Antigenicity of Bovine Cortical Bone. (27268)

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Little has been reported in the past several years on the antigenicity of bone. Bonfiglio, Jeter and Smith(1) demonstrated antigens in an extract of rabbit bone as evidenced by antibody titers in the sera of immunized rabbits. Curtiss, Powell and Herndon(2) failed to find circulating antibodies in rabbits injected with rabbit bone, and Siegrist and Enneking(3) were unable to detect circulating antibodies in hamsters injected with rat femur homogenate.

The experiments reported here were undertaken to explore the antigenicity of bovine cortical bone. Rabbits were immunized with bovine cortical bone and their sera tested for the presence of circulating antibodies to the injected bone.

Methods and materials. Young adult male rabbits weighing 2-3 kg (Albino Farms, Red Bank, N. J.) were used throughout these studies.

Preparation of antigens. a) Whole bovine cortical bone. Cortical bone free of periosteum and marrow, obtained from 175-225 lb Holstein calves, was crushed in an Anderson Bone Mill (The Tower Co., Seattle, Wash.) to a size of .2-.5 mm diameter. Addition of dry ice to the crushed bone kept the pulverization temperature below 30°C. The pulverized cortical bone was sterilized with ethylene oxide. b) Bovine cortical bone extract. An aqueous extract of the pulverized cortical bone was prepared according to the procedure described by Bonfiglio *et al.*(1). The pulverized bone was homogenized with water in a Waring Blendor at 0-4°C for 20 minutes. The mixture was stirred for 18 hours at 4°C, centrifuged, and the supernatant fluid filtered through a Berkefeld candle. The lyophilized aqueous extract of bone contained 2.9% nitrogen.

Immunization. Ten adult male Albino rabbits were immunized by weekly intramuscular injections of 100 mg pulverized bone suspended in 1 ml of Freund's complete adjuvant (Difco) and containing approximately 4 mg of bone nitrogen. Each animal received 24 injections. Ten days following the last injection, the rabbits were bled, the sera collected and stored at –20°C until used.

Serology. Hemagglutination. The antisera were assayed by the hemagglutination reaction according to the procedure of Boyden as modified by Fineberg(4). Sheep red cells were treated with tannic acid and the test antigen adsorbed onto the surface of the red cells. Bovine serum (Nutritional Biochemicals) and the aqueous extract of bovine cortical bone were used as test antigens. Titrations were performed on 2-fold serial dilutions of the rabbit sera and the agglutination patterns read after 2 hours at room temperature. Controls carried out with each experiment included the antigen-treated tanned red cells and normal, untreated red cells. The detailed procedure is identical to that reported by Fineberg(4).

Antibody Absorption. Antibodies to bovine serum and to bovine red cell antigens were absorbed from the rabbit anti-bone sera. Equal volumes of antigen-coated tanned sheep red cells and rabbit anti-bone sera were allowed to react for 2 hours at room temperature. The agglutinated red cells were centrifuged and discarded. The antigens used for absorption were bovine serum, bovine red cell lysate, bovine albumin, bovine gamma globulin and bovine fibrinogen. Absorption of antibodies was considered complete when the serum no longer gave a positive hemagglutination reaction with bovine serum or the bovine red cell lysate, or a precipitin reaction with bovine albumin, bovine gamma globulin or bovine fibrinogen. As additional evidence for complete absorption of antibodies, no precipitin lines developed after 12 days on a Grabar plate when the absorbed serum was tested against bovine serum antigens.

Grabar Immunoelectrophoresis. The number and nature of antibodies in the antisera were determined by the immunoelectropho-

TABLE I. Antigenicity of Bovine Cortical Bone. Demonstration, by the hemagglutination assay, of circulating antibodies in sera of rabbits immunized with bovine cortical bone.

Test sera	Hemagglutination titer: Test antigens: Bovine serum	Red cell lysate	Cortical bone extract
Rabbit anti-cortical bone serum	1:128	1:64	1:32
Idem, absorbed free of bovine serum and red cell antibodies	0	0	1:16

retic method of Grabar(5). This analytical method combines zone electrophoresis in an agar medium with the double diffusion precipitin technic of Ouchterlony. The antigen, bovine cortical bone extract, was placed in the center well of an agar plate and its components resolved electrophoretically (Fig. 1). Electrophoresis was continued for 18 hours with a voltage gradient on the plate of approximately 5-6 volts per centimeter (total potential drop across the 25 × 34 cm plate was approximately 220 volts). The total current per plate was 41 milliamperes. After electrophoresis, the rabbit anti-bovine cortical bone serum was placed in the lower row of wells parallel to the axis of electrophoretic migration and in the upper row of wells was placed the same rabbit anti-bone serum absorbed free of bovine serum and red cell antibodies. The agar plate was stored at room temperature for 12 days during which time the antigen-antibody precipitin lines developed.

