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The Affinity and Temporal Variation of Isoelectric Fractions of Rabbit Anti-Lactose Antibody†

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ABSTRACT: A killed vaccine of *Streptococcus faecalis*, strain N, has been used in rabbits to produce anti-lactose antibody of restricted heterogeneity. The specifically purified anti-lactose antibody preparations have been further fractionated by preparative isoelectric focusing to yield antibody populations exhibiting functional homogeneity. The binding of a monovalent lactose-containing hapten was measured by equilibrium dialysis to provide affinity values for the isoelectric fractions and evidence of homogeneous reactivity. All of the rabbits studied showed a multiplicity of isoelectric fractions with anti-lactose specificity early in the response. The three animals selected for detailed study differed in the range of affinity observed with the isolated fractions reflecting, presumably, a difference in their genetic capability to produce anti-lactose antibody. The maturation of the response in these animals involved the preferential emergence of the isoelectric fractions of maximum affinity. However, only in the case in which the individual expressed a wide range of affinity, 100-fold in the association constant, was the development of a monoclonal response approached. When restimulation was carried out a year after the initial exposure to the vaccine and following the decline of serum antibody, the same isoelectric fractions found in the early response were observed. Their quantitative distribution, however, was modified in favor of a larger fraction of antibody of the higher affinities. The absence of new isoelectric fractions indicated that no significant somatic diversification had occurred during the year of adult life with respect to reactivity with the lactose determinant.

The study of the homogeneous antibody products of single clones of antibody-producing cells has emerged in recent years as a powerful tool for the analysis of the cellular parameters of antibody synthesis. These studies have utilized monofocal splenic fragments cultured *in vitro* (Klinman, 1969) and the selection and propagation *in vivo* of a single antibody-forming clone (Askonas *et al.*, 1970). At the same time the use of bacterial vaccines has become an effective technique for the induction of high levels of rabbit antibody with restricted heterogeneity specific for carbohydrate antigens. With such antibody preparations it has proved possible to isolate homogeneous 7S antibody directed against streptococcal cell wall determinants (Krause, 1970) and against capsular polysaccharides of the pneumococcus (Pincus *et al.*, 1970).

The recent discovery of a strain of *Streptococcus faecalis* (strain N), whose cell wall contains lactose as the immunodominant group (Pazur *et al.*, 1971, 1973), has expanded the utility of bacterial vaccines to the production of anti-lactose antibody of restricted heterogeneity. Although functionally homogeneous antibody has been observed in only one rabbit immunized with *S. faecalis* (see below), the application of preparative isoelectric focusing (Freedman and Painter, 1971) has allowed the separation of purified anti-lactose antibody into homogeneous fractions. We have undertaken to characterize these fractions with respect to their affinity for monovalent hapten and to follow the temporal variations of these monoclonal products over a period of about 1 year. The main purpose of the study was to assess the significance of the affinity of the secreted product of a clone for the natural history of the clone. The emergence of dominant clones, their finite life span and the persistence of clones reveal a high degree of complexity in their selection involving probably both

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TABLE I: Immunization Schedule with *Streptococcus faecalis* Vaccine.

First Course	Rest	Second Course	Rest	Third Course	Rest	Fourth Course	Rest	Fifth Course
Three injections per week	4 weeks	Three injections per week	4 weeks	Three injections per week	2 weeks	Three injections per week		Three injections per week
0.4 mg of cells/injection,[a] first week		0.7 mg of cells/ injection, first week		0.6 mg of cells/ injection, first week		2 mg of cells/ injection		1 mg of cells/injection, first week
1 mg of cells/injection, second week		1 mg of cells/ injection, second week		1 mg of cells/ injection, second week		Immunization continued through 8 weeks (1472), 9 weeks (1480), 10 weeks (1476), 12 weeks (1474 and 1479)	13 weeks (1472) 12 weeks (1480) 11 weeks (1476) 9 weeks (1474 and 1479)	1.5 mg of cells/injection, second week
1.5 mg of cells/injection, third week		1.5 mg of cells/ injection, third week		1.5 mg of cells/ injection, third week				2 mg of cells/injection, third and fourth weeks
2 mg of cells/injection, fourth and fifth weeks		2 mg of cells/ injection, fourth week		2 mg of cells/ injection, fourth week				

[a] 0.2 mg of cells/injection was used for rabbits 1472, 1474, and 1476.

genetic limitations and environmental factors (Haber, 1971; Krause, 1970; Spring *et al.*, 1971).

