

CLONAL DOMINANCE

I. Restricted Nature of the IgM Antibody Response to Group A Streptococcal Carbohydrate in Mice*

BY DAVID E. BRILES‡ AND JOSEPH M. DAVIE

(From the Departments of Pathology and Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110)

Immunization of experimental animals with streptococcal or pneumococcal vaccines often leads to the production of large amounts of homogeneous antibodies (1, 2). While these immunoglobulins have proven useful in structural (3-5) and genetic studies (2-9), the immunologic mechanisms leading to their production remain unclear (2). It is possible that large numbers of specific clones are stimulated initially, as with other antigens, but that immunologic (affinity) maturation (10,11) precedes to the extreme. In the case of the antibody response to hapten-coupled proteins, affinity maturation causes the bulk of the antibody produced late in the response to be made by a minority of the antihapten clones stimulated initially (12, 13). A study by Kimball (14) suggested that affinity maturation may play a role in the production of restricted antibody to Type III pneumococcal carbohydrate. However, these results were unusual since individual animals, upon intensive hyperimmunization over 9 mo, had several cycles of restricted responses, each antibody response of higher affinity than that in the previous cycle.

We have re-examined the role of affinity maturation in the response to polysaccharides by measuring the avidity and specificity heterogeneity of early IgM antibodies in mice immunized with Group A streptococci. When A/J mice are hyperimmunized with this antigen they generally produce 10-30 mg/ml of anti-Group A carbohydrate (GAC)¹ antibody with markedly restricted heterogeneity (7, 9, 15-18). By examining the heterogeneity of antibody throughout a response to GAC, we hoped to gain insight into the mechanism leading to the extreme restriction seen late in the response. Our results indicate that IgM antibodies are restricted both early and late in the immune response to GAC and

* This work was supported by U.S. Public Health Service grants AI-11635 and CA-16032 and by the following companies: Brown & Williamson Tobacco Corporation; Larus and Brother Company, Inc.; Liggett & Myers, Incorporated; Lorillard, a Division of Loews Theatres, Incorporated; Philip Morris, Incorporated; R. J. Reynolds Tobacco Company; United States Tobacco Company; and Tobacco Associates, Inc.

‡Supported by U.S. Public Health Service Postdoctoral Fellowship AI-00685.

¹Abbreviations used in this paper: BSB, borate saline buffer; Con A, concanavalin A; GAC Group A carbohydrate; 2-ME, 2-mercaptoethanol; ϕ -NAGlu, *p*-azophenyl- β -*N*-acetylglucosaminide; PBS, phosphate-buffered saline; PFC, plaque-forming cells.

suggest that clonal dominance in this system is not the result of affinity-driven competition for antigen.

Materials and Methods

Mice. Female A/J, SWR/J, and BALB/cJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine. AKR mice were obtained from National Animal Laboratories, St. Louis, Mo. BSVS mice were bred in our colony from BSVS mice obtained from NIH, Bethesda, Md.

Immunization. Strain J174A Group A streptococci (a gift of Dr. R. C. Lancefield, The Rockefeller University, New York) was used to prepare Group A streptococcal vaccine (19). The dose of vaccine injected was expressed in terms of the μg of rhamnose (20) contained in the cell wall of the bacterium (21). The immunization schedule consisted of i.v. injections of vaccine containing 3, 10, 30, and 30 μg of rhamnose on days 0, 7, 14, and 49, respectively.

Primary immunization with sheep erythrocytes (SRBC) consisted of a single i.v. injection of 0.1 ml of 10% packed SRBC. Hyperimmune responses were obtained by boosting 2 wk later with an identical injection.

Radioimmunoassay of Anti-GAC Antibodies. A modified Farr procedure was employed which used ^3H -labeled GAC (15). GAC was isolated from whole lyophilized streptococci by extraction with formamide (19). The carbohydrate was deacetylated (22) and reacylated with [^3H]acetic anhydride (23). Antibody concentrations of test sera were determined by comparing the amount of [^3H]GAC bound by these sera as compared to standard sera whose antibody concentrations had been determined by quantitative precipitation (19).

Preparation of Antigen- or Hapten-Coupled SRBC. SRBC were coated with GAC by a modification of the method of Pavlovskis and Slade (24). GAC (50 mg) was reacted for 3 days at room temperature with 20 μl of palmitoyl chloride (Eastman Kodak Co., Rochester, N. Y.) dissolved in 4 ml dimethyl formamide and 0.6 ml of pyridine. The palmitoyl-GAC was precipitated and washed with acetone, and subsequently dissolved in 45 ml water. Optimal sensitization of SRBC was obtained by adding 133 μl of the palmitoyl-GAC solution to 1.0 ml of packed SRBC in 8 ml of phosphate-buffered saline (PBS: 0.056 M Na_2HPO_4 ; 0.016 M KH_2PO_4 ; 0.077 M NaCl; pH 7.2). Cells were sensitized for 30 min at 37°C, washed in PBS, and suspended in minimum essential medium (MEM, Grand Island Biological Co., Grand Island, N.Y.). For avidity determinations, 1.0-ml portions of packed SRBC were sensitized with 133, 66, 33, 13 and 7 μl of palmitoyl-GAC solution as above to prepare GAC-SRBC preparations 1, 2, 3, 4 and 5, respectively.

p-Azophenyl- β -*N*-acetyl-glucosaminide (ϕ -NAGlu)-coupled SRBC were prepared as follows: 18 mmol *p*-diazonium- β -*N*-acetyl-glucosamine, prepared from *p*-nitrophenyl- β -*N*-acetyl-glucosaminide (Mann Research, New York) (25) was added to 1.5 ml of 25% SRBC in borate saline buffer (BSB: 0.035 M $\text{Na}_2\text{B}_4\text{O}_7$, 0.08 M NaCl, pH 9.1) and stirred at room temperature for 30 min. The ϕ -NAGlu-SRBC were washed and suspended in PBS.

Detection of Antibody-Secreting Cells. Spleen cells were suspended in a final vol of 0.5 ml of 0.56% agarose (Seakem, Rockland, Maine) in alpha-MEM (Flow Laboratories, Rockville, Md.) which contained 1.5% sensitized SRBC. The suspension was poured on microscope slides which were incubated sequentially for 1 h at 37°C and 1 h in the presence of 5% fresh frozen guinea pig complement in MEM. Indirect plaques were visualized either by adding 2% antimouse IgG sera to the complement solution or by incubating the slides for 1 h with 1% anti-IgG before incubation with complement. The antimouse IgG sera consisted of a mixture of goat anti-IgG₁ and anti-IgG₂ mouse myeloma proteins.

