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Analysis of Cross-Protection between Serotypes and Passively Transferred Immune Globulin in Experimental Nutritionally Variant Streptococcal Endocarditis

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Nutritionally variant streptococci (NVS), which account for 5 to 10% of all cases of streptococcal endocarditis, were recently subdivided into three serotypes. In the past, using a rabbit endocarditis model, I demonstrated that by immunization of rabbits with NVS and by challenge with a strain from the homologous serotype a level of 90 to 100% protection was elicited. In the present study, the level of cross-protection between strains from different serotypes was measured. No cross-protection was demonstrated between serotype I and II or III strains. However, significant cross-protection was observed when serotype II and III strains were analyzed in the model. Since high levels of immunoglobulin G were demonstrated against the surface of the NVS after immunization, passive transfer experiments were initiated. Even at comparable levels of surface immunoglobulin G, none of the rabbits given immune globulin were protected against challenge with a dose of live NVS that equaled 20 times the 50% infective dose. Therefore, it appears that components from the immune system, in addition to humoral components, are required for active protection against NVS endocarditis.

The rabbit endocarditis model devised by Durack et al. (4), as modified by Scheld et al. (12), has been shown to be applicable for studying protection against nutritionally variant streptococcal (NVS) endocarditis through immunization (14). This technique and others have also served to show the role of immunization in the prevention of bacterial endocarditis for streptococci other than the NVS (5,12). Since the NVS now are serotyped and since a preliminary analysis of their cell surface has been completed (13), I decided to investigate the role of immunization in cross-protection between serotypes and the role of passively transferred immune globulin in protection by using the NVS rabbit endocarditis model.

The viridans group streptococci are responsible for 50 to 55% of microbial endocarditis. Among these varied species, the NVS have recently been identified as the main organisms associated with culture-negative endocarditis and are responsible for 5 to 10% of all cases of streptococcal endocarditis (10). NVS are characterized by their growth as satellite colonies around colonies of *Staphylococcus epidermidis* or several other gram-positive or gram-negative bacterial strains (7) and by the presence of a pH-dependent chromophore in their cell wall (15). Recently, I subdivided the NVS into three serotypes by using the techniques of rocket-line immunoelectrophoresis and hemagglutination inhibition (16). During these studies, no common group antigen was found.

In this report, I demonstrate that rabbits immunized with NVS elicit a substantial immunoglobulin G (IgG) response and that these rabbits are protected against challenge with a dose of live bacteria from the same serotype equal to 20 to 60 times the 50% infective dose (ID_{50}). Furthermore, a degree of cross-protection was demonstrated between serotype II and III by using the rabbit endocarditis model. Finally, the passive transfer of immune globulin was not sufficient to protect the rabbits from challenge with live NVS and the subsequent end result of endocarditis.

MATERIALS AND METHODS

Strains. The NVS strains used in this study were isolated from patients with endocarditis and were previously described by us (16). Stock cultures were kept lyophilized or frozen at −80°C. For each experiment, satellite tests were performed to check the purity and stability of each strain.

Growth of bacterial strains. For preparation of inocula and vaccines the NVS were grown in a semisynthetic medium (CDMT) (3). Growth of cultures was measured by using a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) in 18-mm tubes at a wavelength of 650 nm.

Experimental rabbit endocarditis model. Left-sided endocarditis was induced according to modifications of techniques previously described (4,12). Rabbits (2.5 to 3.5 kg) were catheterized by placement of a polyethylene catheter across the aortic valve; the catheter was then permitted to remain in place for an additional 20 min. At this time the strains were injected through the catheter in a volume of 1 ml of CDMT. After an additional 10 min the catheter was removed. The animals were sacrificed at 72 h, and the aortic valves were removed aseptically, homogenized in CDMT, and then plated on THBP (3). In addition, the homogenates also were incubated at 37°C for 24 h and replated. Rabbits were diagnosed positive for endocarditis if both satellite colonies were isolated from the aortic valve and the isolated organisms reacted positively in the chromophore assay.