Materials and Methods

Vaccine. The culture of *S. faecalis* (strain N) was grown in Todd–Hewitt medium for 18 hr at 37°. The culture was then heat treated for 1 hr at 60° in a water bath. Cells were centrifuged and resuspended in PBS (pH 7.4).[1] The last step was repeated three times after which cells were stored in 0.2% formalized-saline for immunization. The sterility of the preparation was checked by plating on nutrient agar. The dry weight concentration of the cells was determined with a Klett–Summerson colorimeter using a value of 100 Klett units for a 0.288-mg/ml cell suspension (Davies *et al.*, 1968).

Immunization. Male New Zealand rabbits (white and brown) were immunized with the vaccine intravenously over a 1-year period according to the schedule shown in Table I. Bleedings were taken the 5th, 6th, or 7th day after the last preceding injection.

Quantitative Antibody Assay. A diheteroglycan of glucose and galactose was isolated from the cell wall of *S. faecalis* by the method of Pazur *et al.* (1971). This polysaccharide was used for quantitative analysis with the antisera. The serum sample (0.5 ml) was mixed with an appropriate amount of diheteroglycan to give complete precipitation. The precipitate was washed with cold PBS several times and dissolved in 0.1 M sodium decyl sulfate. The optical density was measured with a Zeiss spectrophotometer at 280 nm. An extinction coefficient of 14.4 at 280 nm was used for $E^{1\%}$ (Utsumi and Karush, 1964).

Cellulose Acetate Electrophoresis. Cellulose acetate membrane electrophoresis was carried out in a Beckman Microzone apparatus with barbital buffer (pH 8.6), ionic strength 0.075.

Purification of Antibody. The antibody was specifically purified by passing the serum through an immunoadsorbent column prepared by the method of Cuatrecasas *et al.* (1968). In our procedure *p*-aminophenyl β-lactoside (Cyclo Chemical Corp.) was coupled to activated Sepharose. The serum was passed through the column and the latter thoroughly washed with PBS until the optical density of the effluent was negligible. The absorbed antibody was eluted with 0.1 M lactose in PBS. The eluted antibody was first dialyzed against PBS, next against 0.5 M galactose and, finally, was exhaustively dialyzed against PBS. The antibody preparation was generally concentrated by ultrafiltration and stored frozen. A solution of 6 M guanidine hydrochloride was used to wash the immunoadsorbent column after each purification.

Preparative Isoelectric Focusing. Liquid isoelectric focusing was carried out on a preparative scale using an electrofocusing column (about 100-ml capacity) constructed in this laboratory according to Vesterberg and Svensson (1966). Ampholine with a pH range of 5–8 was used in all cases with a stable density gradient of 0–45% sucrose. The sample (about 20–30 mg), concentrated to a volume of 2–3 ml, was applied in the middle of the column. The sample was electrophoresed for 72 hr at 4°. A potential of 300 V was applied for the first 24 hr and 700–750 for the remaining 48 hr. After the electrofocusing was completed the solution was withdrawn from the column and collected in 1-ml fractions with a microfraction collector (Gilson). The absorbance of the fractions was measured at

[1] Abbreviations used are: Lac dye, *p*-(*p*-dimethylaminophenylazo)-phenyl β-lactoside; PBS, 0.15 M NaCl–0.02 M phosphate (pH 7.4).

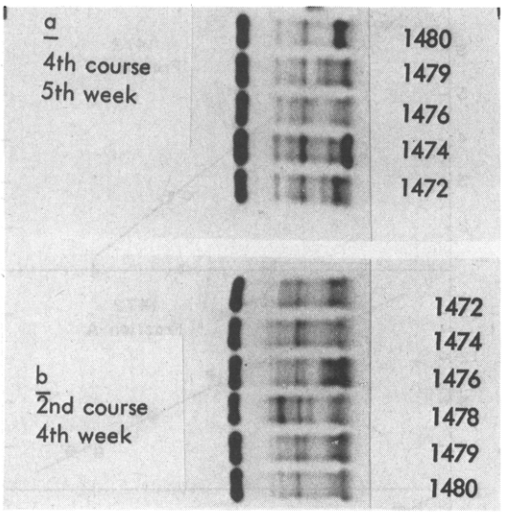

FIGURE 1: Microzone electrophoretic patterns of rabbit antisera produced against *Streptococcus faecalis* vaccine. Dense bands at the extreme left represent serum albumin whereas γ-globulin bands are at the extreme right. Anode is at the left.