IgM plaques were blocked by adding an appropriate dilution of goat anti- μ antiserum to the cell suspension in the agarose. Inhibition of plaques with concanavalin A (Con A, Grade III, Sigma Chemical Co., St. Louis, Mo.) (26) was performed by incubating the slides with various concentrations of Con A in MEM for 1 h at 37°C after an initial 1 h incubation at 37°C and before the incubation with complement and/or facilitating serum. Control slides, not exposed to Con A, were incubated with MEM during this hour. Inhibition of IgM PFC with 2-mercaptoethanol (2-ME) (27, 28) was performed by incubating the slides with various concentrations of 2-ME in BSB for 1 h before exposure to complement. Control slides were incubated in BSB without 2-ME. The slides were then washed several times in saline for 1 h before incubation with complement and/or facilitating antiserum. Plaque diameters were measured, after fixation in 2% glutaraldehyde, under 63 \times

magnification with a graticule that had been calibrated with a stage micrometer (E. Leitz, Wetzlar, Germany).

Determination of Avidity. Relative avidity of antibody secreted by individual cells can be estimated either by inhibition of plaque formation with soluble hapten (12, 29) or, as in this study, by varying the epitope density on the target erythrocyte (30). Thus, high affinity antibody will lyse both lightly and heavily conjugated erythrocytes, whereas low affinity antibody will only lyse heavily conjugated cells. Each spleen cell suspension was plaqued using the five GAC-SRBC preparations, 1, 2, 3, 4 and 5, which varied from high to low epitope density and normal SRBC. Anti-SRBC PFC were subtracted from the number of PFC observed with the GAC-SRBC preparations. The fraction of the maximum number of anti-GAC PFC observed was calculated for each GAC-SRBC preparation. The relative number of PFC in the avidity subgroups 1-2 (lowest avidity), 2-3, 3-4, 4-5, and 5-0 (highest avidity) was calculated by subtracting the relative number of PFC observed with the GAC-SRBC designated by the second number from the relative number of PFC observed with the GAC-SRBC designated by the first number. The relative number of PFC in subgroup 5-0 was the fraction of total PFC observed with the lowest epitope density GAC-SRBC, preparation 5. In some cases, where the mice were not plaqued against GAC-SRBC preparation 5, the highest avidity subgroup was 4-0. A coefficient of restriction was calculated for each avidity subgroup profile using the formula

$$R = 10 \sqrt{\sum_{n=1}^5 S_n^2} - 4.47$$

where R is the coefficient of restriction, S is the fraction of PFC in each of the five individual avidity subgroups. The more unequal the distribution of PFC in the different subgroups, the more restricted the response and the higher the coefficient of restriction. The theoretical minimum and maximum of this function are 0 and 5.53, respectively.

The average avidity of the PFC of an individual spleen or pool of spleens can be calculated from the following sum of products: average avidity = $(S_1 \times 1) + (S_2 \times 2) + (S_3 \times 3) + (S_4 \times 4) + (S_5 \times 5)$, where S_1, S_2, S_3 , etc. are the fraction of PFC in avidity subgroups 1-2, 2-3, 3-4, 4-5, and 5-0, respectively.

Hemagglutination of Anti-GAC Sera. Antisera were titrated in twofold dilutions in 25 μ l 1% fetal calf serum using microtiter apparatus (Cooke Engineering Company, Alexandria, Va.). 25 μ l of 0.75% GAC-SRBC, preparation 2, were added to each antiserum dilution. 2-ME inhibition of IgM was performed by incubating the serum in 0.1 M 2-ME for 1 h before titration.

In Vivo Propagation of Antibody-Producing Cell Clones. This procedure has been previously described (15) and is a modification of that used by Askonas et al. (31).

Results

Kinetics of Anti-GAC Antibody Production. Mice of strains A, AKR, SWR, BALB/c and BSVS produce greatly increased numbers of direct PFC after immunization with streptococcal Group A vaccine. Fig. 1 shows the kinetics of the direct PFC responses for strains A, AKR, and SWR. After three injections the number of PFC in all three strains rose above 1,000 PFC/10⁶ spleen cells. In AKR mice approximately 1% of the nucleated spleen cells secreted anti-GAC antibody after the fourth injection. The larger numbers of PFC seen after injections 3 and 4 were not merely the result of the larger antigen doses, since the same dose given to unprimed mice gave responses of 231, 41, and 0.5 PFC/10⁶ for strains A, AKR, and SWR, respectively.

The relationship of these high direct PFC responses to serum antibody levels in strain A mice is shown in Fig. 2. It can be seen that assays which favor IgM antibody (direct PFC and ME-sensitive HA) and IgG antibody (ME-resistant HA and serum antigen-binding capacity) have similar kinetics. The average

RESTRICTED IgM ANTIBODY

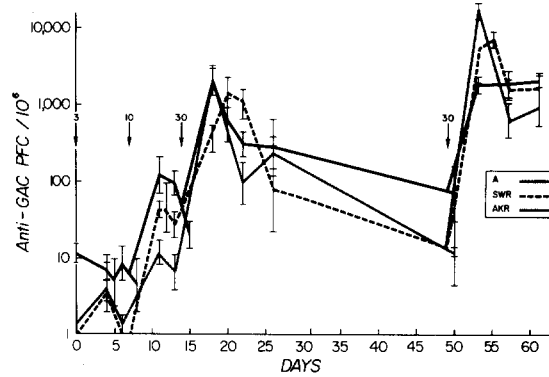


FIG. 1. Kinetics of direct PFC response to GAC of strain A, AKR, and SWR mice. Arrows indicate immunizations at days 0, 7, 14 and 48 with 0.1 ml of Group A vaccine adjusted to contain 3, 10, 30, and 30 μ g of rhamnose, a component of GAC. Each point and brackets are the mean and standard error of values from 5 to 14 mice.

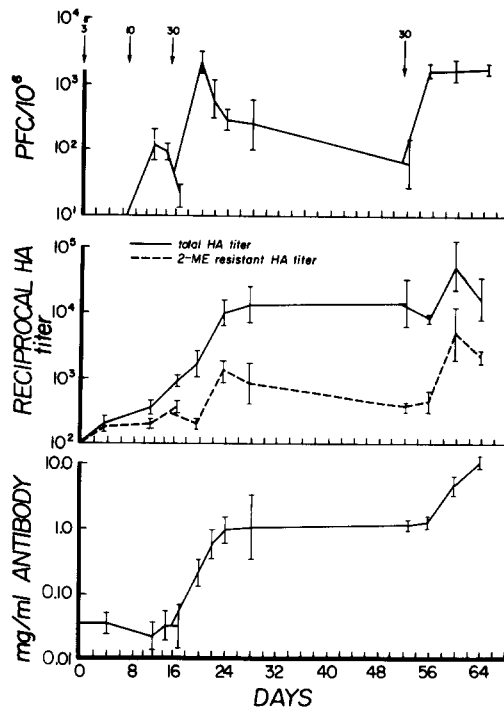


FIG. 2. Anti-GAC response of strain A mice immunized with Group A vaccine, as monitored by PFC/10⁶ spleen cells, serum HA titers and mg antibody/ml serum. The immunization schedule was the same as in Fig. 1.

concentration of antibody increased to 1 mg/ml after the third immunization and to 10 mg/ml after the fourth injection. Studies with AKR and SWR mice showed similar concordance between PFC kinetics and serum antibody. Table I shows the number of direct PFC/10⁶ spleen cells and the concentration of serum