For determination of the ID_{50} of each NVS strain, six rabbits per set were inoculated with bacteria (10^4, 10^5, 10^6, 10^7, and 10^8 CFU). The challenge dosage was preliminarily quantitated by using a previously defined optical density versus viable count curve for ID_{50} and protection studies. Dilutions of the bacteria used in the experiments were made in CDMT and subsequently spot plated (25 μl) on Todd-Hewitt agar plates supplemented with 5% sheep blood and 50 μg of pyridoxal hydrochloride per ml. All counts were done in quadruplicate.

The ID_{50} was calculated by using the method of probit analysis (6), and the significance of the differences of the

TABLE 1. ELISA titer of antibody to NVS in sera from rabbits immunized with a serotype I organism

Antigen (serotype)	Immune status	Mean titer ± SEM	
		IgM	IgG
NVS-47 (I)	Preimmune	<100	<200
	Immune	9,000 ± 913	302,619 ± 39,323
NVS-63 (II)	Preimmune	<100	<200
	Immune	533 ± 91	1,486 ± 223
NVS-69 (III)	Preimmune	<100	<200
	Immune	355 ± 65	556 ± 93

ID_{50}s was determined at the 5% level by using multiple regression analysis and the F test for equality of variances (2). Protection data were analyzed by using the Fisher exact test (2).

Chromophore assay. Streptococci (250 ml) were grown in CDMT as described above and harvested by centrifugation at 10,000 × *g* for 5 min. The supernatant was removed, and the bacteria were suspended in a volume of 2 N HCl equal to that of the pellet. The suspension was then heated at 100°C for 5 min. The appearance of a pink to red color indicated a positive reaction.

Satellite test. NVS were characterized by the satellite test on Trypticase soy agar enriched with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.). The surface was streaked with the suspected NVS culture, overlaid with loops of *S. epidermidis*, and incubated for 18 to 48 h at 37°C. Colonies of the NVS strain grew only adjacent to the *S. epidermidis* colony. It is important only to use commercial plates or at least 2-week-aged blood plates for this assay to function properly.

Preparation of inocula and immunization of rabbits. NVS strains were grown to the stationary phase in CDMT (1 liter) and then sedimented at 1,500 × *g* for 10 min. The bacteria were suspended in 30 to 40 ml of phosphate-buffered saline (PBS; 0.05 M sodium phosphate, 0.15 M sodium chloride [pH 7.4]) and resedimented as described above. This process was repeated three times to remove extracellular contaminants. The bacteria were then suspended in 50 ml of PBS and UV killed.

Bacteria were UV killed by placing the NVS in petri dishes under a UV source for 30 to 45 min. This procedure was followed by five further washes with PBS. The bacteria were finally suspended in 40 ml of PBS, and each preparation was tested for viability by plating the organisms on fresh blood agar plates before the immunization of rabbits (16).

Rabbits were immunized with the above UV-killed whole-cell vaccine intravenously three times weekly for 4 weeks. The rabbits received 0.1 ml per injection during week 1, 0.2 ml during week 2, and 0.3 ml during weeks 3 and 4. All of the above rabbits were bled for both preimmune and postimmune sera for the antibody studies.

Analysis of rabbit sera for antibodies against the immunogen. The antisera were quantitated for bacterial surface antibody by using an enzyme-linked immunosorbent assay (ELISA). The ELISA for detection of antibodies against the NVS was accomplished according to a modification of the procedure of Voller et al. (17) as follows. Bacteria (optical density at 650 nm = 0.5, 1:10 dilution) were coated onto 96-well microtiter plates by using a 0.05 M sodium carbonate buffer, pH 9, containing 0.02% sodium azide. The antigen was incubated at 4°C for 18 h, followed by three washes with 0.05 M sodium phosphate–0.15 M sodium chloride–0.05% Tween 20 buffer (pH 7.4) (PBST). Any exposed sites on the plates were then covered by using a solution of 5% bovine serum albumin (ELISA grade; Sigma Chemical Co., St. Louis, Mo.) in PBST at 37°C for 1 h. Excess bovine serum albumin was removed by three further washes with PBST, followed by incubation of the diluted antisera for 3 h at 37°C. All dilutions were made in 1% bovine serum albumin–PBST. The plates were then washed four times with PBST, and a mixture of peroxidase-conjugated specific antibody to IgG or IgM (Litton Bionetics, Kensington, Md.) was diluted 1:750 in 1% bovine serum albumin–PBST, added to the plates, and incubated for a further 3 h at 37°C. Finally, the plates were washed four times with PBST and one time with PBS and then incubated with *ortho*-dianisidine dihydrochloride for 15 min at 37°C for color development. The plates were read on a Titertek Multiskan MC by using a 450-nm filter. The reciprocal of the last dilution with a reading of 0.1 was considered the titer of the serum.