280 nm. The pH of the fractions was determined at 20° with an expanded scale pH meter (Radiometer, Copenhagen, Model 26). Appropriate fractions were pooled, dialyzed against PBS, concentrated by ultrafiltration and stored for equilibrium dialysis experiments.

Equilibrium Dialysis. The binding affinity of the anti-lactose antibody was determined with microdialysis cells (Eisen, 1971), purchased from Drummond Scientific Co., Broomall, Pa. The hapten used was tritiated Lac dye [*p*-(*p*-dimethylaminophenylazo)phenyl β-lactoside] prepared according to the method described earlier (Karush and Sela, 1967). A volume of 100 μl of specific antibody solution containing ^{14}C-labeled normal rabbit IgG, used as a volume marker, was placed in one compartment. In the other, 100 μl of the hapten solution was placed and equilibrium established in a rotating dialyzer at 25° for 48 hr. After equilibration, hapten concentrations from both sides were measured with 50 μl of sample by liquid scintillation counting in Scintisol (Isolab, Inc., Akron, Ohio). This procedure eliminated the need of casing adsorption corrections and the volume marker allowed for the correction of any volume change during the equilibration process. A 10% quenching correction factor was used to take into account the decrease in the counting efficiency for the bound hapten. Analysis of the binding data was done by the Sips equation (Karush, 1962) with the aid of a computer program developed by Dr. Ivan Otterness. In a few cases the binding affinity of the purified antibody was determined using [^{14}C]lactose as hapten. No volume marker was used in this instance since no casing adsorption correction was needed. The lactose concentration was measured only from the protein-free side. The antibody solutions used in the binding experiments were prepared by combining the same isoelectric fractions obtained from different bleedings of the same animal. This procedure was necessary in order to obtain adequate amounts of antibody.

Results

Observations with six rabbits showed that during the first three courses of immunization the antibody exhibited very little restriction of normal heterogeneity (Figure 1b) and the amounts of antibody produced were also not high in four rabbits (Table II). In the fourth course, however, bleedings taken after 4 weeks of immunization showed the appearance of antibody components with restricted heterogeneity (Figure 1a) and substantial increase in the amount of antibody produced (Table II), except in rabbits 1472 and 1476. The increase was particularly large in the case of rabbit 1480 (~25 mg/ml in the 4th week of bleeding). On repeated immunization for another 4 weeks the restricted banding of the antibody microzone patterns remained almost unchanged and no increase in the amount of antibody was noted. In fact, a significant decrease was observed with rabbit 1480. After a rest period of 2–3 months on reimmunization all the rabbits produced appreciable amounts of antibody although no distinct banding was observed in their antibody microzone pattern. Results obtained with five other rabbits also showed that continuous immunization was not effective in increasing the amount of antibody produced. For maximum levels of antibody a rest period of several weeks is probably necessary.

TABLE II: Level of Anti-Lactose Antibody in Various Bleedings.[a]

Rabbit	First Course, Fifth Week	Second Course, Fourth Week	Third Course, Fourth Week	Fourth Course, Fifth Week	Fourth Course, Ninth Week	Fifth Course, Fourth Week
1472	2.6	5.2	6.4	8.2	6.1	9.2
1474	1.6	1.4	2.7	10.0	11.4	
1476	5.0	7.5	9.9	6.2	4.8	14.4
1478[b]	1.9	2.1				
1479	0.8	2.9	2.7	5.0	6.1	11.6
1480	0.7	2.0	2.4	17.0	2.7	12.5

[a] In milligrams of antibody protein per milliliter of serum. [b] Rabbit 1478 died during the second course.

After observing the general pattern of the nature and amount of antibody produced in these rabbits, we chose three rabbits for further studies using isoelectric focusing and equilibrium dialysis methods. These three rabbits were selected since they exhibited different types of the immune response. Figure 2 shows the liquid isoelectric focusing pattern of the purified antibody preparations obtained from the sera of rabbit 1472 at various time intervals. The liquid isoelectric focusing pattern of normal rabbit IgG has, also, been shown in Figure 3 for comparison. It can be seen in Figure 2 that at first (first course, 5th week) the pattern was dominated by the bands at pH's 6.42, 6.56, and 6.71 on the alkaline side of the pH gradient, although the pH 5.68 band was also strong. In the second course (4th week), however, the higher pH bands diminished considerably whereas the band at pH 5.92, which was relatively weak in the beginning, emerged strongly. The change continued into the 5th week of the 4th course when the bands at the higher pH almost disappeared whereas the bands at pH's 6.01 and 6.10 now predominated. These bands were present in lower amounts in the 1st course and also in the second course as indicated by the shoulders (Figure 2B). The most interesting observation was that after a rest period of 3 months, after which no detectable antibody was present, on reimmunization the higher pH bands, 6.42, 6.56, and 6.71, reappeared in substantial amounts. In fact all of the isoelectric bands which were present in the 1st course could be identified also in the fifth course in spite of a span of about 1 year.

