INFECTION AND IMMUNITY, Oct. 1978, p. 52-56
0019-9567/78/0022-0052$02.00/0

Vol. 22, No. 1
Printed in U.S.A.

Effect of Immunization on Susceptibility to Experimental *Streptococcus mutans* and *Streptococcus sanguis* Endocarditis

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Received for publication 24 April 1978

It has been asserted that humoral immunity is an important potentiating factor in pathogenesis of infective endocarditis, in that prior immunization to certain bacteria may predispose the host to endocarditis caused by those organisms. If so, possible future vaccination of humans with streptococcal antigens for the prevention of dental caries might increase the susceptibility of the population to streptococcal endocarditis. To examine this hypothesis further, we immunized rabbits with killed *Streptococcus sanguis* or *Streptococcus mutans*. After complement-fixing antibody had developed, the rabbits were tested for susceptibility to experimental infective endocarditis. Rabbits with high titers of complement-fixing antibody to the infecting organism developed streptococcal endocarditis less often (13%) than animals with lower titers (69%; $P < 0.0002$). These findings do not support the hypothesis that pre-immunization predisposes to infective endocarditis and lend no credence to the concept that vaccination of human subjects against dental caries might increase their susceptibility to streptococcal endocarditis. On the contrary, the results of these experiments indicate that specific antibody can confer relative immunity to infective endocarditis.

Results of several experimental studies indicate that immunization against *Streptococcus mutans* may offer protection against dental caries (2, 6, 11, 19). Accordingly, attempts to develop an effective vaccine for prevention of caries in humans are now in progress. If such a vaccine could be produced and proved to be effective, it is likely that community-wide administration (at least to children) would be recommended. However, the safety of any vaccine must be established before extensive prophylactic programs can be undertaken. The possibility that immunological cross-reactions existing between *S. mutans* and heart muscle could cause rheumatic fever or a related disease process has been examined elsewhere (20). Another possible undesirable effect that must be considered in this context is that vaccination against alpha-hemolytic streptococci paradoxically may predispose the recipient to develop streptococcal endocarditis.

Specific antibodies often protect the host against infection with the homologous organism. That the opposite may be true for infective endocarditis has been suggested repeatedly since 1919, when Wadsworth (21) observed that horses inoculated with pneumococci to raise antipneumococcal sera sometimes developed pneumococcal endocarditis. Others (12, 16) confirmed Wadsworth's observation, which was interpreted as providing evidence that pre-immunization predisposes to infective endocarditis. This view has been accepted fairly widely (8, 23). For example, Weinstein and Schlesinger (23) listed prior immunization to the infecting organism as one of the four prerequisites for establishment of a subacute endocardial infection, along with a cardiac abnormality causing turbulent blood flow, a sterile platelet-fibrin thrombus, and bacteremia.

Alpha-hemolytic (viridans) streptococci cause approximately half of all cases of bacterial endocarditis (5, 22). *S. sanguis*, the species most often isolated, accounts for about 50% of cases of endocarditis caused by viridans streptococci. *S. mutans* is next in frequency, causing about one-fifth of cases (7). If prior immunization with an organism actually increases susceptibility to endocarditis caused by that organism, the safety of vaccines derived from *S. mutans* inevitably would be questioned, especially if the vaccine also raised cross-reacting antibody to related species such as *S. sanguis*.

Answers to these questions cannot be obtained from human studies at present. Because an animal model offers the only in vivo alternative, we investigated the influence of prior immunization with two species of alpha-hemolytic streptococci on the susceptibility of rabbits to experimental streptococcal endocarditis.

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MATERIALS AND METHODS

Organisms. Two species of alpha-hemolytic streptococci were used. R. T. Evans of the State University of New York at Buffalo supplied *S. mutans* strain 6715, which has been used extensively in studies of immunization against dental caries (6, 19). The second organism was a strain of *S. sanguis* serotype 2 (NCTC 7864), originally obtained from the blood of a patient with endocarditis and employed in previous studies from this laboratory (4, 17).

Immunization of rabbits. To prepare streptococcal antigen, an overnight culture of streptococci in Trypticase soy yeast broth was killed by addition of 0.5% Formalin. After subculture to confirm that no organisms remained viable, the suspension was centrifuged at 1,000 × *g* for 10 min and washed three times in normal saline. The killed streptococci were then suspended in saline at an optical density of 0.5 at 540 nm on a Coleman Junior spectrophotometer and stored at −70°C. Portions of this suspension were thawed and emulsified with equal volumes of Freund complete adjuvant; 1.0 ml was injected subcutaneously over each shoulder of male New Zealand White rabbits weighing 1.5 to 2.0 kg. Subsequently, 0.1 ml of killed streptococci was injected intravenously into each animal at 2-week intervals for a total of at least 6 weeks. The rabbits were bled at intervals; sera were separated and stored at −70°C.