Isolation of globulin. Globulin was isolated and purified from pooled immune and control rabbit antisera according to the procedure of Harboe and Ingild (8). Essentially, 98 to 100% of the IgG was recovered by this technique. All globulin fractions were concentrated sixfold as compared with the initial serum volume by using an Amicon stirred cell with a YM-10 membrane (Amicon Corp., Lexington, Mass.).

RESULTS

Immunization of rabbits with NVS. Rabbits were immunized intravenously with UV-killed whole NVS vaccines (serotype I, 34 rabbits; serotype II, 27 rabbits; serotype III, 26 rabbits). After a 3-week immunization schedule, the rabbit sera were analyzed for specific antibody to the immunizing strain and the prototype strains of each of the other two serotypes by ELISA.

Quantitation of the specific antibody in the sera of rabbits immunized with NVS-47 (serotype I) demonstrated that high titers of antibody were present in the sera of the immune rabbits (IgM, 9,000; IgG, 302,619) as compared with their preimmune sera (IgM, <100; IgG, <200) (Table 1). Furthermore, only low levels of cross-reactive antibodies (IgM and IgG) were developed which reacted with the surface of the prototype strains from serotypes II and III.

When the sera from rabbits immunized with strain NVS-63 (serotype II) were analyzed for antibodies directed against the surface of these organisms, IgM and IgG titers of 1,976 and 490,656 were obtained (Table 2). Again, only minor titers were obtained when the preimmune sera were analyzed. The antibodies cross-reactive with the prototype strains from other serotypes were again much lower than the homologous reactions. However, there appeared to be a significant level of antibodies (IgG, 10,711) cross-reactive with the serotype III prototype strain as compared with the serotype I strain (IgG, 629).

TABLE 2. ELISA titer of antibody to NVS in sera from rabbits immunized with a serotype II organism

Antigen (serotype)	Immune status	Mean titer ± SEM	
		IgM	IgG
NVS-47 (I)	Preimmune	<100	<200
	Immune	475 ± 71	629 ± 102
NVS-63 (II)	Preimmune	<100	<400
	Immune	1,976 ± 337	490,656 ± 73,713
NVS-69 (III)	Preimmune	<100	<200
	Immune	377 ± 157	10,711 ± 2,957

Mean IgM titers of 412 and mean IgG titers of 71,040 were demonstrated against the surface of strain NVS-69 (serotype III) when sera were analyzed from rabbits immunized with this strain (Table 3). Again, only low titers were seen in preimmune sera and when the immune sera were tested against strain NVS-47 (serotype I). However, in these experiments, the sera from rabbits immunized against strain NVS-69 contained significant titers against the surface of strain NVS-63 (serotype II) (IgM, 2,000; IgG, 39,822).

Since sera from the rabbits were demonstrated to contain various levels of antibody cross-reactive between serotypes, these rabbits were used to analyze the cross-protective effect of the antibodies.

Cross-protection between serotypes against endocarditis in rabbits. The rabbits described above were challenged with a dose of bacteria (same or different serotype) equivalent to that required to induce 100% endocarditis (20 to 60 times the ID_{50}) (14). Three days later, the rabbits were sacrificed, the hearts were removed, and the heart valves were analyzed for vegetations and the presence of the challenge strain.

Of the rabbits immunized with NVS-47, a serotype I strain, 90% (9 of 10) were protected when they were challenged with live organisms (Table 4). When rabbits immunized with this strain were challenged with live serotype II or III organisms, none of 15 (0%) and 2 of 9 (22%) rabbits were protected, indicating that the surfaces of the organisms do not express immunologically similar components responsible for protection.

When these studies were repeated with rabbits immunized with strain NVS-63 (serotype II), rabbits challenged with the homologous strain were again afforded protection (11 of 12; 92%). Rabbits challenged with serotype I organisms had little protection (1 of 7; 14%), whereas rabbits challenged with serotype III organisms were afforded significant protection (5 of 8; 63%).