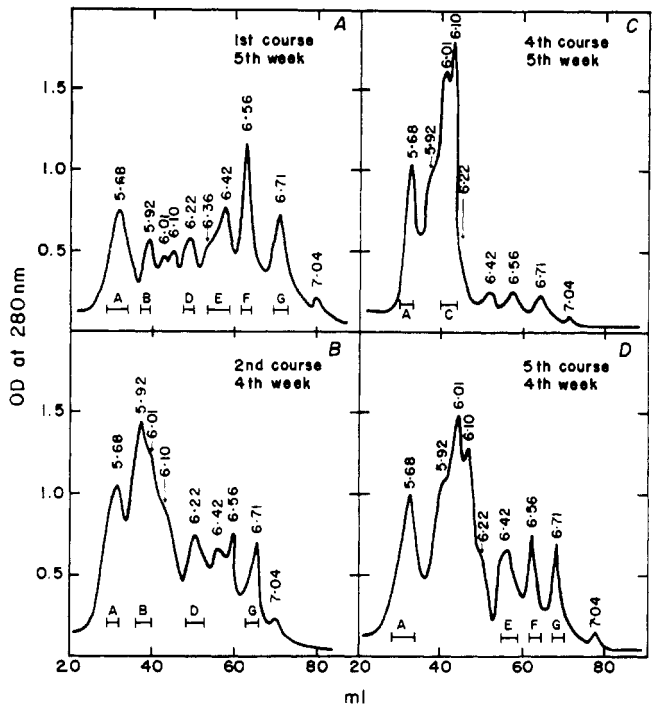

FIGURE 2: The liquid isoelectric focusing elution profiles of purified anti-lactose antibodies of rabbit 1472 in the pH range 5-8. The different isoelectric bands and shoulders are characterized by average isoelectric pH values shown at the top. The individual variation of the pH values was within ±0.01 pH unit of the average value. Fractions under the same isoelectric bands, as marked in the figure, were pooled and used for binding experiments. Anode is at the left.

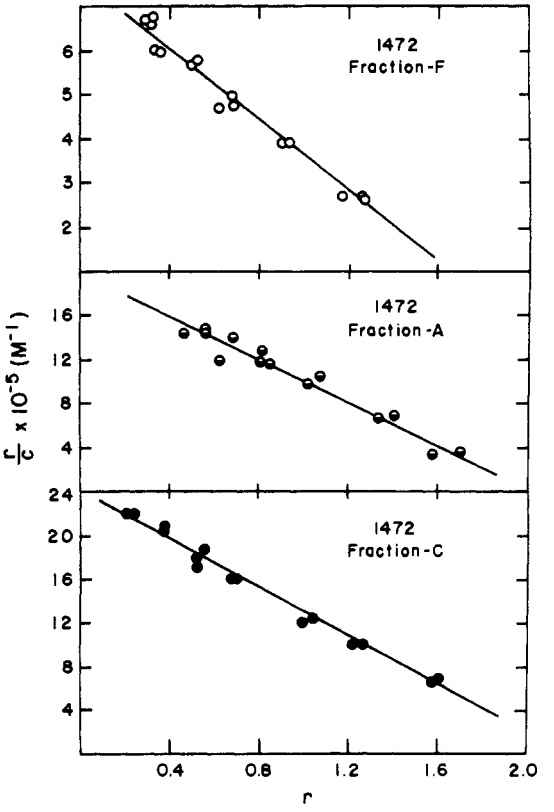

FIGURE 4: Scatchard plots of equilibrium dialysis data for some isoelectric fractions of rabbit 1472 anti-lactose antibodies binding [^{3}H]Lac dye at 25°. Solvent used was 0.02 M phosphate buffer–0.15 M NaCl (pH 7.4).

