*STREPTOCOCCAL GROUP-SPECIFIC ANTIBODIES: OCCURRENCE OF A RESTRICTED POPULATION FOLLOWING SECONDARY IMMUNIZATION**

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Certain rabbits produce high concentrations of γG antibodies to streptococcal carbohydrates after primary intravenous immunization with streptococcal vaccines.[1-3] The electrophoretic behavior of these antibodies and their isolated light chains points to a markedly restricted population of γG molecules. This type of response is not found in all immunized rabbits. It may depend on the structure of the carbohydrate antigen, the intravenous route of immunization, the genetic background of the rabbits, and prior sensitization with the same or a related antigen. The rabbits in the present study did not respond in this way after primary immunization, but they did produce a high level of relatively homogeneous antibodies after secondary immunization. This suggests that in some instances prior sensitization may lead to a restricted population of γG antibodies.

Materials and Methods.—Streptococcal vaccines: The vaccines were prepared from groups A, A-variant, and C streptococci as previously described.[1]

Streptococcal group-specific carbohydrates: Groups A, A-variant, and C carbohydrates were isolated from hot formamide digests of cell walls.[4]

Immunization: New Zealand red rabbits were immunized intravenously 3 times a week for 3 weeks with groups A or A-variant streptococcal vaccines.[1] From 3 to 6 months following the first immunization, second series injections were given 3 times a week for 2 weeks. Sera were collected prior to each immunization period and at weekly intervals throughout immunization. Each sample of blood was collected 4 days after the last intravenous injection of vaccine.

Serological, immunochemical, and electrophoretic methods: These were previously described.[1] Disc electrophoresis was performed at pH 8.3 in acrylamide gels by the method of Davis.[5] Total serum protein was determined by the Biuret method;[6] concentrations of albumin and globulins were calculated from the densitometric scan of the zone electrophoresis pattern and the total protein value.

Results.—Primary intravenous immunization of three rabbits with group A-variant vaccine and three rabbits with group A vaccine did not produce group-specific antibodies with restricted electrophoretic heterogeneity. However, reimmunization three months later did yield antibodies with such restriction in two of the six rabbits. The sera from one of these rabbits were studied in detail, and antibody isolated from the primary response serum was compared to that isolated from the secondary response serum.

Figure 1 shows quantitative precipitin tests on sera collected from a rabbit during the first and second immunizations with group A-variant vaccine. Serum collected on day 24 after the start of primary immunization contained 13 mg/ml of antibodies to group A-variant carbohydrate. On day 98, just prior to re-

