Horse Anti-Pneumococcal Immunoglobulins. II. Specific Mouse Protective Activity. (31414)

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Antibodies of each of the major immunoglobulin classes with similar determinant specificity initiate different biological effects following their combination with antigens(1,2,3), and vary in their capacity for specific neutralization of certain biologically active antigens (4,5).

The virulence of the type I pneumococcus is apparently related to the capacity of its capsular polysaccharide to inhibit phagocytosis(6). The protective effects of immune serum prepared by immunization with whole bacteria has been related directly to its capacity to combine with the capsular polysaccharide(7). It was of interest, therefore, to determine the relative biological effectiveness of the major immunoglobulins in mediating specific protection against fatal infection with pneumococcus, type I.

Isolation of highly purified horse γM, γG and γA antibodies to type I pneumococcal polysaccharide (S1)(8) has permitted quantitative study of their specific mouse protective activity. The results show that the protective effect of the γM antibody is the greatest of the immunoglobulins, and that this class probably accounts for most of the protective activity of whole serum.

Methods. The preparation and the physicochemical and antigenic characteristics of S1 and horse anti-S1 immunoglobulins used have been described(8). Briefly, S1 was extracted from 24-36-hour-old cultures of type I pneumococcus grown in brain heart infusion agar (Difco) enriched with 1% sheep erythrocytes. Sedimentation velocity analysis of the S1 revealed a single component of $s_w = 3.0$ S. Horse anti-S1 antibodies were isolated from hyperimmune serum prepared by immunization of a single horse with multiple injections of whole heat-killed type I pneumococci. Anti-type II pneumococcal serum was prepared in a similar fashion. The anti-S1 immunoglobulins were extracted from a specific precipitate prepared at equivalence and washed with cold saline and dissolved in acetate buffer, 0.05 M, pH 3.5. The insoluble residue, mostly S1, was removed by centrifugation and the eluted antibodies were further purified either by gel filtration through Sephadex G-200 to obtain γM, or by DEAE cellulose to obtain γA and γG proteins(8). Approximately 82% of the antibody protein eluted was γM, and the other 18% consisted of γA and γG proteins. The purity of these antibody preparations was verified by sedimentation analysis in a Model E Spinco analytical ultracentrifuge and by immunoelectrophoresis using a rabbit anti-horse serum protein antiserum. The anti-S1 content of whole serum was determined by quantitative precipitin techniques(9).

Pneumococci were prepared for the *in vivo* assay by serial passage through young adult mice obtained from the Charles River Laboratories, Wilmington, Mass. The heavily encapsulated bacteria were grown in Brain Heart Infusion broth for about 6 hours at 37°C, counted in a Coulter particle counter, and adjusted to a concentration of 1000 organisms/ml in 0.15 M NaCl. Equal volumes of this bacterial suspension and the test reagents or controls were mixed and a 0.2 ml aliquot injected intraperitoneally into the mice. The animals were returned to the cage and the number of survivors in each experimental group was recorded after 36 hours. To evaluate the effect of the reducing agent, 2-mercaptoethanol (2-ME) upon the activity of the immunoglobulins, the samples were dialyzed against 0.1 M 2-ME in phosphate buffered saline, pH 7.2 for 18 hours at 4°C. At room temperature, 0.02 M iodoacetamide,

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TABLE I. Protection Against Type I Pneumococcus Infection in the Mouse by Purified Horse Anti-S1 Immunoglobulins.

Immunoglobulin preparation	Conc of antibody (μg/ml)	No. surviving/No. in group
Normal horse serum	0	0/15
Anti-SII serum	0	0/6
Anti-S1 serum	10.0 to .01	26/26
" "	.001	5/9
" "	.0001	0/5
" " 2-ME reduced	10.0 to .1	0/15
γM antibody	10.0 to .001	39/40
" "	.0001	2/5
" "	.00001	0/4
" " 2-ME reduced	1000 to 100	10/10
" " " "	10.0 to .1	0/25
γG antibody	1000 to 100	10/10
" "	10.0	6/9
" "	1.0	0/6
" " 2-ME reduced	10.0	3/5
" " " "	1.0	0/5
γA antibody	100 to 10.0	15/15
" "	1.0	9/10
" "	.1	5/9
" "	.01	0/6
" " 2-ME reduced	1.0	5/5
" " " "	.01	0/5