Table III shows the equilibrium dialysis results obtained with the purified antibody fractions of rabbit 1472. In Figure 4 Scatchard plots (Scatchard, 1949) of the binding data obtained with some of the isoelectric fractions are shown. The results demonstrate that the antibody fractions, obtained by preparative isoelectric focusing method, are functionally homogeneous apart from their electrophoretic uniformity. Fraction B shows some functional heterogeneity which might be due to the presence of the pH 6.01 and 6.10 shoulders and also incomplete separation from the pH 5.68 band. These results may be

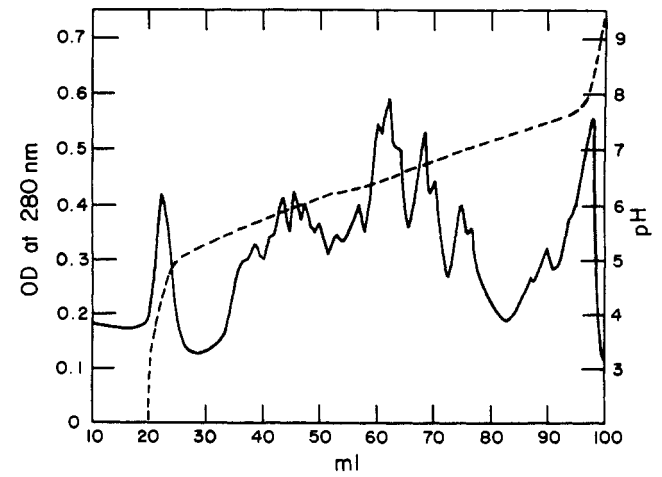

FIGURE 3: The liquid isoelectric focusing elution profile of normal rabbit IgG in the pH range 5–8. Solid line denotes absorbance at 280 nm whereas dashed line represents pH gradient. Anode is at the left.

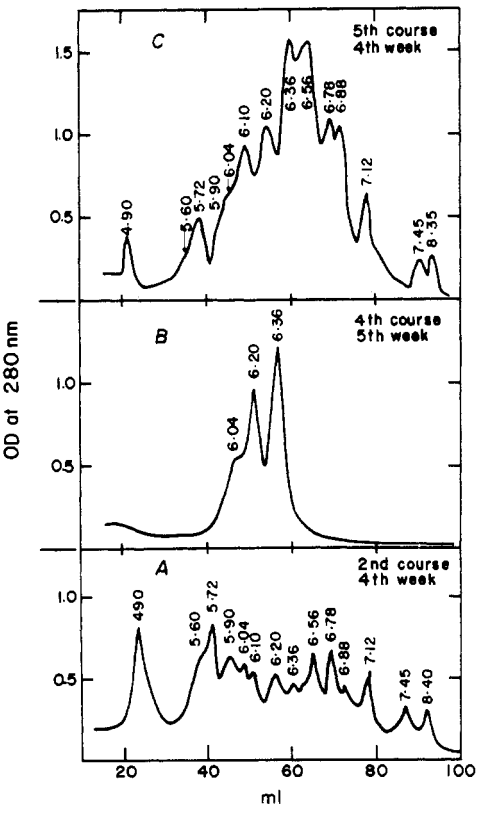

FIGURE 5: The liquid isoelectric focusing elution profiles of purified anti-lactose antibodies of rabbit 1480 in the pH range 5–8. The different isoelectric bands and shoulders are characterized by the average isoelectric pH values shown at the top. Anode is at the left.

TABLE III: Binding Results for Purified Anti-Lactose Antibody and Isoelectric Fractions Obtained by Equilibrium Dialysis at 25°.

Rabbit	Fraction	Isoelectric pH	Hapten	Ass. Constant K (M^{-1})	Heterogeneity Index, a
1472	Purified antibody (first course, fifth week)		Lac dye	0.67×10^6	0.72
	A	5.68	Lac dye	1.00×10^6	1.02
	B	5.92	Lac dye	1.56×10^6	0.82
	C	6.01, 6.10	Lac dye	1.34×10^6	1.03
	D	6.22	Lac dye	0.77×10^6	0.99
	E	6.42	Lac dye	9.98×10^6	1.02
	F	6.56	Lac dye	0.38×10^6	0.97
	G	6.71	Lac dye	0.95×10^6	1.00
	A	5.68	Lactose	0.62×10^5	0.98
1476	I	5.56	Lac dye	6.42×10^4	0.79
	II	5.91	Lac dye	5.16×10^4	0.70
	III	6.00, 6.14	Lac dye	2.40×10^4	0.91
	IV	6.32	Lac dye	3.60×10^4	0.98
	V	6.48, 6.55	Lac dye	5.09×10^4	0.99
	VI	6.72	Lac dye	7.00×10^4	0.93
1480	Purified antibody (second course, fourth week)		Lac dye	2.20×10^4	0.66
	Purified antibody (fourth course, fifth week)		Lac dye	2.07×10^6	0.98
	Purified antibody (fifth course, fourth week)		Lac dye	2.09×10^5	0.73
	Purified antibody (fourth course, fifth week)		Lactose	0.97×10^5	1.01

compared with those obtained with the unfocused purified antibody of rabbit 1472 (first course, 5th week). The latter showed a relatively low value of the Sips heterogeneity index (Table III).