Results. It was demonstrated by the hemagglutination assay (Table I) that the sera of rabbits immunized with fresh bovine cortical bone contained antibodies to bovine serum, bovine red cell lysate and to the bovine cortical bone extract. The sera absorbed free of bovine serum and bovine red cell antibodies failed to elicit a response against bovine serum and a bovine red cell lysate but gave definite titers against the cortical bone extract. These studies definitely indicate the presence of antigens in bovine bone unrelated to its serum and red cell content.

The immunoelectrophoretic method of Grabar was used to determine the number and the nature of the antigens involved in the above antigen-antibody reactions. In Fig. 1 may be seen the presence of a number of distinct precipitin lines occurring after electrophoresis as a result of the interaction of components of the bovine cortical bone extract and the rabbit anti-bovine bone serum. When this same rabbit anti-bone serum was absorbed free of bovine serum and bovine red cell antibodies, 2 faint but distinct precipitin lines still remained. This demonstrates the presence of 2 antigens in an aqueous extract of cortical bone separate and distinct from the serum and red cell content of that bone. It has not been established whether these antigens are "true bone antigens" derived from the osteocyte or the collagen of bone. It is possible that leucocytes, blood vessels and nerves present in bone could contribute to its antigenicity. Although the relative amounts of antibodies were not determined quantitatively, it is apparent from the hemagglutination and the immunoelectrophoretic data that the major antigenicity of the cortical bone is in its serum and red cell content.

Discussion. The nature of the antigens involved in graft rejection is open to question. Evidence has been published by Billingham, Brent and Medawar(6) that the critical transplantation antigens are present in the nuclear fragments. Careful experiments by these same authors(7) have shown that deoxyribonucleic acid itself has no antigenic ac-

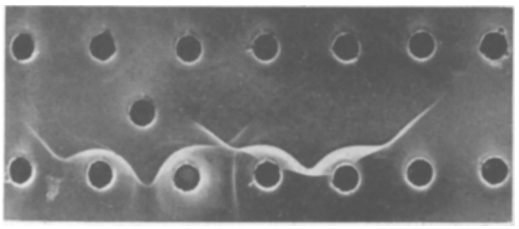

FIG. 1. Grabar immunoelectrophoresis. Center well, bovine cortical bone extract. Lower row of wells, rabbit anti-bovine cortical bone serum. Upper row of wells, rabbit anti-bovine cortical bone serum absorbed free of bovine serum and bovine red cell antibodies.

tivity and that "the substances responsible for the specificity of the transplantation antigens are probably similar to the blood group mucoids". There is an indication that the blood group A and B antigens are involved in the sloughing of homogenous skin grafts and clouding of corneal transplants(8). The presence of these antigens in human cancellous bone and their absence from human cortical bone has been observed by Weinberg, Makin, Nelken and Gurevitch(9). Curtiss, Chase and Herndon(10) showed that a blood group A antigen-antibody interaction had no demonstrable effect on the acceptance of an iliac bone graft. Thus the successful transplantation of bone, in contrast to soft tissues, occurs even in the presence of known antigen-antibody interaction.

Cortical bone is relatively acellular and reports of apparently successful transplants made with autogenous and homogenous bone (11,12) would suggest the low order of antigenicity of bone tissue. Therefore, it is not surprising that most of the antigenicity of cortical bone observed in the present study comes from its blood content rather than from its cellular composition. If the antigens of bovine serum and bovine red cells could be removed from the bovine cortical bone, it is possible that an immunologically unreactive bone could be made available clinically for heterogenous bone transplantation.

Summary. Rabbits were immunized with finely ground, pulverized bovine cortical bone. It was demonstrated that the antibodies in the sera of these rabbits reacted with the bovine serum and the bovine red cell antigens present in the bone; however, after absorbing from these rabbit sera the bovine serum and bovine red cell antibodies, hemagglutination titers of low order still persisted. Grabar immunoelectrophoresis confirmed the results obtained by the hemagglutination assay and indicated the presence of 2 distinct antigens in bovine cortical bone unrelated to its serum and red cell content.

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Hypersensitivity to Chemical Allergens I. Use of Allergen-Erythrocyte Conjugates in Detection of Antibodies.* (27269)

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Despite the many advantages afforded by the use of simple chemicals in studies of hypersensitivity, critical investigations have been limited by the absence of accurate and sensitive methods for ascertainment of small amounts of antibody. Most reports(1,2,3) concerned with proof of antibody production to chemical allergens (*e.g.*, picryl chloride, citraconic anhydride, etc.,) have been based on results obtained by *passive cutaneous anaphylaxis* (PCA). While this method is presently considered more sensitive for detection

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