TABLE I
 Comparison of PFC and Serum Antibody Produced by Five Strains of Mice upon
 Immunization with Streptococcal Group A Vaccine

Mouse strain	Days after injection III				Days after injection IV			
	4		12		4		12	
	GAC PFC/10 ⁶	Anti-GAC	GAC PFC/10 ⁶	Anti-GAC	GAC PFC/10 ⁶	Anti-GAC	GAC PFC/10 ⁶	Anti-GAC
		<i>mg/ml</i>		<i>mg/ml</i>		<i>mg/ml</i>		<i>mg/ml</i>
A	2,250* (1.4)‡	0.213 (1.6)	270 (2.3)	1.09 (3.1)	1,850 (1.3)	1.38 (1.2)	2,040 (1.2)	11.30 (1.2)
AKR	1,980 (1.5)		235 (1.6)	0.92 (1.2)	16,800 (1.3)	0.51 (1.2)	980 (1.8)	10.00 (1.8)
SWR	351 (1.5)		79 (3.7)	0.28 (1.6)	5,300 (2.7)	0.45 (1.8)	1,700 (1.6)	5.10 (1.2)
BALB/c	1,900 (1.4)						1,086 (1.5)	1.84 (1.6)
BSVS	63 (1.6)						39 (3.6)	0.03 (1.3)

* Geometric mean of values obtained from 4 to 14 mice.

‡ Standard error term is the number by which the geometric mean must be multiplied or divided to yield the upper and lower bounds of the standard error respectively.

anti-GAC antibody for five inbred strains at various time points in the immunization sequence. Compared to the other four strains of mice, BSVS mice failed to show large numbers of direct PFC or serum antibody.

Class of Antibody Secreted by PFC. Animals hyperimmunized with streptococcal vaccines characteristically produce high concentrations of IgG antibody (7, 9, 15–18). However, indirect (facilitated) PFC were never observed in any of the mouse strains in numbers greater than 10% of the number of direct plaques, even after the fourth injection when large amounts of IgG antibody were produced. This is demonstrated in Table II where three hyperimmune A/J mice were examined 10 days after their fifth injection of Group A vaccine. In none of these mice were significant numbers of indirect plaques observed, in spite of the fact that the serum concentration of anti-GAC antibody ranged from 30–41 mg/ml. The bulk of the antibody in the sera of these three mice was shown to be IgG after isolation and immunoelectrophoresis as described previously (7, 15). Since, in rabbits, Read and Braun (32) have shown that both IgM and IgG anti-GAC PFC are direct PFC, it was crucial to our studies to provide additional characterization of the mouse direct PFC.

There are three simple techniques which have been used to inhibit selectively IgM PFC, anti- μ serum, Con A, and 2-ME. These three reagents were tested against four splenic PFC populations: direct (IgM) anti-SRBC PFC from mice 4 days after primary immunization with SRBC, indirect (IgG) anti-SRBC PFC from mice hyperimmunized with two or three biweekly injections of SRBC, direct anti-GAC PFC 4 days after primary immunization with streptococcal vaccine, and direct PFC from GAC hyperimmune mice 10–15 days after the fourth immunization. By all criteria, the anti-GAC PFC are IgM (Fig. 3). Thus, both the anti-GAC and the anti-SRBC direct PFC were completely inhibited at 0.014 M 2-ME, while indirect anti-SRBC were only slightly affected. Similarly, the Con A and anti- μ serum both inhibited the anti-GAC and direct anti-SRBC PFC at much lower concentrations than were required to inhibit indirect anti-SRBC PFC. Fig. 3 B and C also illustrate the fact that 4-day primary and hyperimmune anti-GAC PFC gave inhibition curves that did not differ significantly from each other. The data in Fig. 3 were all derived from strain A mice. Results with AKR

TABLE II
*Direct and Indirect Anti-GAC PFC Responses of Three Mice Hyperimmunized with Group A Vaccine**

Mouse	Anti-GAC mg/ml	Direct anti-GAC PFC/10 ⁶	Indirect anti-GAC PFC/10 ⁶	Indirect/ Direct
1	30	2,860	128	0.04
2	41	1,030	62	0.06
3	35	960	96	0.10

* Mice were injected i.v. with doses of vaccine containing 3, 10, 30, 30, and 30 μ g of rhamnose, as a constituent of the GAC in the cell wall, on days 0, 7, 14, 49, and 110, respectively, and bled 12 days after the last injection.

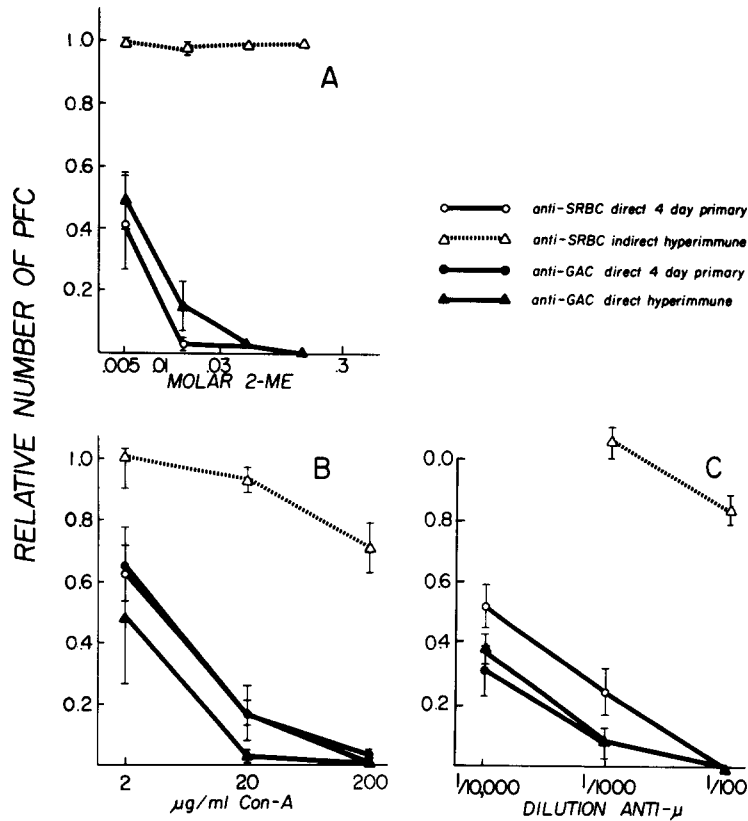


FIG. 3. Inhibition of anti-GAC and anti-SRBC PFC in strain A mice by 2-ME, Con-A, and anti- μ serum. See text for immunization details.

and SWR mice were essentially identical, which indicates that the PFC in these mice similarly secreted IgM anti-GAC antibody.