As noted above the binding data were analyzed according to the Sips distribution function (Karush, 1962) to give the association constant (K) and heterogeneity index (a). The corresponding values listed in Table III were obtained using the actual protein concentration, determined from the optical density at 280 nm, and a molecular weight of 160,000. This procedure was preferred to the alternative of using an extrapolated value of the antibody concentration because the latter method tends to obscure heterogeneity. Some sample calculations with a molecular weight of 150,000 daltons did not alter the values of K and a significantly.

The temporal variation in the response of rabbit 1480 is shown by the isoelectric focusing patterns of the purified antibody during different courses of immunization (Figure 5). The antibody response at first was very poor and the isoelectric pattern of the antibody (second course, 4th week) was very heterogeneous with about 13–14 bands. It remained almost the same until the fourth course when the antibody level started to rise sharply. In the 4th and 5th weeks of the fourth course the sera contained 25 and 17 mg/ml of antibody, respectively, and the isoelectric pattern had only two major bands and a shoulder. In fact, this antibody preparation behaved like a functionally homogeneous protein as indicated by the equilibrium dialysis results (Figure 6 and Table III). Also, there was a 100-fold increase in the binding affinity compared to the affinity of the antibody obtained earlier which was characterized by functional heterogeneity. On continued immunization the antibody level declined and the rabbit was rested for about 12 weeks during which period the antibody content dropped almost to zero. After the rest period when the rabbit was challenged again with the vaccine, it started production of appreciable amounts of antibody with considerable degree of heterogeneity in the isoelectric pattern (Figure 5C). Again, the interesting point to note was that the isoelectric bands which were present in the early stages of immunization reappeared after the rest period.

In Figure 7 isoelectric patterns of the antibody preparations obtained from the rabbit 1476 at various time intervals are shown. As expected from the microzone pattern, the antibody preparation obtained from the first course (5th week) of immunization showed a restricted number of isoelectric bands. Only three major bands could be observed (6.00, 6.14, and 6.32) although there were indications of some minor bands

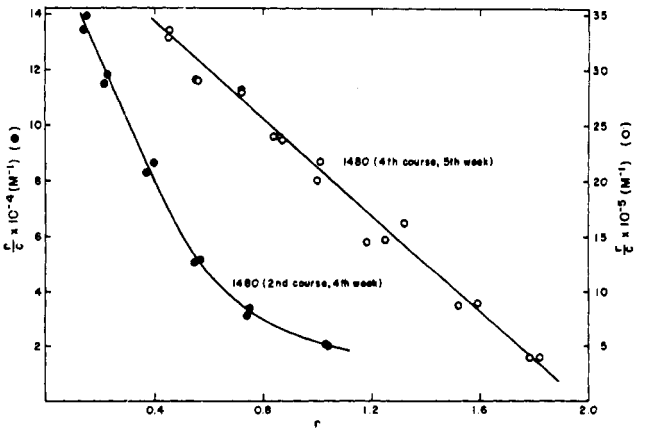

FIGURE 6: Scatchard plots of equilibrium dialysis data for purified anti-lactose antibodies of rabbit 1480 binding [^{3}H]Lac dye at 25°.