Results similar to those seen with serotype II-immunized rabbits were obtained with serotype III-immunized rabbits. Again, rabbits challenged with organisms from the homologous serotype were protected (10 of 10; 100%), whereas those challenged with serotype I organisms were unprotected (1 of 8 protected; 13%). Of interest was the finding that, of the eight rabbits challenged with the serotype II strain, five (63%) were protected.

Statistical analysis of the results indicated that the rabbits immunized with strains from the same serotype were significantly protected as compared with unimmunized controls ($P < 0.001$; data not shown). Furthermore, the experiments which compared serotype I organisms versus serotype II and III organisms (and vice versa) indicated a significant difference in the absence of cross-protection. Finally, there appeared a degree of cross-protection with rabbits immunized with serotype II organisms and challenged with serotype III organisms (and vice versa), in that no significant difference was demonstrated by statistical analysis between homologous protection and cross-protection. These studies indicated that strains from serotype II and III contain an immunologically related surface-protective component.

TABLE 3. ELISA titer of antibody to NVS in sera from rabbits immunized with a serotype III organism

Antigen (serotype)	Immune status	Mean titer ± SEM	
		IgM	IgG
NVS-47 (I)	Preimmune	<100	<200
	Immune	256 ± 47	<200
NVS-63 (II)	Preimmune	<100	<200
	Immune	2,000 ± 693	39,822 ± 8,869
NVS-69 (III)	Preimmune	<100	<200
	Immune	412 ± 91	71,040 ± 6,854

TABLE 4. Analysis of protection and cross-protection of rabbits against NVS endocarditis by immunization with heat-killed whole cells[a]

Immunogen (serotype)	Challenge organism (serotype)	No. protected/ no. challenged (%)	P
NVS-47 (I)	NVS-47 (I)	9/10 (90)	
	NVS-63 (II)	0/15 (0)	<0.001
	NVS-69 (III)	2/9 (22)	<0.01
NVS-63 (II)	NVS-47 (I)	1/7 (14)	<0.002
	NVS-63 (II)	11/12 (92)	
	NVS-69 (III)	5/8 (63)	NS[b]
NVS-69 (III)	NVS-47 (I)	1/8 (13)	<0.001
	NVS-63 (II)	5/8 (63)	NS
	NVS-69 (III)	10/10 (100)	

[a] The rabbits were analyzed for the presence of titers of antibody to the cell surface components before they were challenged with a dose 20- to 60-fold larger than the ID_{50} of exponential-phase cells grown in CDMT. Rabbits were considered positive for endocarditis if the challenge organism was isolated from the heart valve 3 days after inoculation.

[b] NS, Not significantly different from the protection rate observed with homologous challenge.

Passive transfer of serum and globulin components. Since antibody levels against the surface of the organisms were high in rabbits that were protected, experiments were designed to analyze whether this protection can be transferred between rabbits. Preliminary experiments indicated that specific antibodies in serum from immunized and protected rabbits injected intravenously into naive rabbits decreased slowly over the 72 h required for the protection model. Therefore, rabbits were injected intravenously with high-titer serum (serotype I; 5 ml, 4 animals; 10 ml, 2 animals; 15 ml, 1 animal). After 30 min, the rabbits were challenged with NVS-47 (25 ID_{50}s) and were checked after an additional 72 h for endocarditis. The challenge organism was demonstrated to be present in all of the rabbits except one, indicating that protection was not transferred (Table 5, rabbits 1 to 7).

Since the possibility existed that insufficient antibody was transferred due to volume considerations, the globulin fraction was isolated from pooled high-titer immune rabbit sera. Preliminary experiments indicated that intraperitoneal injec-

TABLE 5. Analysis of the effect of passive transfer of antibody on protection by using the rabbit endocarditis model

Rabbit no.	Pretreatment (no. of ml, route)[a]	Challenge (no. of ID_{50}s)	Endocarditis
1	Immune serum (5, i.v.)	25	+
2	Immune serum (5, i.v.)	25	−
3	Immune serum (5, i.v.)	25	+
4	Immune serum (5, i.v.)	25	+
5	Immune serum (10, i.v.)	25	+
6	Immune serum (10, i.v.)	25	+
7	Immune serum (15, i.v.)	25	+
8	Immune globulin[b] (16, i.p.)	20	+
9	Immune globulin[b] (16, i.p.)	20	+
10	Control globulin[b] (16, i.p.)	20	+

[a] i.v., Intravenous; i.p., intraperitoneal.