Test for CF antibody. Rabbit sera were coded and tested for complement-fixing (CF) antibody without prior knowledge of the rabbits' history of immunization. Standardized suspensions of heat-killed *S. mutans* and *S. sanguis* were prepared in isotonic veronal buffer containing 0.0005 M $MgCl_2$, 0.00015 M $CaCl_2$, and 0.1% bovine serum albumin. Guinea pig complement was used at a concentration of 2.5 50% hemolytic complement units per ml. Sheep erythrocytes were sensitized in standard fashion (14) and then diluted 1:7. The complement fixation test was performed with microtiter plates and microdiluters (Cooke Engineering Co., Alexandria, Va.) as follows. A 25-μl amount of veronal buffer was added to all the wells of the microtiter plate. Next, 25 μl of each rabbit serum was added to the first well in each row, from which serial twofold dilutions were made. A 25-μl amount of guinea pig complement was then added to each well, followed by 25 μl of either *S. mutans* or *S. sanguis* suspension. Appropriate controls for antigen, antibody, complement, and sheep erythrocytes and for hemolytic activity of rabbit sera were included for each test. The test reactants were mixed in the wells by gently tapping the microtiter plates and were incubated at 4°C for 18 h, after which 25 μl of sensitized sheep erythrocytes was added to all the wells. The plates were incubated at 37°C for 20 min, and the sheep erythrocytes in the wells were allowed to settle. The titer of CF antibody was recorded as the dilution of serum in the last well that contained settled erythrocytes.

Test for susceptibility of endocarditis. Male New Zealand White rabbits weighing 1.5 to 2.5 kg were made susceptible to endocarditis by placing a polyethylene catheter into the left side of the heart, as described previously (4, 17). Between 24 and 72 h after placement of the catheter, living streptococci were injected intravenously. Because alterations in host susceptibility to infection should be easiest to detect near the 50% infective dose, inocula were chosen accordingly. The 50% infective dose for this strain of *S. sanguis* in the rabbit model is approximately 10^5 colony-forming units (17). Preliminary experiments with *S. mutans* 6715 indicated that the 50% infective dose for that strain was approximately 10^7 colony-forming units. The two species of streptococci were grown overnight in Trypticase soy yeast broth, spun down, and washed twice in normal saline. The suspension was adjusted to an optical density of 0.3 ± 0.05 at 540 nm and diluted with saline to give the desired inoculum. The number of organisms actually injected in each experiment was determined by incorporating 0.5 ml from 10-fold dilutions of the inoculum with sheep blood in Columbia (Difco) agar pour plates and counting colonies after incubation at 37°C for 36 to 48 h. The mean inoculum of *S. sanguis* was 3.0 ± 3.1 (standard deviation, $n = 8$) × 10^5 colony-forming units and of *S. mutans* was 9.6 ± 5.5 (SD, $n = 5$) × 10^6 colony-forming units.

Three to 5 days after inoculation with streptococci, the animals were killed, and the vegetations were excised and homogenized in Trypticase soy yeast broth. Vegetations were cultured to determine whether they contained alpha-hemolytic streptococci by incorporating 0.5-ml aliquots of 10-fold dilutions of the homogenized vegetation in blood agar pour plates, as described previously (17). Colonies were counted after incubation of the plates for 36 to 48 h at 37°C.

Statistical analysis. Because the CF antibody titers for rabbit sera had been determined by doubling dilutions, the results were converted to $\log_2$, and the arithmetic mean, standard deviation, and degrees of freedom were calculated for each group. The mean titers for each group were then compared by using Student's *t* test. The incidence of endocarditis in normal rabbits was compared with the incidence in immunized rabbits by chi-square analysis in 2 × 2 tables.

RESULTS

Antibody titers in normal rabbits. The mean CF antibody titer against *S. sanguis* and *S. mutans* in the serum of normal rabbits was slightly higher than the anticomplementary titer of the same sera ($P < 0.01$ and $P < 0.05$, respectively; Table 1). This finding is consistent with the presence of a low level of preexisting "natural" antibody to these organisms.