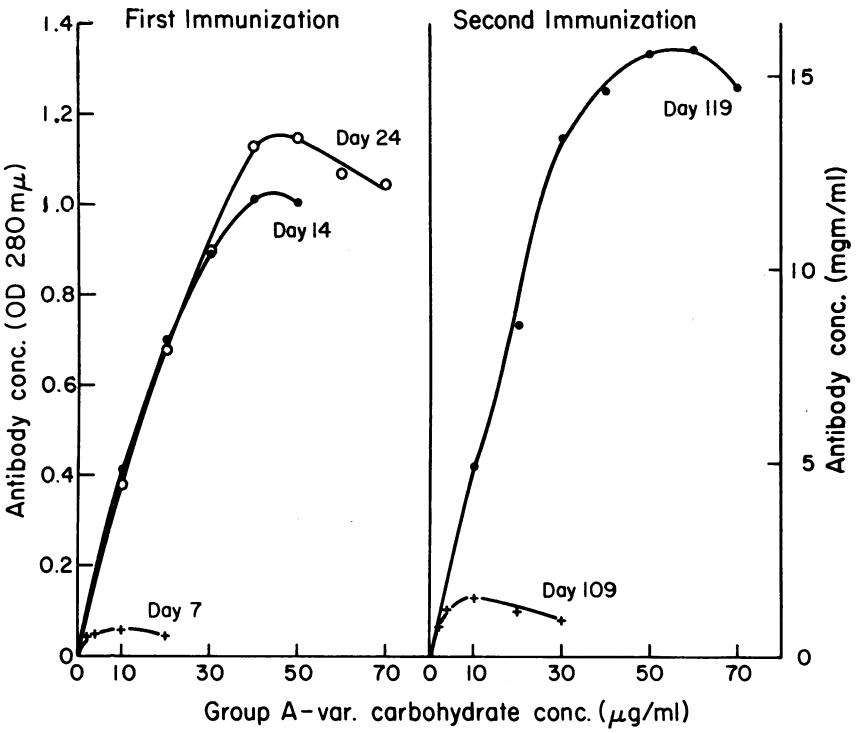

FIG. 1.—Levels of group-specific antibodies in a rabbit immunized with group A-variant vaccine. Quantitative precipitin tests on sera from the first and second immunization periods. The antibody content of 0.1 ml of serum, which was recovered from the precipitate, was redissolved in 0.1 *N* NaOH and measured spectrophotometrically. The amount of antibody precipitated at each antigen concentration is given both in terms of optical density of the redissolved precipitates (*left scale*) and antibody content of the whole serum as calculated from the optical density data (*right scale*).

immunization, no precipitating antibody was detectable. The second immunization course consisted of six injections over a two-week period. After this reimmunization, serum collected on day 119 contained 16 mg/ml of antibodies to group A-variant carbohydrate.

In Figure 2, the zone electrophoretic patterns of primary response serum (24-day) and secondary response serum (119-day) are compared. The patterns of the sera collected prior to both immunization periods are also shown. The γG following the primary immunization is electrophoretically heterogeneous. The γG following the second immunization, on the other hand, contains considerable material with restricted electrophoretic mobility. Figure 3 shows the microzone electrophoretic patterns of the two immune sera before and after absorption with an equivalent concentration of A-variant carbohydrate. Absorption removed 50 per cent of the γG from 24-day serum and 70 per cent of that from 119-day serum.

Immunoelectrophoresis revealed that antibodies to A-variant carbohydrate in primary response serum were more heterogeneous than those in secondary re-

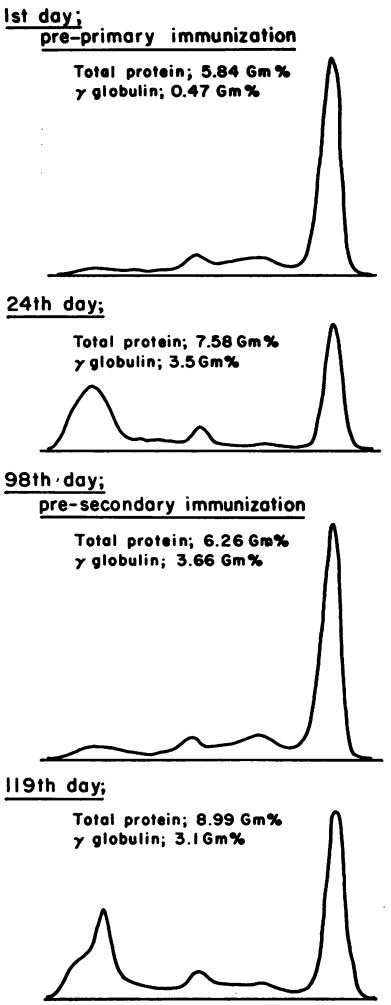

FIG. 2.—Densitometric scans of electrophoretic patterns of group A-variant sera collected before and after the primary and secondary immunization periods.

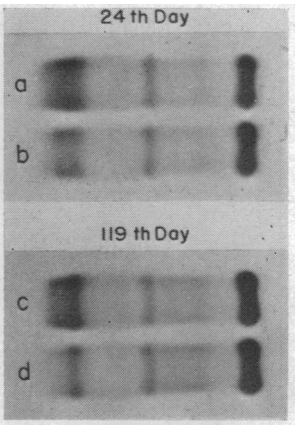

FIG. 3.—The electrophoretic patterns of the group A-variant sera before and after absorption at equivalence with group A-variant carbohydrate. Primary response serum (24-day); pattern *a* before, and pattern *b* after absorption. Secondary response serum (119-day): pattern *c* before, and pattern *d* after absorption.