Avidity Restriction of IgM PFC. The relative avidities of anti-GAC IgM antibodies produced by different populations of PFC have been compared by plaquing spleen cells from individual mice against SRBC coated with various

amounts of GAC. Avidity comparisons are possible since both high and low avidity anti-GAC antibodies would be expected to plaque GAC-SRBC coated with a high density of GAC, whereas only the high avidity antibodies would be expected to form plaques in SRBC coated with a low density of GAC. This point is illustrated in Fig. 4 which shows the avidity data for the anti-GAC response of two AKR mice. In Fig. 4 A the relative number of PFC for each of the GAC-SRBC preparations is plotted. The PFC population depicted by the solid line has cells which produce relatively high avidity antibody since almost 60% as many PFC were observed with the GAC-SRBC bearing the lowest density of GAC as with

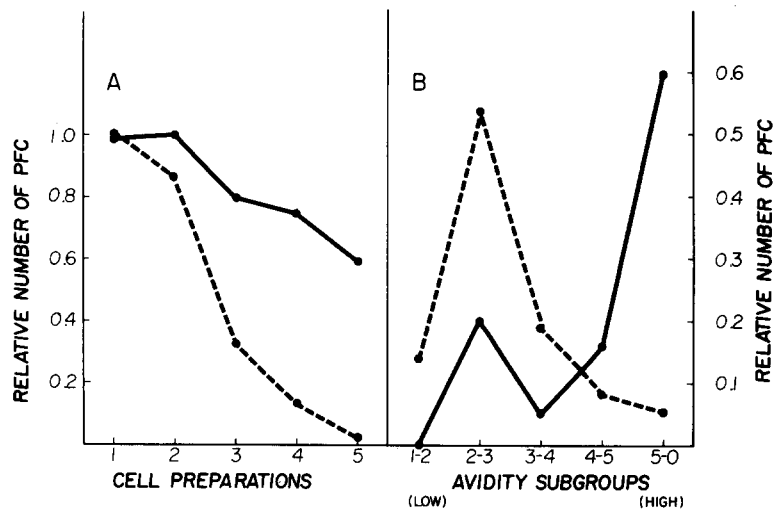


Fig. 4. (A). Relative number of anti-GAC PFC obtained using GAC-SRBC preparations 1-5 with immune spleen cells from two AKR mice. GAC-SRBC preparations 1, 2, 3, 4, and 5 are coated with decreasing amounts of GAC. (B). Avidity subgroups calculated from the data in Fig. 4 A, for the PFC of the two AKR mice. The subgroups are of increasing avidity going from 1-2 to 5-0.

the SRBC bearing the highest density GAC. On the other hand, the cell population producing low avidity anti-GAC IgM (dashed line) produced almost no plaques with the lowest epitope density GAC-SRBC. The differences in avidity of the PFC of these two mice is made even more clear by plotting the data in terms of avidity subgroups, where the number of additional PFC visualized by each successively higher density of GAC on the SRBC are expressed as a fraction of the total number of PFC (Fig. 4 B). This treatment of the data subdivides the total PFC population from a given spleen into five subgroups based on their avidities. The average avidity for each spleen (see methods) are 4.4 and 2.4 for the high and low avidity spleens, respectively.

Fig. 5 shows the avidity subgroup profiles for 16 strain A mice which had been immunized for different time intervals. It is apparent from the shape of the curves and from the coefficient of restriction (see methods) that the IgM anti-GAC responses of most of the individual mice show restricted heterogeneity of their avidities. For example, the lowest avidity subgroup, 1-2, contains over

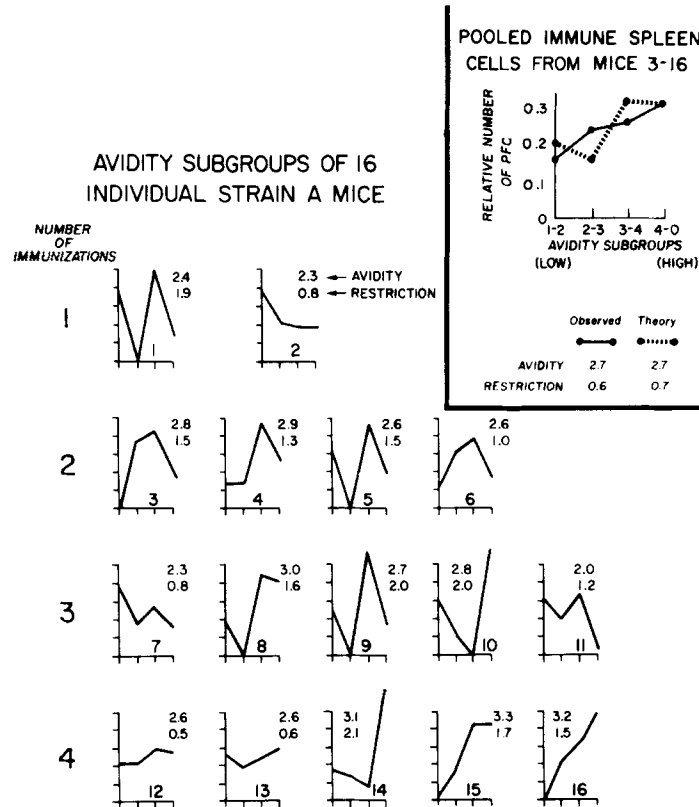


FIG. 5. Avidity subgroups for 16 strain A mice 4 days after the 1st, 2nd, 3rd, and 4th immunizations with streptococcal Group A vaccine. Average avidities and coefficients of restriction are given for each curve. Also shown are the avidity subgroups for a pool of immune spleen cells from mice 3-16.

30% of the PFC from mice 1,2,5,7,10, and 11 but none of the PFC from mice 3, 15 and 16. Likewise, avidity subgroup 2-3 comprises over 30% of the PFC response of mice 3 and 6, but none of the response in mice 1, 5, 8, and 9. Subgroup 3-4 which is a major subgroup of about two thirds of these mice comprised none of the response of mouse 10 and was less than 10% of the response of mouse 14. Similar contrasts can be made for subgroup 4-0.

Avidity differences obtained by this method were shown to be reproducible by mixing spleen cells with different avidities to obtain mixtures that possessed a more heterogeneous distribution of PFC. An example of this type of analysis is seen in Fig. 5, where the predicted and observed avidity intervals are plotted for a pool of anti-GAC spleen cells from 14 mice (mice 3-16). The coefficient of restriction has been calculated for the pool as well as for the mice making up the pool. These values along with average avidities are given along side each profile. The greater heterogeneity of PFC avidity for the pool as compared to that for the individual mice is apparent from a comparison of the shapes of the avidity subgroup profiles as well as a comparison of the coefficients of restriction for the PFC pool and the individual mice. It can be seen that the theoretical values for the pool compare favorably with the observed values.

A second demonstration of the reliability of the avidity assay involved the transfer of immune spleen cells to sublethally irradiated recipients. If the avidity assay were accurately measuring avidity we would expect all of the recipients from a given donor's immune spleen cells to produce PFC with similar avidities. In the experiment depicted in Fig. 6 spleen cells from two donor mice were injected i.v. into four sublethally irradiated recipients. Each recipient was immunized and assayed 4 days later. It is clear that the avidity profiles within a group of recipients are indistinguishable.