[b] Globulin preparations were concentrated sixfold during preparation.

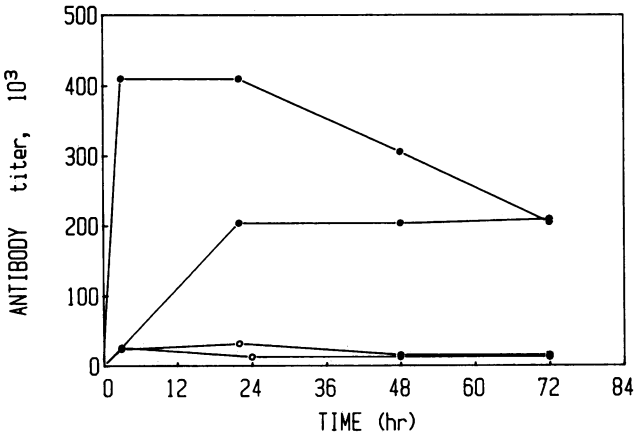

FIG. 1. Time course analysis of levels of anti-NVS surface antibody in the serum of rabbits injected with immune globulin. Rabbits were given immune globulin (16 ml) intraperitoneally, and then their serum antibody titers against NVS were monitored by using an ELISA. Symbols: ●, rabbits 8 and 9, immune globulin; ○, rabbits 10 and 11, control globulin.

tion of the globulin fraction required 2 to 3 h for equilibration with the serum (data not shown). Therefore, rabbits were injected with either immune globulin (serotype I) or control globulin (serotype III) (16 ml, sixfold concentrated) intraperitoneally, and the globulin was permitted to equilibrate for 3.5 h. At this time the rabbits were challenged with 20 ID_{50}s of live organisms (NVS-47). Analysis of the heart valves after 72 h indicated that each of the rabbits had contracted endocarditis and therefore was not protected (Table 5, rabbits 8 to 10). Analysis of periodic bleedings from these rabbits showed that sufficient antibody was present for protection as compared with earlier studies (Fig. 1; Table 4). Therefore, these data indicate that a factor(s) other than antibody is required for complete protection against endocarditis.

DISCUSSION

Immunization of rabbits with UV-killed NVS has been shown to be protective in a rabbit endocarditis model. Immune rabbits (30 of 32; 94%) were protected when challenged with 20 to 60 ID_{50}s of the homologous organism. However, only 1 of 30 nonimmunized control rabbits did not contract endocarditis 3 days after challenge, indicating that the results were significant at a level of $P < 0.001$. Furthermore, there appeared to be no cross-protection between serotypes except for serotypes II and III (Table 4; 63%). This level of protection was present when the challenge was in either direction.

Previous analysis of the surface structures of the NVS by me (13) demonstrated that serotype II and III strains express an immunologically similar protein on their cell wall. Furthermore, only strains from these serotypes were found to contain galactosamine as part of their cell walls, which was lacking in the cell walls of serotype I strains (13). One or both of these common components of serotype II and III could account for the cross-protection data presented in this paper. Final confirmation of this will have to await the purification of the cell wall components and their analysis as possible protective antigens. A further possibility for the cross-protection data is the presence of a ribitol teichoic acid in the cell walls of all NVS tested (serotypes I, II, and III) (13). Since there was no cross-protection between serotype I and the other serotypes, this cell wall polymer could be responsible for cross-protection only if strains from serotypes II and III had a unique side group on the ribitol backbone (i.e., galactosamine). These studies are presently under way in this laboratory.

Analysis of the antibody response after immunization of the rabbits with NVS demonstrated that high titers of IgG directed against the surface structures of the organisms were present in the sera (Tables 1 to 3). The IgM titers were low, as would be expected in hyperimmunized rabbits. Furthermore, analysis of the titers of anti-NVS surface IgM or IgG in individual rabbits demonstrated no correlation with cross-protection between serotypes II and III.