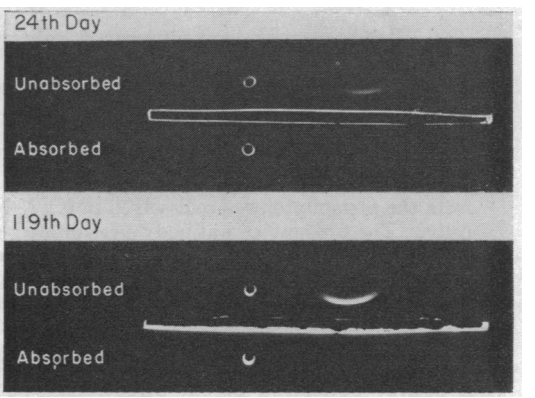

FIG. 4.—Immunoelectrophoresis of primary response (24-day) and secondary response (119-day) sera. *Upper frame:* top well, unabsorbed 24-day serum; bottom well, absorbed serum. *Lower frame:* top well, unabsorbed 119-day serum; bottom well, absorbed serum. Group A-variant carbohydrate added to both upper and lower troughs.

sponse serum. Unabsorbed sera and sera absorbed at equivalence with A-variant carbohydrate were added to the wells and A-variant carbohydrate to the troughs. The results are depicted in Figure 4. While a double arc reflecting heterogeneity was formed by 24-day serum, a single major arc was formed by 119-day serum.

Before describing the electrophoretic properties of the light chains of the secondary response antibodies that point to electrophoretic homogeneity, it will be

useful to comment first on evidence of this sort in the case of previously described primary response antibodies.[1] An occasional rabbit produces group-specific antibodies during the primary response which exhibits an unusual restriction of electrophoretic mobility. Furthermore, the light chains of these antibodies migrate in a sharp distinct zone in either acid or alkaline starch gel electrophoresis.[2] The light chains of one of these antibodies have been studied further by disc electrophoresis. Antibodies to group C carbohydrate after four weeks of primary immunization were recovered from a specific immune precipitate by previously described methods.[1] Light chains were isolated from the reduced and alkylated antibody by the method of Fleischman *et al.*[7] Light chains were similarly isolated from normal rabbit γG. Disc electrophoresis patterns of these light-chain preparations are illustrated in Figure 5. The light chains of normal rabbit γG are distributed in at least six bands, whereas the antibody light chains are predominantly concentrated in a major and minor band. Similar electrophoretic studies were performed on antibodies from the primary and secondary response group A-variant sera depicted in Figure 1.

Light chains of primary response anti-A-variant antibodies (24-day serum), secondary response antibodies (119-day serum), and normal γG were isolated as described in the paragraph above. Light chains from normal γG and primary response antibodies migrated as a diffuse smear in acid urea starch gel electrophoresis, whereas the light chains of secondary response antibodies migrated primarily in a restricted band. The disc electrophoresis patterns of the light chains of normal rabbit γG and antibodies from primary and secondary response sera are shown in Figure 6. The light chains from 24-day serum are distributed in at least five major bands, whereas the light chains from 119-day serum are mainly in one band. In this respect, the light chains of secondary response antibodies resemble those of antibodies to group C carbohydrate from a primary response serum. The light chains of myeloma proteins may also have as few as two or three bands.[8]

Figure 7 shows the zone electrophoretic patterns of sera collected from a rabbit during primary and secondary immunization with group A vaccine. The γG in the primary response serum (22-day) is diffuse, whereas that in the secondary response serum (203-day) is primarily concentrated in a major band. The primary response serum contained 7.6 mg/ml of γG and 2.6 mg/ml of antibody to group A. The secondary response serum contained 16.6 mg/ml of γG of which 12.1 mg/ml was antibody to group A. Thus, antibodies to group A comprise approximately 75 per cent of the total γG in the secondary response serum. These antibodies are primarily directed against terminal β N-acetyl glucosaminide residues of the group A carbohydrate[9] since their precipitation with antigen is readily inhibited by N-acetyl glucosamine.

Discussion.—Studies on the chemistry of antibodies would receive a major impetus if reproducible means were available to produce antibodies sufficiently homogeneous for detailed chemical analysis. A major impasse thus far has been the marked heterogeneity of the immune globulins following immunization. As an alternative approach to the problem, the myeloma proteins, which are remarkably homogeneous, have been examined for antibody activity. Metzger

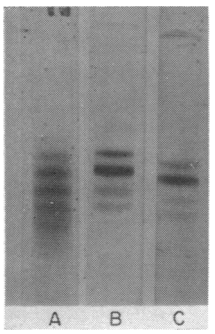

FIG. 5.