It has been suggested (33) that the rate of antibody secretion could seriously influence the apparent avidity measurements using plaque assays, so that cells secreting large amounts of antibody may be difficult to inhibit, regardless of the

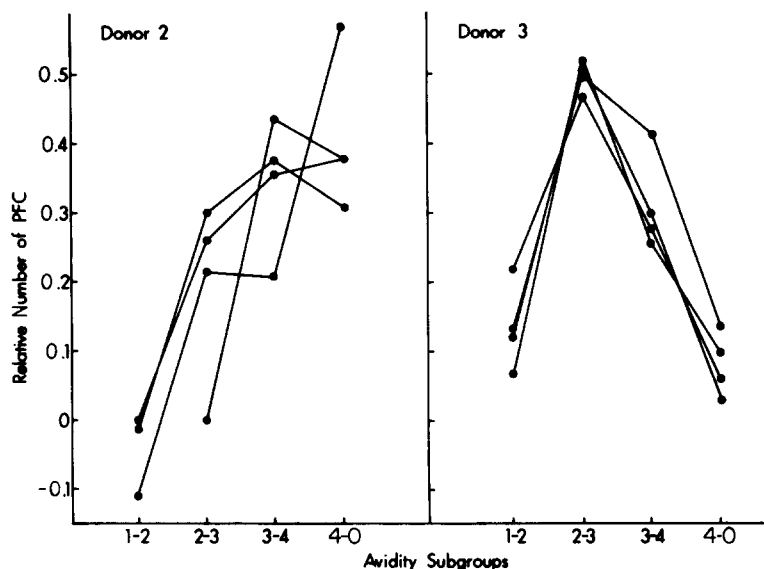


FIG. 6. Avidity subgroups of recipients of immune spleen cells from two strain A donor mice harvested 10 days after hyperimmunization with four injections of Group A vaccine (see Materials and Methods). Recipient strain A mice were given 500 rads X-radiation 1 day before cell transfer. The spleens of the recipient mice were plaqued 4 days after injection with an amount of Group A vaccine containing 30 μ g of rhamnose. The recipients of donor 1 produced no more than background PFC.

avidity of the antibody. We therefore measured the plaque sizes in several of the mice producing restricted anti-GAC PFC (Fig. 7). The data shown were obtained with strain A mice; essentially identical findings were obtained with SWR mice. Plaque size was found to vary inversely with average avidity. These results indicate that in general the avidity differences described were not due to different average rates of immunoglobulin secretion from PFC of different mice, since, by our assay, "high" avidity antibody producers make small PFC as expected (28). One possible exception was the PFC depicted in the lower left of Fig. 7. The plaques of this mouse were unusual in that they showed incomplete lysis and were present only at the highest epitope densities. These rare PFC may represent clones producing less than the usual amounts of antibody per cell.

Specificity Restriction of IgM PFC. Restriction in the heterogeneity of IgM

RESTRICTED IgM ANTIBODY

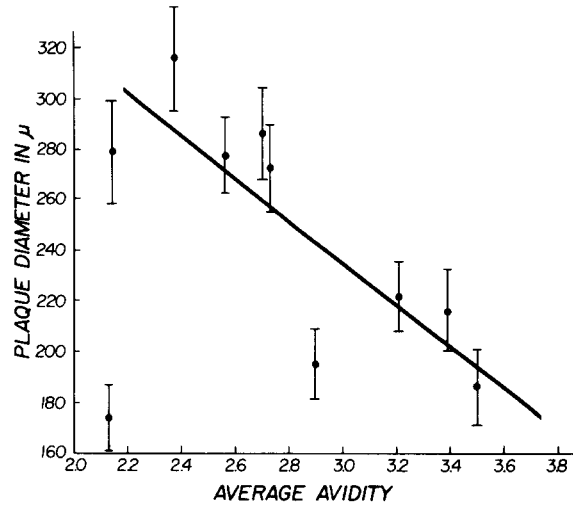


FIG. 7. Comparison of average plaque diameter and average avidity of anti-GAC PFC from 10 A/J mice immunized with Group A streptococcal vaccine. The correlation coefficient of the plaque diameter with average avidity for all 10 mice is -0.42 . If the point at the lower left is omitted from the calculation, the correlation coefficient is -0.83 . These two correlation coefficients are statistically different from zero at 10% and 1%, respectively.

anti-GAC antibodies was also demonstrated by plaquing the spleens of mice immunized with Group A vaccine against SRBC coated with ϕ -NAGlu, a haptenic determinant which cross-reacts to varying degrees with anti-GAC antibodies (34). The results for 13 A/J mice at three stages in the immunization schedule are given in Table III. In 10 of these mice, less than 33% of anti-GAC antibody-secreting cells formed plaques with ϕ -NAGlu-SRBC. In three mice 49, 66, and 96% of the anti-GAC cells formed plaques on ϕ -NAGlu-SRBC. This result is not a reflection of the avidity of the antibody for GAC (Table III), but probably represents variability among the immunodominant determinants detected by different anti-GAC antibodies.

Kinetics of Avidity Restriction. In all five strains of mice tested restriction in the heterogeneity of avidity was seen, even in the earliest anti-GAC responses (Fig. 5). The lack of increase in restriction or avidity of the IgM response with time is illustrated in detail for strain A in Fig. 8, where the average coefficients of restriction and average avidities are measured throughout the course of immunization. No significant changes were seen in this strain or with other strains examined. Average coefficients of restriction and avidities for the five mouse strains are shown in Table IV. While all strains produced about equally restricted IgM antibodies, significant differences exist between strains with respect to the average avidities of the antibodies. Thus, BSVS and SWR mice produce significantly higher avidity IgM antibody than do strains A or AKR ($P < 0.001$).

Discussion

The usefulness of the plaque assay in the evaluation of the relative avidity and specificity of an antibody response is apparent. In addition to the obvious ease

TABLE III
Specificity of Strain A PFC for GAC and ϕ -NAGlu

No. of im- munizations	Mouse	PFC/10 ⁶ spleen cells*		Anti-GAC avidity‡	ϕ -NAGlu/ GAC ratio §
		Anti-GAC	Anti- ϕ -NAGlu		
2	1	656	23	2.73	0.04
	2	358	111	3.11	0.31
	3	237	116	2.86	0.49
3	4	246	2	2.80	0.01
	5	521	8	2.23	0.02
	6	1,054	59	3.58	0.06
	7	129	85	2.36	0.66
	8	424	407	2.38	0.96
4	9	2,833	765	2.34	0.27
	10	679	115	2.50	0.17
	11	833	125	2.70	0.15
	12	5,172	362	3.08	0.07
	13	1,643	444	4.04	0.27

* All mice were plaqued on the same day; the last immunization was 4, 46 and 13 days previously for mice given 2, 3, and 4 immunizations, respectively. For anti-GAC PFC the spleens were plaqued against the preparation of GAC-SRBC with the highest density at GAC. For anti- ϕ -NAGlu spleens were plaqued against *p*-azophenyl- β -*N*-acetyl-glucosamine coupled SRBC.

‡ Average avidities were determined as described in text.

§ Ratio of the numbers of PFC obtained with ϕ -NAGlu-SRBC divided by the number of PFC obtained with GAC-SRBC.