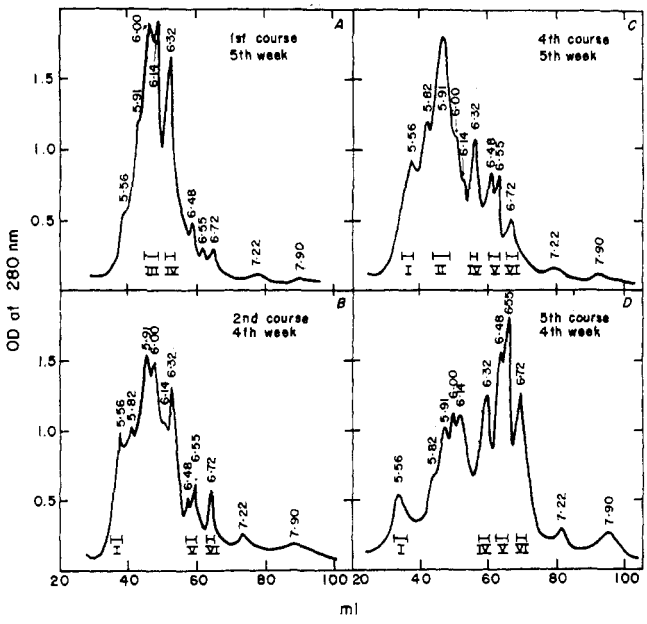

FIGURE 7: The liquid isoelectric focusing elution profiles of purified anti-lactose antibodies of rabbit 1476 n the pH range 5–8. Explanatory markings are the same as used in Figure 2. Fractions under the same isoelectric bands, as marked in the figure, were pooled and used for binding experiments. Anode is at the left.

in the higher and lower pH ranges. In the second course, however, the pattern became more complex as minor bands on the higher and lower pH side gained in intensity. This trend continued into the fourth course (5th week) and the pH 5.91 band emerged very strongly. After a rest period of 11 weeks, the isoelectric pattern remained as complex as before, although the bands in the higher side of the pH gradient were dominant. However, as observed in the other two cases, no new bands emerged after the rest period. The binding data of some of the isoelectric fractions of this rabbit are presented in Table III and, except for two fractions, they indicated functional homogeneity. These two fractions were not well separated from the adjoining fractions (Figure 7) and therefore may be expected to exhibit some degree of functional heterogeneity.

Discussion

Since much of the significance of the results described above rests on the presumed monoclonal origin of the isoelectric fractions, the basis for this view requires critical examination. The argument for its validity rests upon two related experimental observations. Because of the *a priori* possibility that the products of two antibody-producing clones might exhibit the same isoelectric pH, the findings of Freedman and Painter (1971) are of great importance. The first observation was that the isoelectric fractions of purified anti-hapten antibody from the rabbit exhibited molecular homogeneity by several criteria. These included light chain banding, N-terminal amino acid sequence, and allotype distribution. The second observation relates to the functional homogeneity found in most of the isoelectric fractions of anti-lactose antibody. The conjunction of identical values of the isoelectric pH and of the association constants for two clones, molecular properties which are evidently not closely linked, is highly unlikely and much less so for several pairs of clones in an individual rabbit. We may, therefore, tentatively, but with considerable assurance, regard the association constant of a functionally homogeneous isoelectric fraction as a phenotypic marker of a molecularly homogeneous population.

The bulk of the binding results (Table III) were obtained with the Lac dye and involved, therefore, the nonspecific interaction of the aglycoside, *p*-(*p*-dimethylaminophenylazo)-phenyl, with the antibody. The use of the Lac dye was dictated by the relatively low affinity of the binding of lactose and allowed binding measurements to be made over a wide range of occupancy of the antibody sites including near saturation. Under these circumstances there exists the possibility that the nonspecific contribution of the aglycoside to the binding affinity might vary among the homogeneous antibody fractions and thereby render ambiguous our conclusions regarding the maturation of affinity. Although this uncertainty cannot be entirely discounted, the fact that the ratio of association constants for Lac dye and lactose is 16 for fraction A of rabbit 1472 and 21 for the high-affinity antibody of rabbit 1480 indicates that the nonspecific contribution exhibits only minor variations. This conclusion is further supported by the finding that with equine IgM antibody induced with the *S. faecalis* vaccine the corresponding ratio of affinities at 4° was 24 (Kim and Karush, 1973).

The three rabbits studied in detail in our investigation appear to represent three distinct patterns of genetic capability for response to the lactosyl determinant. The pattern that lends itself most readily to a straightforward interpretation is that exhibited by rabbit 1480. In this case, as may be seen from Figure 5 and Table III, the early response, observed 12 weeks after initial immunization, is characterized by a highly heterogeneous population of antibody dominated by the contributions of the low-affinity clones. It should be noted, however, that isoelectric fractions corresponding to the later high-affinity clones can be detected at this stage although we have not demonstrated rigorously that these are high-affinity fractions. At the next stage, 14 weeks later, the antibody level had risen 12-fold to 25 mg/ml of serum and the population showed functional homogeneity with a 100-fold increase in the association constant. Subsequently, after a 12-week rest, restimulation resulted again in a highly heterogeneous response in which 13 of the original 14 isoelectric fractions could be identified. There was, however, the important difference that the apparently high-affinity clones contributed a larger fraction of the antibody population in the recall response than they did in the initial response. This enhanced contribution is seen in the 10-fold increase of the average association constant compared to that found in the initial response (2.09×10^5 M^{-1} *vs.* 2.20×10^4 M^{-1}).