Results of immunization. Sera obtained from 12 rabbits at 2, 3, and 4 weeks after immunization with *S. mutans* were tested for CF antibody. Between 2 and 3 weeks, there was a sharp rise in CF antibody titer against the homologous organism, while a small but significant rise in titer against *S. sanguis* was also observed. In this group of 12 animals, the anticomplementary titer did not rise significantly after immunization (Fig. 1). Subsequently, sera drawn 4 to 10 weeks after immunization with *S. sanguis* (48 rabbits) or with *S. mutans* (76 rabbits) were compared with sera from 95 nonimmunized rab-

TABLE 1. *CF antibody titers in normal rabbits and rabbits immunized with S. sanguis or S. mutans, expressed as mean $\log_2$ CF titer ± standard error of the mean*

Rabbit group	CF titer to *S. sanguis*		CF titer to *S. mutans*		Anticomplementary titer	
	Mean ± SEM[a]	*P* value vs normal	Mean ± SEM	*P* value vs normal	Mean ± SEM	*P* value vs normal
Normal (n = 95)	4.21 ± 0.10[b]		4.07 ± 0.08[c]		3.87 ± 0.07	
Immunized with *S. sanguis* (n = 48)	7.42 ± 0.16	<0.001	4.40 ± 0.11	<0.01	4.19 ± 0.12	<0.01
Immunized with *S. mutans* (n = 76)	5.46 ± 0.15	<0.001	8.00 ± 0.12	<0.001	4.54 ± 0.11	<0.001

[a] SEM, Standard error of the mean.
[b] Higher than anticomplementary titer, $P < 0.01$.
[c] Higher than anticomplementary titer, $P < 0.05$.

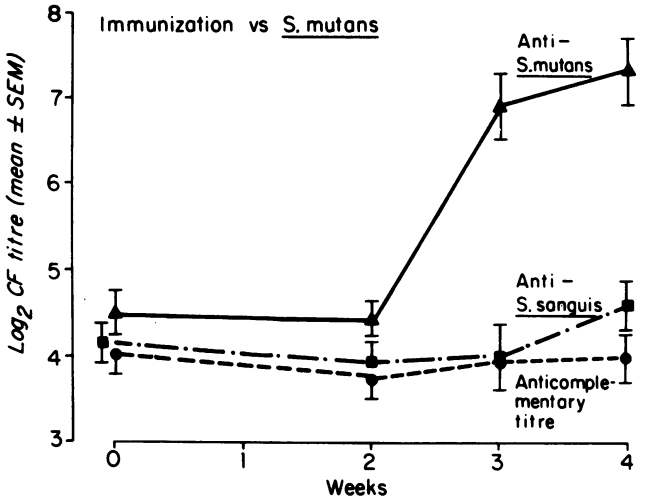

FIG. 1. *Results of immunization of rabbits with S. mutans, expressed as $\log_2$ CF titer to S. mutans and S. sanguis and anticomplementary titer at 0, 2, 3, and 4 weeks. SEM, Standard error of the mean.*

bits (Table 1). Immunization resulted in a 32- to 64-fold increase in titer of specific CF antibody against the homologous organism. There was a smaller but significant increase in titer against the other species of streptococcus in each case, consistent with some cross-reaction with the related species. A significant rise in anticomplementary titers also occurred, consistent with the formation of circulating immune complexes, possibly consisting of antibody and streptococcal antigen (Table 1).

Susceptibility of normal and immunized rabbits to endocarditis. Forty-two of 71 normal rabbits (59%) developed *S. sanguis* endocarditis, compared with 29 of 73 rabbits previously immunized with this organism (40%; Table 2). This difference was significant at a probability level of less than 0.05; immunization with *S. sanguis* therefore appeared to confer some protection against endocarditis in this model. However, the group of rabbits immunized with *S. mutans* developed endocarditis with the same frequency as normal animals (49 and 50%, respectively; Table 2).

Not all of the immunized animals developed high titers of antibody. Rabbits with CF titer less than 1:512 (including both normal rabbits and those immunized rabbits that did not develop high CF titers) developed streptococcal endocarditis more often (69%) than rabbits with CF titers of 1:512 or higher (13%, $P < 0.0002$; Table 3). The protective effect of a CF titer of ≤1:512 was significant for both *S. sanguis* and *S. mutans* ($P < 0.03$ and $P < 0.02$, respectively; Table 3).

DISCUSSION

Early studies of the immune response to bacterial endocarditis were stimulated by the hope that treatment with antisera might benefit patients with this disease. CF, opsonic, and agglutinating antibodies to the infecting bacteria were detected in sera from patients with infective endocarditis (9, 10, 13); titers rose as the disease

progressed (18). In 1962 Williams and his colleagues (24) discovered that tests for rheumatoid factor were positive in approximately 50% of patients with subacute bacterial endocarditis. Rheumatoid factor increased in frequency after 6 weeks of illness and reverted to normal after successful treatment (15, 24). Recently, Bayer et al. demonstrated circulating immune complexes in 97% of sera from patients with infective endocarditis; these complexes disappeared after treatment (1). It appears, then, that titers of both specific and nonspecific antibodies are likely to rise during the course of infective endocarditis and to decrease after cure.