0.1 ml aliquots of the indicated antibody-containing solution were mixed *in vitro* with an equal volume of a suspension of freshly grown pneumococci containing 1×10^2 organisms/ml. The mixture was then injected intraperitoneally into adult mice and the survival of the animals was recorded in 36 to 48 hr.

dissolved in phosphate buffer saline, pH 7.2, and 0.15 M NaCl were successively substituted in the dialysate. These conditions have been shown to reduce intact γM to subunits of a molecular weight of about 190,000 without significant contamination by incompletely reduced material(10).

Results. The purity of the horse anti-S1 antibodies used in these experiments has been previously described(8). Sedimentation analysis of a 0.5% protein solution dissolved in 0.1 M sodium phosphate, pH 6.8, revealed a single homogeneous peak for each immunoglobulin preparation after ultracentrifugation at 56,140 RPM for 200 minutes. With a rabbit anti-horse serum protein serum, each antibody preparation showed a single precipitin arc following immunoelectrophoresis.

As has been reported previously(11), 100 pneumococci per experimental animal served as a reliable 100% lethal dose. The results obtained in 3 representative experiments are shown in Table I. Dilution of the anti-S1 serum to a precipitating antibody concentration of 0.001 μg/ml conferred protection to about 50% of mice. This result is consistent with previous studies using whole anti-S1 serum in a similar assay system(7). The specificity of this protective effect is confirmed by the lack of protection given by normal horse serum, saline and anti-SII serum.

Gamma M anti-S1 immunoglobulin was 10 times more biologically active than precipitating antibody compared on the basis of antibody protein content. As little as 0.00001 μg (0.1 ml of 0.0001 μg/ml) of the γM preparation protected some of the mice. In contrast, concentrations of 0.1 μg/ml of the γA and 10.0 μg/ml of the γG anti-S1 immunoglobulins were required with this inoculum to demonstrate a similar protective effect.

Following treatment of whole anti-S1 serum with 2-ME, no protective activity was retained. An approximately one million-fold decrease in the protective activity of the γM was found after treatment with 2-ME. No significant change in the activity of the γA and γG anti-S1 immunoglobulins followed similar mild reduction and alkylation.

Discussion. Despite their similar determinant specificity, anti-S1 antibodies showed strikingly different activity in the conferring protection against infection with living type I pneumococci. This difference in specific protective activity between the horse anti-S1 γM antibody and the other immunoglobulin classes is greater than has previously been reported for rabbit anti-*Salmonella typhimurium* "O" antibodies(3). Conceivably, this difference in the specific activity of antibacterial antibodies could be explained by effects secondary to the greater complexity of the surface antigens of *Salmonella typhimurium,* or to the pathophysiological effects provoked by lipopolysaccharide. The S1-immunoglobulin interaction is complicated by neither of these factors. Following combination with specific antibody, the capsular polysaccharide determinant is no longer capable of effective interference with phagocytosis and intracellular destruction of the live pneumococci(6).

The difference between the molecular weight of the γM, γG and γA antibodies does not provide a direct explanation for the approximate 1,000- to 10,000-fold differences in their protective activity. One possibility that may explain this phenomenon is the difference in the total number of combining sites of the γM as compared to the γG and γA proteins. Thus, 6 active combining sites, recently reported for a rabbit anti-hapten γM antibody(12), might be required to form bacterial-protein complexes of a critical size in order to induce efficient phagocytosis. Another explanation for the magnitude in difference in activity of the immunoglobulins relies upon the structural differences of these proteins. For this argument, one would have to postulate that there are specific receptor sites on the μ chain that interact with phagocytes. Such a critical area on the μ chain might be opened for direct interaction with phagocytic cells following combination of the antibody with its antigen, or perhaps through secondary interactions with a complement component.