The time course of behavior of rabbit 1480 can be accounted for in terms of a competition for antigen among high-affinity antigen-reactive cells, low-affinity antigen-reactive cells, and serum antibody as has been suggested by Kimball (1972) from his analysis of the response of rabbits to a pneumococcal vaccine. In his study it was demonstrated that the appearance of high levels of anti-type III antibody with restricted heterogeneity was accompanied by substantial increase in the affinity of binding of an oligosaccharide derived from type III capsular polysaccharide. According to this analysis the increase of circulating antibody results in a decreasing level of available antigen and, thereby, leads to the selective stimulation of high-affinity cells and associated decay of the low-affinity clones. The autocatalytic character of this selection would then account for the emergence of one or a few antibody-producing clones of highest affinity. Whether the three high-affinity isoelectric fractions of Figure 5B arise from a single clone is not

clear although a common origin is consistent with their functional homogeneity and the multiplicity of banding often seen with myeloma proteins (Awdeh *et al.*, 1970).

The subsequent response to restimulation after the circulating antibody level has become negligible revealed a new feature of the oligoclonal response. The enhanced contribution of the high-affinity clones suggests that the relative number of antigen-reactive cells of high affinity (Askonas and Williamson, 1972) was greater at this stage than it was in the earliest response. This redistribution of the antigen-reactive population would be in accord with the preceding selection of high-affinity clones. However, there remains the possibility that primary high-affinity-reactive cells are more susceptible to inhibition by antigen than are high-affinity memory cells (Klinman, 1972). In this case, the selective inhibition of high-affinity clones in the initial response would be less significant in the recall response. It may be noted that the rise and fall of antibody-secreting clones in the course of immunization, as exemplified by rabbit 1480, has also been inferred from the change of idiotypic specificities of anti-hapten antibody during immunization (Spring *et al.*, 1971).

One further conclusion may be drawn from the pattern of response of rabbit 1480 regarding the relation of restricted response to the genetic capability of the individual. The view that restricted or monoclonal response is associated with a more limited number of structural genes for appropriate heavy and light chains than is the case with individuals not exhibiting such response (Eichmann *et al.*, 1971) is not in accord with the complexity of the early response of rabbit 1480 (Figure 5A).

In contrast to the highly selected response observed with rabbit 1480, the other two rabbits, apparently, expressed a more limited genetic capability with respect to range of affinity. This limited range is, however, substantially different between them. Thus for rabbit 1472 the association constants for the isoelectric fractions measured fall in the high-affinity range of 4×10^5 to 16×10^5 M^{-1} and for rabbit 1476 the six fractions measured average about 20-fold lower, ranging from 2.4×10^4 to 7×10^4 M^{-1}. The limitation that is thereby imposed on selection with respect to affinity would account for the fact that a highly restricted response similar to that of rabbit 1480 has not been observed with rabbits 1472 and 1476. There was, to be sure, a changing distribution of the relative quantities of the various isoelectric fractions but in each bleeding of rabbit 1472 nine isoelectric components could be identified and, similarly, with rabbit 1476 there were 10 or 11 components in each bleeding.

The changes in the distribution of the isoelectric focusing patterns did, nevertheless, appear to reflect an affinity-dependent selection process. Thus with rabbit 1472 the development of the peaks at pH's 5.92 (B), 6.01 (C), and 6.10 (C), after the first course of immunization, is correlated with the fact that these fractions exhibited the highest affinity. With rabbit 1476 the peaks at pH's 5.56 (I), 5.91 (II), 6.48 (V), 6.55 (V), and 6.72 (VI) became more significant after the first course. These again represent the fractions with highest affinity among the six measured. In both rabbit restimulation after prolonged rest resulted in the appearance of all the original fractions, indicating that the same array of antigen-reactive clones was present as that available in the initial response. However, as observed with rabbit 1480, the high-affinity antibody-producing clones were quantitatively more significant in the recall response.

Our results lead to the view that a highly restricted or monoclonal response to bacterial vaccines can be expected only if one or a few high-affinity antibody-producing clones are potentially available. Under these circumstances selective stimulation can occur although it is not clear why such selection should be associated with abnormally high antibody levels. Finally, the fact that only the earlier isoelectric fractions appeared after a lapse of 1 year suggests that no new anti-lactose clones were generated during this period. Thus, if somatic mutation is the basis for the generation of diversity, this process did not appear to contribute to the antibody-forming potential with respect to the antigen under study during the year of adult life involved in our observations.

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