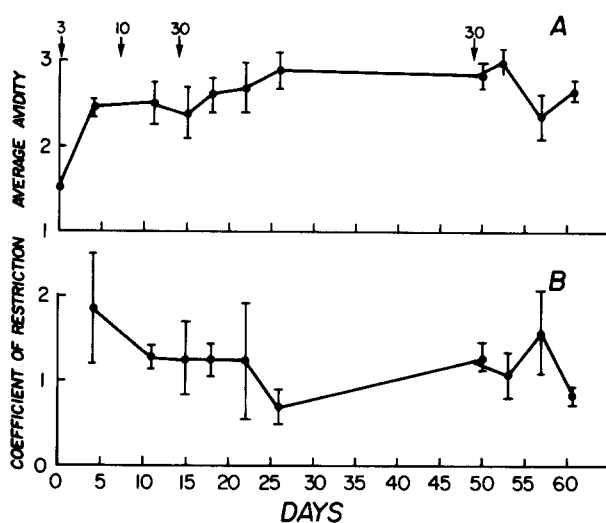


FIG. 8. (A). Mean and standard errors of the average avidities of anti-GAC PFC from spleens of strain A mice before immunization, and at 10 times during immunization with streptococcal Group A vaccine. The immunization schedule and mice are the same as those for Fig. 1. (B). Coefficients of restriction of the anti-GAC PFC from the immunized mice whose mean avidities are plotted in Fig. 8 A.

RESTRICTED IgM ANTIBODY

TABLE IV
Average Avidity and Coefficient of Restriction of Direct Anti-GAC
PFC from Five Inbred Mouse Strains

Mouse strain	No. of Mice	Avidity	Coefficient of restriction
BSVS	9	3.57 ± 0.25	1.91 ± 0.24
SWR	28	3.15 ± 0.10	1.38 ± 0.14
BALB/c	10	2.91 ± 0.20	1.10 ± 0.24
A	43	2.68 ± 0.09	1.15 ± 0.10
AKR	39	2.59 ± 0.07	1.34 ± 0.12

* *P* values by Student's *t* test for avidity average comparisons:

BSVS vs. AKR,	<0.001
BSVS vs. SWR,	<0.1
SWR vs. AKR,	<0.001
SWR vs. A,	<0.001
SWR vs. BALB/c,	<0.4
BALB/c vs. AKR,	<0.05

and speed that the method offers, it is possible to characterize antibody responses before significant serum concentrations of antibody are achieved. At the same time, the present study demonstrates an obvious deficiency of the plaque technique, i.e., a dependence on the hemolytic capacity of an antibody. Thus, animals which possessed greater than 10 mg/ml of specific IgG anti-GAC antibody had insignificant numbers of IgG PFC, regardless of epitope density on the red cells, facilitating sera, etc. It is probable that this was due to the low affinity of the IgG antibody, because isoelectric focusing of the serum antibody, which concentrated the antibody to higher levels than achieved around a single antibody-secreting cell, resulted in the hemolysis of GAC-SRBC.² On the other hand, it is theoretically possible that cells secreting very high affinity antibody will not be detected as PFC since plaque size, as shown here, is inversely proportional to the avidity of the antibody secreted. Thus, it is likely that a fraction of total IgG and possibly IgM-secreting cells are ignored in the analysis of the PFC response to most antigens.

Since the pioneering studies of Kunkel, Kabat, and colleagues with human antipolysaccharide antibodies it has been clear that polysaccharides often elicit antibodies with unusually restricted heterogeneity (35-37). In addition, hyperimmunization with polysaccharide-containing bacterial vaccines often leads to massive amounts of these restricted antibodies (1, 2). The studies reported here extend these findings by demonstrating that mice which are producing large amounts of relatively homogeneous IgG antibody also are producing large amounts of restricted IgM antibody. For example, in AKR mice hyperimmune to GAC, approximately 1% of nucleated spleen cells secrete IgM antibody. Of the five strains of mice tested, only BSVS mice failed to produce large amounts of either IgM or IgG antibody (18). It is interesting that the IgM antibodies produced by BSVS are the highest in average avidity of the strains tested.

²Briles, D. and J. Davie. Detection of isoelectric focused antibody by autoradiography and hemolysis of antigen-coated erythrocytes. A comparison of methods. Manuscript submitted for publication.

Multiple hypotheses (2, 14, 38) have been advanced to account for the large amounts of restricted antipolysaccharide antibody, but none is totally satisfactory. In some antipolysaccharide responses, such as that of mice to the phosphorylcholine moiety of pneumococcal C carbohydrate, all mice within an inbred strain make antibodies with the same binding site specificity and the same idiotype (39, 40). In this case, it is clear that mice have a genetically limited repertoire of precursor cells with specificity for phosphorylcholine (41, 42). With other restricted antipolysaccharide antibody responses, such an explanation will not suffice. For example, in the immune response to streptococcal group or pneumococcal type carbohydrates, where continued immunization with bacterial vaccines leads to the production of large amounts of restricted IgG, antipolysaccharide antibodies, it has been shown that individual animals (1, 2) or inbred lines² are capable of producing many different antibodies, but generally restrict their response to one or two major components.

It has been suggested that affinity maturation may in fact be responsible for the restricted antibody response to polysaccharides (2, 14, 38). By this mechanism, which has been shown to operate in the antibody response to proteins and hapten-protein conjugates (10-13), the initial antibody response is heterogeneous and includes both low and high affinity antibodies. With time, the concentration of antigen decreases so that only those clones with high affinity receptors are stimulated to secrete antibody (13). The result of this precursor cell selection is a gradual rise in the affinity of serum antibody. Kimball (14), in a study of the antibody response to type III pneumococcal carbohydrate in individual rabbits, concluded that indeed affinity maturation takes place with this antigen. However, it is not clear that the maturation he observed was responsible for the restricted antibody response since even the earliest antibodies he studied (1 wk after immunization) were already highly restricted, but of low affinity. It is equally possible that the gradual increase in affinity was a reflection of other control mechanisms resulting from the intensive immunization protocol where animals received weekly injections of vaccine for 9 mo.

Our results are not consistent with the hypothesis that affinity maturation is responsible for the restricted response to Group A carbohydrate. We found that individual mice produced IgM antibodies which were restricted in terms of avidity and specificity at all times of observation. It was clear that no change in average avidity of the IgM antibody occurred with time or after multiple immunizations. There are several possible interpretations of our findings in relation to the appearance of the massive, homogeneous IgG response seen with hyperimmunization: (a) There may be no memory induced with the early immunization so that each injection of vaccine randomly stimulates new dominant clones. Therefore, the late homogeneous IgG antibody may be unrelated to the early IgM antibodies we have studied. (b) Memory might be induced among the IgM-responding cell populations, so that all IgM antibodies of an individual animal are derived from the initial dominant clone, while the homogeneous IgG antibody may be derived from different precursors. It is clear from hapten-protein studies that the requirements for the stimulation of IgM and IgG production are different (43, 44). It is therefore conceivable that the regulation of IgM and IgG antibody production is also different. A further extension of this possibility is that avidity maturation may be present in the IgG response to GAC but is not reflected in the IgM response (45) because the clones which develop the IgM, and which fail to switch to IgG production, may be short lived, and therefore are continually replaced by clones of random avidity. (c) The clones which give rise to the early

restricted IgM antibodies may be the same clones which secrete massive amounts of IgG antibody after hyperimmunization. This possibility, which we favor, can be distinguished experimentally from the others since it would be expected, in this case, that early IgM and late IgG antibodies from individual animals would share idiotypic (variable region) determinants. Studies are underway in this laboratory to resolve this important question.

