺ ions (as detected by Nessler's reagent). The preparation was freeze-dried by use of the Virtis Freeze-Drier (Virtis Co. Inc., Gardiner, N.Y.).

Cellulose and starch-gel electrophoresis of papain. The composition of the 3a-1 fraction was determined by cellulose acetate and starch-gel electrophoresis. The modified method ofMarsh, Jolliff, and Payne (7) was used for starch-gel electrophoresis. The starch
was manufactured by Connaught Medical Laboratories, Toronto, Canada. The concentration of starch was 13.6 g per 100 ml of diluent. Amido Black 10 B was used as the stain.

Cellulose and starch gels were scanned by use of a Photovolt Densitometer (model 542) equipped with a 525-nm filter.

The nitrogen content of the 3a-1 fraction of papain was determined by the Kjeldahl method.

Rabbit tolerance test. The maximal nonlethal dose of the 3a-1 fraction was determined. The end point was that concentration which, when injected intravenously in six consecutive doses, every other day, caused no lethality. A dose of 10 mg per injection fulfilled these requirements.

Urinary aminopolysaccharide determination of papain-treated animals. Urinary aminopolysaccharides were isolated by the method of DiFerrante and Rich (1), and the hexuronamide acid content was determined by the carbazole method of Dische (2). Animals were injected intravenously in the lateral ear vein with 10 mg of the 3a-1 fraction of papain. Urination of the animals was induced by two 50-mg injections of Diamox (acetazolamide, Lederle Laboratories, Pearl River, N.Y.) in the lateral ear vein.

Starch-gel electrophoresis of serum. The same procedure was used as that outlined under cellulose and starch gel electrophoresis of papain.

Identification of serum proteins by starch-gel electrophoresis. Tentative identification of rabbit serum proteins was kindly supplied by Pentex, Inc., Kankakee, Ill.

Determination of the immunosuppressive activity. For the studies of the suppressive effect of the 3a-1 papain fraction upon antibody synthesis, three antigens were used, i.e., sheep red blood cells (SRBC), horse serum, and Salmonella choleraesuis. All rabbits were injected intravenously. Blood was withdrawn after heart puncture.

Thirty-four rabbits were used for the hemolysin series. Seven rabbits were used as controls, and SRBC inoculations were given on day 0 and on days 2, 14, 20, and 28. Bleedings and antibody tests were completed on days 10, 19, 26, 34, and 52. The average titers of these seven sera are shown in Fig. 4A.

Another group of seven rabbits (Fig. 4B) and a third group of eight animals (Fig. 4C) were similarly immunized (same quantity of inoculum and same injection and bleeding schedule). With the smaller group, however, beginning 10 days after the last SRBC injection, five papain injections of 10 mg each were administered every other day for a period of 10 days. With the larger group, the rabbits were given five papain injections of 10 mg each every other day for a period of 10 days, followed by the SRBC injections 10 days after the last papain injection.

For the final papain study with the SRBC antigen, seven rabbits were immunized on day 0 and on days 2, 6, 12, 18, and 36. However, 12 to 18 hr before each SRBC injection (with the exception of the injection on the 36th day), 10 mg of papain was injected. Bleedings and hemolysin determinations were completed on days 9, 16, 20, and 38. Average titers of the seven serum samples are shown in Fig. 4D. A control group of five rabbits was similarly immunized, but with the exclusion of the papain injections (Fig. 4E).

A group of 22 rabbits were used for the bacterial agglutinin studies. Five animals were used as controls and were injected on day 0 and on days 2, 8, 12, 18, 36, and 58. Bleedings and agglutinin tests were completed on days 10, 16, 20, 40, and 62. The titers representing the average of the five rabbit sera are shown in Fig. 5A.

Another group of five animals was similarly immunized, but, 8 days after the fifth antigen injection, five papain injections of 10 mg each were injected every other day for a period of 10 days (Fig. 5B). The final two groups of six rabbits also received the same quantity of antigen and followed the same injection and bleeding schedule. One of the groups, however, received five papain injections of 10 mg each every other day for a period of 10 days, the last papain injection falling 10 days before the first bacterial-antigen injection (Fig. 5C), and the other group (Fig. 5D) received the 10-mg papain injections 12 to 18 hr preceding each bacterial-antigen injection.

