Staphylococcal Immunity: Production of Staphylococcal Hemagglutinins in Rabbits Receiving Staphylococcal Vaccine.* (25501)

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Patients with focalized staphylococcal disease often fail to demonstrate significant humoral antibody against staphylococci or staphylococcal products. Neter and his associates have recently demonstrated that hemagglutination technics are of sufficient sensitivity to allow detection of antistaphylococcal antibody in human gamma globulin and to a lesser extent in pooled human sera (1). The present study was undertaken to determine the nature and extent of serologic response of rabbits to a staphylococcal bacterial cell vaccine, utilizing a hemagglutination test for measurement of serum antibody. The vaccines employed, the methods evolved for performing hemagglutination tests, antigens used in the procedure, and the nature of antibody response are reported here.

Materials and methods, vaccines. The Giorgio strain of staphylococcus was used for preparation of the 2 vaccines employed.‡ *One vaccine* was prepared from surface growth obtained on 15 cm meat infusion agar plates heavily seeded with 0.85% saline suspension prepared from 18 hour culture of staphylococci. Following incubation for 18 hours at 37°C, 10 ml saline was added to each plate, and the thick suspension obtained was pooled and centrifuged at 1200 rpm for 1 hour. The sedimented microorganisms were twice washed with 70 ml saline and resuspended in 200 ml saline. The resultant homogeneous suspension containing approximately 10 billion microorganisms/ml was placed in 60 ml rubber stopper bottles and sterilized by heating in 60°C. water bath for one hour on 3 separate days. The pH of final vaccine was 6.9. *A second vaccine* was prepared from 4 hour cultures. Culture plates similarly seeded were harvested after 4 hours incubation at 40°C. The growth obtained was removed in 125 ml of hemagglutinin buffer (Difco pH 7.3) and well shaken in Erlenmeyer flask containing glass beads. The bacterial mass obtained from such young cultures was viscous and slimy, which made washing difficult. After centrifugation, most of supernatant was removed, and an attempt made to produce a homogeneous suspension from the glutinous material by 2 additional washings with 120 ml buffer. The resultant volume of bacterial cells was suspended in 175 ml of buffer in 500 ml Erlenmeyer flask, and the preparation heated at 60°C for one hour with constant agitation. During this heating, the viscosity gradually disappeared. This preparation was pipetted into 60 ml bottles, shaken, and reheated at 60°C for 15 minutes to attain sterility. The pH of final vaccine was 7.3. *Immunization of rabbits.* Male albino rabbits weighing 4 to 5 kilos were used. Animals were housed in individual cages and received rabbit pellets, lettuce, carrots, and water *ad lib.* In initial experiments, 4 rabbits were injected at intervals of 7 to 10 days with increasing amounts of 18 hour vaccine (0.2 to 1.0 ml). A different route of administration (intravenous, subcutaneous, intraperitoneal, and intradermal) was used in each rabbit using equal total quantities of vaccine. A second series of 6 rabbits were also injected by various routes with increasing doses of 4 hour vaccine. The first injection was 0.5 ml, each succeeding injection increasing by 0.5 ml to sixth and final injection of 3 ml. Two rabbits received vaccine intravenously, 2 subcutaneously, and 2 intraperitoneally. Rabbits were bled from the ear prior to first injection and at appropriate intervals thereafter. Sera were

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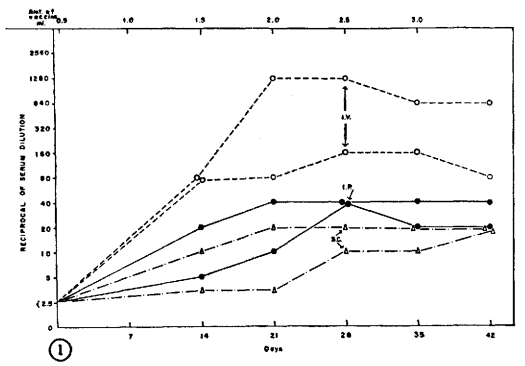
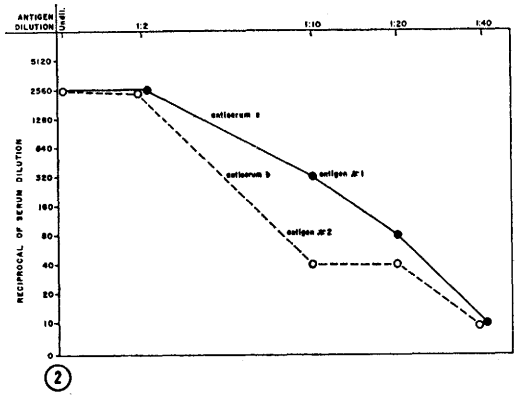
‡ This phage group III is lysed by bacteriophages 47/53/54/75/VA4, and has been used extensively in experimental studies within this institution(2).