In addition to these recognized responses, preexisting antibody may play a special role in the pathogenesis of endocarditis. Kerr (8) wrote that the presence of "a high degree of immunity... to cause localization of the bacteria on the endocardium" was one of three major factors leading to development of subacute endocarditis. More recently, Weinstein and Schlesinger listed "a high titer of agglutinating antibody for the infecting organism" as one of four main predisposing factors responsible for initiation of subacute endocarditis (23).

The conclusions reached by these authors appear to be based partly upon theoretical considerations and partly upon reports of endocarditis occurring in immunized animals. In theory, organisms circulating in the blood stream might be agglutinated by preexisting antibody, perhaps facilitating their adherence to valves, and raising the inoculum size at that site (23). Alternatively, circulating bacteria might be opsonized by preexisting antibody, leading to their uptake by phagocytes situated on the endocardium. We are unaware of any direct evidence indicating that any of these mechanisms actually plays a part in pathogenesis of endocardial infection. Neither is there reason to believe that the antibodies observed in patients with endocarditis reflect preexisting high levels of immunity rather than a normal response to infection. Indeed, some patients had no demonstrable humoral response to infection, even though subacute endocarditis had been present for many weeks (10, 15, 24).

Reports of infective endocarditis occurring in immunized animals (12, 16, 21) have been cited as direct evidence supporting the contention that prior immunization predisposes to endocarditis (8, 23), but critical review of these reports raises some doubts. Wadsworth initiated this hypothesis when he observed that seven of eight horses immunized with killed pneumococci for the purpose of raising antisera, and subsequently inoculated repeatedly with live organisms, had vegetative endocarditis at necropsy (21). Unfortunately, no nonimmunized control animals were available. Mair produced pneumococcal endocarditis in 7 of 10 rabbits by injecting live organisms after pre-immunization with killed pneumococci, again without reported controls (12). Wright was unable to confirm this finding in a larger group of rabbits; endocarditis developed in only 6 of 58 animals in his series (25). Miller, following a suggestion by Wadsworth, injected live meningococci into horses previously immunized with killed meningococci for production of antisera. Only 14 of 110 horses developed meningococcal endocarditis (16). The proportion of infections achieved by Wright and by Miller was not significantly higher than the rates of infection that have been achieved by injecting bacteria intravenously into normal animals (3).

Critical examination of the evidence from experimental animals, therefore, does not substantiate the hypothesis that a preexisting high level of humoral immunity is required for the development of infective endocarditis. We conclude that a significant role for antibody in predisposing the host to develop infective endocarditis can be neither proved nor disproved by any previous evidence available from the literature.

The present study investigated the influence

TABLE 2. *Effect of immunization on susceptibility to experimental endocarditis*

Organism	Rabbits	No. infected/total	% Infected	χ^2	P
S. sanguis	Normal	42/71	59	4.69	<0.05
	Immunized	29/73	40		
	Total	71/144	49		
S. mutans	Normal	19/38	50	0.01	NS[a]
	Immunized	21/43	49		
	Total	40/81	49		

[a] NS, Not significant.

TABLE 3. *Susceptibility to experimental endocarditis related to CF titer against the infecting organism*

Organism	Rabbits	No. infected/total	% Infected	χ^2	P
S. sanguis	<1:512	63/90	70	5.00	<0.03
	≥1:512	1/6	17		
S. mutans	<1:512	16/25	64	5.44	<0.02
	≥1:512	1/9	11		
Total	<1:512	79/115	69	15.04	<0.0002
	≥1:512	2/15	13		

of preexisting humoral immunity on susceptibility to infective endocarditis in an experimental model. Rabbits immunized with *S. mutans* developed CF antibody, and lower levels of cross-reacting antibody to a related streptococcus. Although the incidence of *S. mutans* endocarditis was the same in the total immunized group as in normals, a small subgroup with CF titer of 1:512 or higher was significantly less likely to develop endocarditis than animals with lower titers. Immunization with *S. sanguis* resulted in a significant protective effect for the whole group; protection was even greater in that subgroup with CF titers of 1:512 or higher.

The results of this study do not substantiate the concept that preexisting antibody predisposes to infective endocarditis and by extension do not support the thesis that immunization against *S. mutans* for prevention of dental caries might increase susceptibility of the population to infective endocarditis caused by *S. mutans* or other viridans streptococci. On the contrary, high levels of CF antibody to streptococci conferred relative immunity to endocarditis in this experimental system.

It seems unlikely that immunization of patients with valvular heart lesions to prevent endocarditis will ever be feasible, because the disease may be caused by any one of a variety of organisms. However, if vaccines derived from streptococci should come into general use in the future, it will be interesting to study the effect of vaccination on the incidence of streptococcal endocarditis in patients at risk.

ACKNOWLEDGMENTS

We thank Karen Peterson, Charles Wick, and Mike Edwards for technical assistance.

This investigation was supported by Public Health Service grant HL 17751 from the National Heart and Lung Institute.

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