A group of 25 rabbits was immunized with horse serum. Six rabbits served as controls, and were inoculated on days 2, 8, 12, 17, and 42. Bleedings and precipitin tests were completed on days 11, 18, 26, and 46 (Fig. 6A). With the same quantity of inoculum and the same injection and bleeding schedule, six rabbits received an additional 10 mg of papain on alternate days after antigen injections (Fig. 6B), six received 10 mg of papain on alternate days before antigen injection (Fig. 6C), and seven were injected with 10 mg of papain 12 to 18 hr before each antigen was injected (Fig. 6D).

Antigens. SRBC were prepared from fresh defibrinated whole sheep blood; the final concentration was 5% (v/v).

Heat-inactivated horse serum was used as the soluble antigen. A 1-ml amount of Merthiolate (1:1,000) was added to 9.0 ml of serum.

The bacterial antigen S. choleraesuis was grown on nutrient agar at 37 C; physiological saline with 5% neutral Formalin was used to flush the bacterial growth from the surface. The final concentration was approximately 1.2 X 10^9 cells per milliliter.

Determination of antibody titer. Hemolysin titer was determined by the procedure of Smadel (9).

Bacterial agglutinin titer was determined by the cross-titration of 0.5 ml of dilutions of bacteria with 0.5 ml of heat-inactivated antisera dilutions. The results represent the reciprocal of the highest dilution of antisera reacting with 5.2 X 10^9 cells per milliliter. Mixtures were incubated for 1 hr at 37 C and overnight at 4 C.

Serum precipitin titer was determined by the cross-titration of 0.5 ml of antigen dilutions with 0.5 ml of heat-inactivated antisera dilutions. Mixtures were incubated for 1 hr at 37 C and overnight at 4 C. The results reflect the reciprocal of the highest dilution of antisera reacting with the 1:4 dilution of antigen. Controls were maintained for all tests.
RESULTS

Electrophoretic and nitrogen analysis of 3a-1 fraction of papain. Analysis of the 3a-1 fraction of papain by cellulose acetate and starch-gel electrophoresis (Fig. 1A) revealed three protein bands. The analysis of the crude papaya latex by Kimmel and Smith (5) was included to show the removal of three other contaminating plant proteins (Fig. 1B). Results of 10 determinations showed the nitrogen content of the 3a-1 fraction to be 12.5 ± 0.5%.

Rabbit tolerance test. Lethal doses of the 3a-1 fraction caused death 2 min after injection. Necropsy revealed hemorrhagia in lungs, heart, arteries, and renal tissue.

Determination of the urinary aminopolysaccharide (APS) content. Urine controls contained approximately 6 to 7 µg of APS per ml during the course of the experiment (Fig. 2). Experimental urines contained 5 to 6 µg of APS per ml during the 10 hr prior to treatment. A 50-mg amount of Diamox was administered at 15 hr prior to treatment, which elevated the APS content fourfold (20 µg/ml). Test animals were injected intravenously with 10 mg of papain at zero-time. At 7 hr, test animals showed an eight-fold increase (43 µg/ml) in APS compared with controls. By 14 hr, the urine APS content was normal.

Starch-gel electrophoresis of serum. Examination of the serum protein of 18 animals 72 hr after the injection of 10 mg of papain indicated that no abnormal proteins were present. Identification of bands was kindly supplied by Pentex, Inc.) However, the serum proteins studied 12 to 18 hr after 10-mg papain injections indicated α-1 lipoprotein and fetuin elevations at 4 and 7 hr. By the 22nd hr, these fractions had returned to normal.

Comparison of results obtained from the urine assay (Fig. 2) and the starch-gel analysis (Fig. 3) between 4 and 7 hr showed fairly good correlation regarding synchrony of appearance of pathologies.

Immunosuppressive activity. The immunological response to the SRBC antigen is presented in Fig. 4A to E. The 12 control animals (Fig. 4A and E) had peak titers of 1:4,000. No apparent titer differences were observed when papain was administered 10 days after the last SRBC injection (Fig. 4B). For example, a 1:1,142 titer was observed with this papain group, whereas the control groups showed titers of 1:1,542 and 1:1,520.
However, a 3-fold titer reduction was observed when the animals received papain 8 to 10 days before antigen inoculation (Fig. 4C), and a 10-fold decrease when papain was given 12 to 18 hr before the antigen (Fig. 4D).