stored at −20°C and inactivated at 56°C for 30 minutes before use. *Staphylococcal antigens.* Four different antigenic preparations were prepared from the Giorgio strain grown as already described. Antigen A was prepared from 18 hour vaccine used for immunization of the first group of rabbits. The vaccine was centrifuged at 2,000 rpm for 2 hours and the slightly cloudy supernatant was used directly as the antigen. Antigen B differed only in that the washed staphylococci were killed by boiling for 1 hour prior to centrifugation. Antigen C was prepared from 4 hour cultures. The bacteria were recovered from plate surfaces in buffer, shaken in an Erlenmeyer flask without washing, and heated to 60°C for 15 minutes. Following centrifugation at 2,200 rpm for 2 hours, the sterile yellow supernatant obtained was utilized as antigen. Antigen D: Three separate crude polysaccharide preparations were made from the Giorgio strain utilizing the method of Keogh, North, and Walburton(3). The first and most potent preparation was prepared from 18 hour cultures. After centrifuging to remove a fine precipitate, the final product was dissolved in Hanks' solution. This crude polysaccharide was slightly opalescent. The other 2 preparations were prepared from twice washed 4 hour cultures, and the gelatinous sediments were used for preparation of polysaccharide antigen. These final products dissolved in buffer solution were water clear after centrifugation. Bacterial antigens were also prepared from 5 heterologous strains of heat killed coagulase positive staphylococci in the same manner as outlined for antigen B. These microorganisms had all been isolated from human infections, produced yellow pigment, hemolysins, and fermented mannite. They belonged to each of the 4 phage groups and were lysed by the following bacteriophages: Strain 1, 29; Strain 2, 55/3A/3B/3C; Strain 3, 6; Strain 4, 42B; Strain 5, 80/81. In addition, one coagulase negative strain (MAM), which has been utilized extensively in our other studies(4), was similarly utilized in production of antigen. *Hemagglutinin tests.* Polyphil sheep red cells§ were washed 3 times with saline or Difco hemagglutinin buffer. (In the first experiments, saline was used to wash and suspend the red cells, subsequently only buffer was used.) To washed packed red cells in Erlenmeyer flask, undiluted antigen was added sufficient to make a 2.5% or 1.25% red cell suspension. The suspension was thoroughly shaken and incubated at 37°C for 40 minutes. The modified red cells thus obtained were centrifuged, washed 3 times with 10 ml buffer, and resuspended in 9.75 ml of buffer. In experiments in which 1.25% red cells were utilized, an aliquot of the modified suspension of 2.5% red cells was diluted 1 to 2 with buffer to obtain a 1.25% red cell concentration which had been exposed to the same amount of antigen. Serums from normal or immunized rabbits were serially diluted with buffer (or saline) and 0.2 ml of each dilution pipetted into 7.5 × 0.8 cm agglutination tubes. 0.2 ml of washed 2.5% or 1.25% modified red cells was added to each tube and tubes immediately mixed. All tubes were incubated at 37°C for 40 min. The resulting agglutination reactions were read grossly after centrifugation at 1000 rpm for one minute. *Controls* of modified red cells and buffer, normal red cells and buffer, and inactivated immune sera and normal red cells were included in each test. Control tubes containing normal red cells and buffer were consistently negative. Six of 10 sera obtained from 10 normal rabbits showed hemagglutination at a dilution of 1:2, all sera were consistently negative at greater dilution. Conversely, 2 of 6 immune sera titrated with normal red cells showed hemagglutination in the initial 1:2 tube, but all were negative at higher serum dilution.

Results. 1. *Characteristics of hemagglutinin response to vaccination.* Rabbits receiving staphylococcal bacterial cell vaccines prepared from 4 to 18 hour cultures responded to immunization in a predictable manner. Hemagglutinins were generally detectable at initial bleeding 14 to 22 days following start of weekly vaccinations, rising to maximal titers at 30 to 40 days. Hemagglutinin titers then remained relatively constant or declined slowly over 42 to 98 days observation, despite

§ Obtained weekly from Carworth Farms, New City, N. Y.





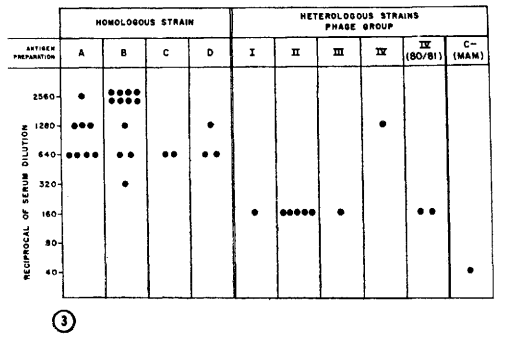


FIG. 1. Hemagglutinating antibody response to staphylococcal vaccine.