Since various investigators have now shown that rabbits can be preimmunized for protection against endocarditis (1, 11, 14), the question arises whether antibody or antibody and complement are sufficient for clearing the organisms by the reticuloendothelial system. One would expect that the antibody inhibits the bacteria from adhering to the heart valve and that antibody- or complement-coated bacteria are cleared by the immune system. By using this endocarditis model, it can be seen that the data indicate that antibody and complement alone are insufficient in protecting the animals. No protection was demonstrated even when hyperimmune levels of antibody were transferred to the naive animals (Table 5; Fig. 1). Other investigators using drugs to deplete various cell populations have provided evidence that monocytes are involved in protection (9). One could speculate from my data and theirs that in the immune animals the monocytes process the organisms on the heart valve for presentation to previously activated T cells. The T cells then commence production of soluble mediators for the recruitment of macrophages to clear the bacteria from the valve. However, in light of this, the lack of protection in passively immunized animals could be explained by the absence of primed T cells before the endocarditic process begins.

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LITERATURE CITED

1. **Adler, S. W., II, D. S. Selinger, and W. P. Reed.** 1981. Effect of immunization on the genesis of pneumococcal endocarditis in rabbits. Infect. Immun. **34**:55–61.
2. **Armitage, P.** 1971. Statistical methods in medical research. John Wiley & Sons, Inc., New York.
3. **Bouvet, A., I. van de Rijn, and M. McCarty.** 1981. Nutritionally variant streptococci from patients with endocarditis: growth parameters in a semisynthetic medium and demonstration of a chromophore. J. Bacteriol. **146**:1075–1082.
4. **Durack, D. T., P. B. Beeson, and R. G. Petersdorf.** 1973. Experimental bacterial endocarditis. III. Production and progress of the disease in rabbits. Br. J. Exp. Pathol. **54**:142–151.
5. **Durack, D. T., B. C. Gilliland, and R. G. Petersdorf.** 1978. Effect of immunization on susceptibility to experimental *Streptococcus mutans* and *Streptococcus sanguis* endocarditis. Infect. Immun. **22**:52–56.
6. **Finney, D. J.** 1962. Probit analysis, p. 39. Cambridge University Press, Cambridge.
7. **Frenkel, A., and W. Hirsch.** 1961. Spontaneous development of L forms of streptococci requiring secretions or other bacteria or sulphydryl compounds for normal growth. Nature (London) **191**:728–730.

8. **Harboe, N., and A. Ingild.** 1973. Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand. J. Immunol. **2**:161–164.
9. **Meddens, M. J. M., J. Thompson, W. C. Bauer, J. Hermans, and R. van Furth.** 1983. Role of granulocytes and monocytes in experimental *Staphylococcus epidermidis* endocarditis. Infect. Immun. **41**:145–153.
10. **Roberts, R. B., A. G. Krieger, N. L. Schiller, and K. C. Gross.** 1979. Viridans streptococcal endocarditis: the role of various species, including pyridoxal-dependent streptococci. Rev. Infect. Dis. **1**:955–966.
11. **Scheld, W. M., J. H. Thomas, and M. A. Sande.** 1979. Influence of preformed antibody on experimental *Streptococcus sanguis* endocarditis. Infect. Immun. **25**:781–785.
12. **Scheld, W. M., J. A. Valone, and M. A. Sande.** 1978. Bacterial adherence in the pathogenesis of endocarditis. Interaction of bacterial dextran, platelets, and fibrin. J. Clin. Invest. **61**: 1394–1404.
13. **van de Rijn, I.** 1985. Quantitative analysis of cell walls of nutritionally variant streptococci grown under various growth conditions. Infect. Immun. **49**:518–522.
14. **van de Rijn, I.** 1985. Role of culture conditions and immunization in experimental nutritionally variant streptococcal endocarditis. Infect. Immun. **50**:641–646.
15. **van de Rijn, I., and A. Bouvet.** 1984. Characterization of a pH-dependent chromophore from nutritionally variant streptococci. Infect. Immun. **43**:28–31.
16. **van de Rijn, I., and M. George.** 1984. Immunochemical study of nutritionally variant streptococci. J. Immunol. **133**:2220–2225.
17. **Voller, A., D. Bidwell, and A. Bartlett.** 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506–512. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology. American Society for Microbiology, Washington, D.C.