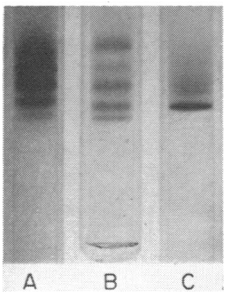

FIG. 6.

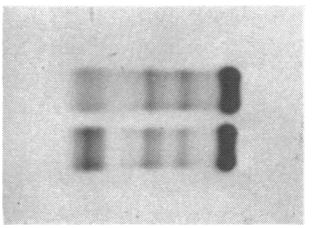

FIG. 7.

FIG. 5.—Disc electrophoresis of light chains. (*A*) Isolated from normal γG; (*B*, *C*), isolated from primary response group C antibodies. *C* is one half the concentration of *B*.

FIG. 6.—Disc electrophoresis of light chains. (*A*) Isolated from normal γG; (*B*) isolated from anti-A-variant antibodies of primary response (24-day) serum; and (*C*) isolated from anti-A-variant antibodies of secondary response (119-day) serum. The three samples were run independently. The gels are aligned in the photograph to facilitate comparison.

FIG. 7.—The zone electrophoretic patterns of group A primary and secondary response sera. *Upper frame:* primary response, 22-day serum. *Lower frame:* secondary response, 203-day serum.

and Stone described a macroglobulin with activity directed against the human Fc fragment.[10] Eisen *et al.*[11] reported a myeloma protein that binds dinitrophenyl (DNP). While the antibody properties of these proteins are of special interest, methods for experimentally stimulating homogeneous antibodies in animals would have an advantage over the infrequent chance occurrence of human paraproteins with antibody activity.

An occasional rabbit may produce antibodies during primary immunization that have considerably restricted electrophoretic heterogeneity.[1,2] The present studies indicate that secondary immunization may increase the availability of such antibodies. Two out of six rabbits that produced heterogeneous antibodies during primary immunization produced antibodies with restricted heterogeneity upon subsequent secondary immunization. This may provide an experimental approach for imposing restraints on the diversity of a specific antibody population. If this proves to be the case, these antibodies may be useful in further studies on the chemistry and immunology of rabbit immune globulins.

The most striking evidence pointing to restraints on the electrophoretic diversity of the antibodies was obtained by disc electrophoresis of the light chains. The light chains of normal γG form six to eight bands in disc electrophoresis. The light chains of the two anticarbohydrate antibodies were primarily confined to one or two major bands. This contrasts with the light chains of rabbit antibodies to arsanilic acid or DNP,[12] which showed multiple components on disc electrophoresis.

Evidence for the uniformity of the antibodies described in this report would be strengthened if it were shown that they possessed only one antigenic type of light chain. A preponderance of one allotype in the anticarbohydrate antibodies

over that of a heterozygous rabbit's preimmune γG would also indicate restriction of the antibody population. Such studies are currently under way in collaboration with Dr. Charles Todd.

Eisen and co-workers[13] found that antibodies to DNP proteins formed early in the immune response are more homogeneous than those formed later. These early antibodies may have arisen from a restricted population of cells that had been previously exposed to a cross-reacting antigen.[14, 15] Our results differ in that the antibodies formed earlier in the immune response are more heterogeneous than those formed later. A possible explanation is that the rabbits in the present study had relatively little previous exposure to a cross-reacting antigen. The initial injection could thus be a true primary immunization forming many varieties of antibodies to the complex antigen. It is more difficult to explain the relatively homogeneous secondary response in rabbits that gave a heterogeneous primary response. The frequent large doses of the same antigen used in secondary immunization may have rendered some cell lines immunologically unresponsive, leaving a limited cell population that forms a relatively restricted population of antibodies.

Summary.—Primary immunization of rabbits with streptococcal vaccines sometimes produces relatively homogeneous populations of antibodies to the group-specific carbohydrate. When this type of response does not follow primary immunization, it may be elicited by secondary immunization. The relative homogeneity of these later antibodies is shown by the electrophoretic patterns of the purified antibodies and of their light chains.

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