FIG. 2. The influence of antigen dilution on the detection of hemagglutinating antibody.

FIG. 3. Hemagglutination titers obtained with different antigen preparations tested against a single high titer serum.

weekly injections of increasing quantities of vaccine.

In general, the intravenous route appeared to provoke a swifter hemagglutinin response which rose to higher levels than was seen following vaccination by intracutaneous, subcutaneous, or intraperitoneal routes. One experiment in which 6 rabbits were injected weekly *via* different routes with increasing amounts of 4 hour bacterial cell vaccine is pictured in Fig. 1. Hemagglutinin titers recorded represent serum dilutions at which 2+ or greater hemagglutination reactions were observed.

2. *Effect of variations in concentration of sheep erythrocytes on hemagglutinin titer.* In experiments performed against a serum of high titer it was found that 1.25% red cell concentration yielded optimal results. End-point titers were consistently 1 or 2 tubes higher utilizing this concentration of cells than those obtained with a standard 2.5% red blood cell suspension.

3. *Influence of antigen concentrations of hemagglutinin titer.* Variations in concentration of antigen used in sensitizing red blood cells produced marked differences in subsequent demonstration of hemagglutinin. Dilutions of antigen greater than 1:2 consistently reduced sensitivity of the hemagglutinating test procedure. A typical experiment is pictured in Fig. 2. Progressive dilution of 2 different antigens resulted in progressive decline in apparent antibody titer from 1:2560 to 1:10 when sensitized red cells were subsequently tested against 2 sera of high titer. Undiluted antigen was thus employed in standard test procedure.

4. *Influence of variations in antigen on detection of hemagglutinin.* Hemagglutination reactions obtained with 4 different Giorgio antigen preparations indicated that, in general, antigens prepared from supernatants from heat-killed 18 hour cultures produced hemagglutinin titers of the same order (A and B, Fig. 3). Antigens prepared from heat-killed 4 hour cultures (C, Fig. 3) and crude polysaccharide preparations (D, Fig. 3) produced a 2 to 4 fold reduction in apparent end point titers, but these differences were not of statistical significance and suggested that these preparations contained similar antigenic products.

Use of supernatants from living 4 hour cultures consistently produced hemolysis of red blood cells, thus rendering such antigens unsatisfactory for test procedure. In contrast, no hemolysis was obtained when 18 hour culture supernatants were employed.

Study of this phenomenon suggested that

such hemolysis resulted from presence of staphylococcal exotoxin in young culture preparations. Four hour cultures were consistently of lower pH (7.4) than 18 hour cultures (pH 8.2 to 8.4), suggesting that exotoxin was inactivated by progressive alkalinization of older cultures. Growth of staphylococci in 20% CO_2 for 18 hours with maintenance of pH 7.4, known to increase the yield of staphylococcal exotoxin(5,6), resulted in striking hemolysis of red blood cells when such 18 hour cultures were used for sensitization. Thus production of a satisfactory antigen required either prolonged growth or heating to inactivate the hemolysin produced by growing staphylococci.

Antigens prepared from 5 heterologous strains of coagulase-positive staphylococci yielded, with one exception, definite but significantly lower titers of hemagglutinating activity against a standard high titer serum. The single coagulase negative strain showed a low but definite cross reactivity. These results are portrayed in Fig. 3.

Discussion. Studies on staphylococcal immune mechanisms in the past have been hampered by lack of a test procedure of sufficient sensitivity to allow detection of antibody response to staphylococcal infection or staphylococcal vaccine preparations. Our experiments indicate that variations in concentration of red blood cells or antigens employed in the procedure produced striking alterations in results obtained, rendering careful standardization of the test procedure necessary for reproducible results. Similarly, staphylococcal exotoxin elaborated by certain strains required inactivation by heat or prolonged growth to prevent interfering hemolysis in the test system. The hemagglutination test appears to offer promise as a sensitive system for detection of antibody substances produced in response to certain staphylococcal products.

Summary. 1. Injections of 2 staphylococcal bacterial cell vaccines resulted in prompt antibody response in normal adult rabbits which could be detected by a simple hemagglutination test. Maximal antibody titers were reached in 30 to 40 days with weekly injections. The intravenous route yielded maximal antibody production. 2. Variations in concentration of red blood cells or antigen employed in sensitizing red cells produced marked alterations in the hemagglutinating end point when tested against standard high titer sera. Careful standardization was thus required to obtain reproducible results. 3. Sensitizing antigen prepared from various heterologous coagulase positive staphylococci of different phage types yielded cross reactions at lower dilutions against sera obtained from vaccinated animals. Antigen prepared from a single coagulase negative strain cross reacted with high titer serum only at low dilutions.

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