Regardless of the relationship of the IgM response to the restricted IgG antibody, it is unclear why only some GAC-specific clones are stimulated to make IgM antibody. Transfer studies (15) have established that a responding clone inhibits, in some fashion, the response of other precursor cells (46). It is by no means clear whether this effect is mediated through active control by, for example, specific suppressor cells (47), or by more passive means such as by massive, random expansion of individual clones so that their progeny may constitute the bulk of potential precursors at subsequent exposures to antigen. These crucial questions remain to be explored.

Finally, it is possible that the restriction of the response and its magnitude are not related events. Many antigens have been described now which give restricted but often meager responses. These include antigens with a repeating unit structure, such as dextran (48) and dinitrophenyl-coated artificial membranes (49). It may be that such molecules, because of their repetitive nature, are exceedingly efficient at triggering individual cells. At the same time, many of these molecules are tolerogenic (50), possibly for the same reason, so that "clonal dominance" may in fact be, as Haber suggested, a complex and random balance between stimulation and tolerization (51). The magnitude of a response is itself dependent on genetically controlled factors (52) which could have multiple levels of control. These could include the development of suppressor functions (53), participation of accessory cells (54), metabolism of antigenic determinants (50), antibody feedback (55), etc., each of which could contribute to the regulation of the amount of antibody produced. Therefore, the seemingly simple, and highly repeatable, phenomenon of clonal dominance as seen in the response to bacterial polysaccharides may be multifactorial. Answers to many of the basic questions concerning the nature of clonal dominance should provide insight not only to our knowledge about the immune response to polysaccharides, but also to our appreciation of the complex mechanisms which control the immunologic machinery.

Summary

The IgM antibody response of mice to the streptococcal group A carbohydrate (GAC) was measured. With most strains tested, large amounts of IgM antibody were produced; in AKR mice, over 1% of the total nucleated spleen cells secreted IgM anti-GAC antibody after hyperimmunization. The relative avidity of the antibody was estimated by a modification of the Jerne plaque assay where spleen cells from individual mice were tested against erythrocytes with varying GAC epitope density. These studies showed that the earliest, as well as latest, IgM antibodies produced were highly restricted in avidity heterogeneity. No evidence of affinity maturation was seen upon hyperimmunization. These data favor the conclusion that the restricted IgG response seen in mice hyperimmunized to GAC is not the result of affinity driven competition for antigen among precursor cells.

These studies profited from discussions with Dr. Latham Claflin, Dr. Stanley Read, and Mr. Robert Warren. We would like to thank Suzanne Murphy for excellent technical assistance.

Received for publication 19 February 1975.

References

1. Krause, R. M. 1970. The search for antibodies with molecular uniformity. *Adv. Immunol.* **12**: 1.
2. Haber, E. 1971. Homogeneous elicited antibodies: Induction, characterization, isolation and structure. *Ann. N.Y. Acad. Sci.* **190**: 285.
3. Braun, D. G., and J.-C. Jaton. 1974. Homogeneous antibodies: induction and value as probe for the antibody problem. *Curr. Top. Microbiol. Immunol.* **66**: 29.
4. Chen, K. C. S., T. J. Kindt, and R. M. Krause. 1974. Amino-acid sequence of an allotype b4 light chain from a rabbit antibody to streptococcal carbohydrate. *Proc. Natl. Acad. Sci. U.S.A.* **71**: 1995.
5. Strosberg, A. D., K. J. Fraser, M. N. Margolies, and E. Haber. 1972. Amino acid sequence of rabbit pneumococcal antibody. I. Light-chain cysteine-containing peptides. *Biochemistry.* **11**: 4978.
6. Eichmann, K., A. S. Tung, and A. Nisonoff. 1974. Linkage and rearrangement of genes encoding mouse immunoglobulin heavy chains. *Nature (Lond.)*. **250**: 509.
7. Briles, D. E., and R. M. Krause. 1974. Mouse strain-specific idiotypy and interstrain idiotypic cross-reactions. *J. Immunol.* **113**: 522.
8. Kindt, T. J., A. L. Thunberg, M. Mudgett, and D. G. Klapper. 1974. A study of V_H region genes using allotypic and idiotypic markers. In *The Immune System, Genes, Receptors, Signals*. E. E. Sercarz, A. Williamson, and C. F. Fox, editors. Academic Press, Inc., New York. 69.
9. Cramer, M., and D. G. Braun. 1974. Genetics of restricted antibodies to streptococcal group polysaccharides in mice. I. Strain differences of isoelectric focusing spectra of Group A hyperimmune antisera. *J. Exp. Med.* **139**: 1513.
10. Eisen, H. N., and G. W. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochemistry.* **3**: 996.
11. Siskind, G. W., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. *Adv. Immunol.* **10**: 1.
12. Davie, J. M., and W. E. Paul. 1972. Receptors on immunocompetent cells. V. Cellular correlates of the "maturation" of the immune response. *J. Exp. Med.* **135**: 660.
13. Davie, J. M., and W. E. Paul. 1973. Immunological maturation. Preferential proliferation of high affinity precursor cells. *J. Exp. Med.* **137**: 201.
14. Kimball, J. W. 1972. Maturation of the immune response to type III pneumococcal polysaccharide. *Immunochemistry.* **9**: 1169.
15. Briles, D. E., and R. M. Krause. 1972. Mouse antibodies to Group A streptococcal carbohydrate; use of idiotypy to detect inbred strain specificity and to monitor spleen cell transfer in syngeneic mice. *J. Immunol.* **109**: 1311.
16. Braun, D. G., B. Kindred, and E. B. Jacobson. 1972. Streptococcal Group A carbohydrate antibodies in mice: evidence for strain differences in magnitude and restriction of the response, and for thymus dependence. *Eur. J. Immunol.* **2**: 138.
17. Eichmann, K. 1972. Idiotypic identity of antibodies to streptococcal carbohydrate in inbred mice. *Eur. J. Immunol.* **2**: 301.
18. Briles, D. E. 1973. Studies on the inheritance of idiotypic markers of mouse antibody to streptococcal Group A carbohydrate and on other genetic factors which influence the immune response. Ph.D. Thesis. The Rockefeller University, New York.
19. McCarty, M., and R. C. Lancefield. 1955. Variation in the groupspecific carbohydrate on Group A streptococci. I. Immunochemical studies of the carbohydrates of variant strains. *J. Exp. Med.* **102**: 11.
20. Dische, Z., and L. B. Shettles. 1948. A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. *J. Biol. Chem.* **175**: 595.