The agglutinin response is indicated in Fig. 5A to D. The control group showed an average peak titer of 1:2,046 (Fig. 5A). Less than a twofold increase in titer (1:1,152) occurred when papain followed the antigen injections (Fig. 5B). The average titers of these rabbits characteristically showed lower bacterial agglutination titers. When compared with the control group, therefore, this small difference was not significant. When papain preceded the antigen injections by 10 days (Fig. 5C) and by 12 to 18 hr (Fig. 5D), 25- and 85-fold titer reductions, respectively, were observed. Suppression of the precipitin antibody was also apparent (Fig. 6A-D), but only with rabbits which received papain 12 to 18 hr prior to the antigen. Peak titers in the control animals (Fig. 6A) occurred after the third horse serum injection (1:149). No significant titer differences were observed.
when the papain injections followed the antigen injections, or when the booster titers (Fig. 6B) were compared with the control-group titers. The two groups gave respective titers of 1:90 and 1:80. Similarly, no appreciable comparative titer differences were noticeable in the series in which papain was injected 10 to 12 days before the antigen injections (Fig. 6C). A 35-fold reduction, however, was again apparent when the papain was injected before the antigen (Fig. 6D).

**DISCUSSION**

The 3a-1 papain fraction was prepared from crude papaya latex. The predominant fractions prepared from papaya latex have been reported to be lysozyme (10), papain protease (5), and chymopapain (3). Fraction 3a-1 injected into our experimental animals contained all three substances, but was particularly rich in chymopapain.

Rabbits injected intravenously with the papain fraction, followed within 12 to 18 hr by antigen inoculations, showed the most marked immunosuppression. Under the experimental conditions outlined, synthesis of hemolysin (SRBC), agglutinin (S. choleraeaus), and precipitin (horse serum) was impaired (Fig. 4D, 5D, and 6D). Furthermore, when papain was given 8 to 10 days after the antigen inoculations, normal antibody development occurred (Fig. 4B, 5B, and 6B). When the series of papain inoculations preceded two of the antigen injections (SRBC and S. choleraeaus) by 10 to 12 days, reductions in antibody titers were noticeable (Fig. 4C and 5C).

Although no adequate explanation is available at present for the effectiveness of the 3a-1 papain fraction in the suppression of the immune response, certain considerations appear to be worth mentioning: e.g., factors relative to the influence of papain upon the antigen, upon complete antibody molecules, and upon antibody-forming cells.

In vitro mixtures of papain, particularly with SRBC and horse serum, would markedly alter the antigens, resulting in complete lysis of SRBC and proteolysis of horse serum proteins. However, we feel that the immunosuppressive effect is not the result of the in vivo action of papain upon the antigens. First of all, the in vivo indexes of papain activity must be considered. The aminopolysaccharide concentration (Fig. 2) and the α-1 lipoprotein and fetuin measurements (Fig. 3) indicate that maximal concentrations occurred 6 to 7 hr after intravenous inoculation. Antigens were introduced 10 to 11 hr after maximal papain activity occurred. Furthermore, previous studies (6) have shown that, after intravenous injection of papain, rather extensive depletion of tracheal, nasal, and auricular cartilage occurs, indicating saturation of these sites with papain. Admittedly, however, definite proof of papain alteration of antigen in vivo is not available.

Our observations also indicate that once antibody was demonstrated against SRBC, S. choleraeaus, and horse serum antigens, five series of intravenous papain injections on alternate days failed to destroy or modify the specificity of the antibodies. The antibody titer of these animals (Fig. 4B, 5B, and 6B) was essentially similar to the controls (Fig. 4A, 5A, and 6A). Furthermore, when the routine hemotoxylin and eosin staining technique was used after paraffin embedding of tissue, examination of experimental rabbit kidneys and spleen tissues (removed 6 to 8 hr after intra-
venous papain injections) exhibited no detectable histological alteration from similar control tissue.

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LITERATURE CITED