The relationship between the total amount of antigen bound by the antibodies and preparation of bacteria for opsonization is not a simple one as indicated by the effect of 2-ME on the 3 classes of antibody. The subunits of γM anti-S1 had low activity in this *in vivo* assay in that it required concentrations of 10 to 100 μg/ml of the reduced γM as compared to 0.0001 μg/ml of the intact γM. However, quantitative studies using coprecipitation with an anti-horse immunoglobulin serum have shown that the 2-ME produced subunit of γM anti-S1 binds antigen to the same extent as the intact, unreduced molecule(8). The 2-ME treated whole serum showed no activity despite the observation that about 82% of isolated antibody protein was γM. The complete loss of activity of serum antibody activity as compared to partial loss of purified γM activity cannot be adequately explained, but may in part be due to interactions of reduced fragments with other serum proteins. In any case, the loss of protective activity of immune serum through reduction is consistent with the hypothesis that the protective activity of the serum is due primarily to its content of γM anti-S1.

It would seem, therefore, for the limited number of systems studied, that antibodies of the γM class have comparatively high specific antibacterial activity(3,4). Considering its rapid production in the spleen(13) this high order of effectiveness suggests that γM specific bacteriocidal and opsonizing antibodies may be critical in early phases of defense against infection, particularly bacteremia(14). In contrast, it has been shown that γG and γA classes of immunoglobulins appear to be much more effective than γM in neutralizing the activity of less complex, low molecular weight antigens such as diphtheria toxin and lysozyme(5). These experimental observations provide further evidence for heterogeneity in the biological consequences of interaction between antigenic determinants and the various immunoglobulins. Such functional heterogeneity may have evolved because of the survival value inherent in an ability to have protection against a wide variety of noxious agents.

Summary. The mouse protecting activity of isolated and purified γM, γA, and γG pneumococcal polysaccharide, type I antibodies was compared. The specific protective activity of γM anti-S1 was found to be approximately 1000 times as much as γA, and 100,000 times that of γG. The γM activity appears to account for the protective action of whole anti-pneumococcal serum.

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Uptake of Dihydrostreptomycin by Intact Splenic Cells Cultured from Immunized Rabbits and its Binding to the 40S Ribosomal Subunit.* (31415)

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Streptomycin (SM) bound to the 30S subunit of ribosomes from SM-sensitive *Escherichia coli*(1) and, with polyuridylic acid as a messenger, caused decreased incorporation of phenylalanine and increased incorporation of isoleucine, thus suggesting that the SM bound to ribosomes altered translation of the information of the messenger(2). Pestka *et al*(4) and Kaji and Kaji(5) showed that SM affects the binding of transfer RNA to the ribosomal template complex and changes the recognition of RNA codons. In support of this hypothesis, Schwartz(3) demonstrated that in cell-free extracts of *E. coli* SM modified the information contained in the RNA of the bacteriophage F_2 in such a way as to cause synthesis of altered viral coat protein, and Bissell(6) found that in the presence of SM or neomycin *E. coli* produced an altered β-galactosidase that had lost its enzymatic activity but still cross-reacted with antiserum to the normal enzyme. SM affected the synthesis of antibody in cultures of splenic and lymph node cells from rabbits immunized with the RNA bacteriophage MS-2 by causing the production of 7S antibody with altered immunological specificity in that it did not combine with complete infectious phage but still combined with incomplete noninfectious phage(7,8). It was suggested that SM may bind to the ribosomes of splenic cells and cause the synthesis of altered antibody by mechanisms similar to those described for bacteria. This paper reports that dihydrostreptomycin (DHSM), which acts similarly to SM in both *E. coli*(9) and splenic cells (7,8), readily penetrates intact splenic cells from immunized rabbits *in vitro* and binds to the 40S subunit of the 76S splenic ribosome. This is the first demonstration that antibiotics of the SM family can bind to similar intracellular sites in mammalian and bacterial cells.

Methods. The preparation of MS-2 phage antigen, immunization of rabbits, and preparation of splenic cell cultures have been previously described(7). Cells from immunized animals were used because they contain more ribosomes than cells from unimmunized ones(10). Tritiated DHSM was the generous gift of Dr. Charles Rosenblum of Merck, Sharp and Dohme Research Laboratories, Rahway, N. J. It was used in preference to SM because it was less likely to exhibit nonspecific binding(11).

For measuring uptake of H^3-DHSM by intact cells, splenic cells were removed from the main culture, washed twice in minimal essential medium (MEM)(12), and resuspended in MEM to a cell count of 2×10^6/ml. H^3-DHSM was added to a final

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