21. Krause, R. M., and M. McCarty. 1961. Studies on the chemical structure of the streptococcal cell wall. I. The identification of a mucopeptide in the cell walls of Groups A and A-variant streptococci. *J. Exp. Med.* **114**: 127.
22. Kristiansen, T. L. Sundberg, and J. Porath. 1969. Studies on blood group substances. II. Coupling of blood group substance A to hydroxyl-containing matrices, including aminoethyl cellulose and agarose. *Biochim. Biophys. Acta.* **184**: 93.
23. Kuhn, R., and W. Kirschenlohr. 1956. Darstellung von *N*-acetyl-lactosamine (4- β -D-galaktopyranosyl-desoxy-2-acetamino-D-glucopyranose) aus lactose. *Annalen der Chemi.* **600**: 135.
24. Pavlovskis, O., and H. D. Slade. 1969. Adsorption of ³H-fatty acid esters of streptococcal Groups A and E cell wall polysaccharide antigens by red blood cells and their effect on hemagglutination. *J. Bacteriol.* **100**: 641.
25. Chesebro, B., and H. Metzger. 1972. Affinity labeling of a phosphorylcholine binding mouse myeloma protein. *Biochemistry.* **11**: 766.
26. Nordin, A. A., H. Cosenza, and W. Hopkins. 1969. The use of concanavalin A for distinguishing IgM from IgG antibody producing cells. *J. Immunol.* **103**: 859.
27. Hosono, M., and S. Muramatsu. 1972. Use of 2-mercaptoethanol for distinguishing between IgM and IgG antibody producing cells of mice immunized with bovine γ globulin. *J. Immunol.* **109**: 857.
28. Jerne, N. K., C. Henry, A. A. Nordin, H. Fuji, A. M. C. Koros, and I. Lefkovits. 1974. Plaque forming cells: methodology and theory. *Transplant. Rev.* **18**: 130.
29. Andersson, B. 1970. Studies on the regulation of avidity at the level of the single antibody forming cell. *J. Exp. Med.* **132**: 77.
30. Pasanen, V. J., and O. Mäkelä. 1969. Effect of the number of haptens coupled to each erythrocyte on haemolytic plaque formation. *Immunology.* **16**: 399.
31. Askonas, B. A., A. R. Williamson, and B. E. G. Wright. 1970. Selection of a single antibody-forming cell clone and its propagation in syngeneic mice. *Proc. Natl. Acad. Sci. U.S.A.* **67**: 1398.
32. Read, S. E., and D. G. Braun. 1974. *In vitro* antibody response of primed rabbit peripheral blood lymphocytes to Group A variant streptococcal polysaccharide. *Eur. J. Immunol.* **4**: 422.
33. North, J. R., and B. A. Askonas. 1974. Analysis of affinity of monoclonal antibody responses by inhibition of plaque-forming cells. *Eur. J. Immunol.* **4**: 361.
34. McCarty, M. 1958. Further studies on the chemical basis for serological specificity of Group A streptococcal carbohydrate. *J. Exp. Med.* **108**: 311.
35. Kunkel, H. G., M. Mannik, and R. C. Williams. 1963. Individual antigenic specificity of isolated antibodies. *Science (Wash. D. C.)*. **140**: 1218.
36. Edelman, G. M., and E. A. Kabat. 1964. Studies on human antibodies. I. Starch gel electrophoresis of the dissociated polypeptide chains. *J. Exp. Med.* **119**: 443.
37. Allen, J. C., H. G. Kunkel, and E. A. Kabat. 1964. Studies on human antibodies. II. Distribution of genetic factors. *J. Exp. Med.* **119**: 453.
38. Ghose, A. C., and F. Karush. 1973. The affinity and temporal variation of isoelectric fractions of rabbit anti-lactose antibody. *Biochemistry.* **12**: 2437.
39. Claflin, J. L., R. Lieberman, and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine. II. Idiotypic specificity and binding characteristics of antiphosphorylcholine antibodies. *J. Immunol.* **112**: 1747.
40. Claflin, J. L., and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine. IV. Idiotypic uniformity of binding site-associated antigenic determinants among mouse antiphosphorylcholine antibodies. *J. Exp. Med.* **140**: 673.
41. Claflin, J. L., R. Lieberman, and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine. I. Specificity, class, and idiotype of phosphorylcholine-binding receptors on lymphoid cells. *J. Exp. Med.* **139**: 58.

42. Cosenza, H., and H. Köhler. 1972. Specific suppression of the antibody response to receptors. *Proc. Natl. Acad. Sci. U.S.A.* **69**: 2701.
43. Mäkelä, O. 1970. Analogies between lymphocyte receptors and the resulting humoral antibodies. *Transplant. Rev.* **5**: 3.
44. Naor, D., S. Morecki, and G. F. Mitchell. 1974. Differential induction of anti-trinitrophenyl plaque-forming cell responses to lightly and heavily conjugated trinitrophenylated heterologous and autologous erythrocytes in mice. *Eur. J. Immunol.* **4**: 311.
45. Kim, Y. D., and F. Karush. 1973. Equine anti-hapten antibody. VII. Anti-lactoside antibody induced by a bacterial vaccine. *Immunochemistry.* **10**: 365.
46. Askonas, B. A., and A. R. Williamson. 1972. Dominance of a cell clone forming antibody to DNP. *Nature (Lond.)*. **238**: 339.
47. Tada, T., and T. Takemori. 1974. Selective roles of thymus-derived lymphocytes in the antibody response. I. Differential suppressive effect of carrier-primed T cells on hapten-specific IgM and IgG antibody responses. *J. Exp. Med.* **140**: 239.
48. Blomberg, B., W. Geckeler, and M. Weigert. 1972. Genetics of the antibody response to dextran in mice. *Science (Wash. D. C.)*. **177**: 178.
49. Uemura, K., J. L. Clafin, J. M. Davie, and S. C. Kinsky. 1975. Immune response to liposomal model membranes: restricted IgM and IgG anti-dinitrophenyl antibodies produced in guinea pigs. *J. Immunol.* **114**: 958.
50. Howard, J. G. 1972. Cellular events in the induction and loss of tolerance to pneumococcal polysaccharides. *Transplant. Rev.* **8**: 50.
51. Chen, F. W., A. D. Strosberg, and E. Haber. 1973. Evolution of the immune response to type III and VIII pneumococcal polysaccharides. *J. Immunol.* **110**: 98.
52. McDevitt, H. O., and B. Benacerraf. 1969. Genetic control of specific immune responses. *Adv. Immunol.* **11**: 31.
53. Gershon, R. G. 1974. T cell control of antibody production. *Contemp. Top. Immunobiol.* **3**: 1.
54. Wiener, E., and A. Bandieri. 1974. Differences in antigen handling by peritoneal macrophages from the Biozzi high and low responder lines of mice. *Eur. J. Immunol.* **4**: 457.
55. Uhr, J. W., and G. Möller. 1968. Regulatory effect of antibody on the immune response. *Adv. Immunol.* **8**: 81.