Qualitative and Quantitative Aspects of the Human Antibody Response to Streptococcal Group A Carbohydrate

STANFORD T. SHULMAN and ELIA M. AYOUB

From the Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida 32610

ABSTRACT The lack of impressive quantitative differences in antibody to various streptococcal extracellular and cellular antigens among patients with acute rheumatic fever, acute glomerulonephritis, and following uncomplicated streptococcal infection has prompted investigation of qualitative aspects of the antibody response among these patients. By using a radiolabeled antigenically univalent hapten derived from streptococcal A carbohydrate, affinity of serum antibody to A-carbohydrate (A-antibody) was studied by an ammonium sulfate precipitation technique. The data obtained demonstrate average association constants (K_0s) of acute rheumatic fever patient sera to be significantly lower than those of acute glomerulonephritis or streptococcal infection patients ($P < 0.02$ and $P < 0.001$, respectively). Further analysis of the data from hapten binding studies documents the fact that the radioimmune precipitin assay for the determination of A-antibody level is little influenced by K_0 but directly correlates with the concentration of antibody binding sites.

These data suggest that qualitative differences in A-antibody are present between rheumatic and nonrheumatic individuals. It is unclear whether the finding of low-affinity A-antibody among acute rheumatic fever patients reflects a generalized phenomenon or one restricted to the A-antibody-A-carbohydrate system.

INTRODUCTION

Previous studies of the host response to infection with group A β-hemolytic streptococci in man have demonstrated that patients with acute rheumatic fever (ARF)[1] and acute glomerulonephritis (AGN) manifest elevated serum antibody titers to a wide variety of streptococcal antigens (1–10). These findings have provided the major evidence establishing the fact that group A streptococcal infection is related to the pathogenesis of these two diseases. While quantitative differences in the magnitude of the antibody responses to various streptococcal extracellular antigens in patients with ARF and AGN have been found during the acute stages of these diseases, available data have not supported a definitive role for the antibodies to these extracellular products in the pathogenesis of the nonsuppurative streptococcal sequelae (11). Although studies of the antibody response to group A streptococcal cell wall carbohydrate have shown little difference in magnitude of response in patients with ARF and AGN (6, 8), the persistence of elevated levels of antibody to group A streptococcal cell wall carbohydrate (A-antibody) in sera of patients with chronic inactive rheumatic valvular heart disease has suggested a pathogenetic relationship (8, 12).

The lack of striking differences in the quantitative aspects of the antibody response to streptococcal antigens among patients with ARF and AGN prompted our investigation of certain qualitative aspects of serum A-antibody in these individuals. Prior studies have demonstrated that one qualitative parameter, antibody affinity, could be assessed by using an antigenically univalent radiolabeled hapten (α hapten) prepared from group A streptococcal carbohydrate (A-carbohydrate) (13). The present study was undertaken to investigate the possible presence of differences in affinity of serum A-antibody during the acute stages of ARF, AGN, and following uncomplicated streptococcal infection. In addition, the

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[1] *Abbreviations used in this paper:* A-antibody, antibody to group A streptococcal cell wall carbohydrate; AGN, acute glomerulonephritis; anti-DNase B, anti-deoxyribonuclease B; ARF, acute rheumatic fever; ASO, anti-streptolysin O; K_0, average association constant; LCM, lymphocytic choriomeningitis; PCA, passive cutaneous anaphylaxis.

molar concentration of A-antibody binding sites was determined (13), and the relative influence of the average association constant (K_0) and binding site concentration upon the A-antibody level was also studied.

METHODS

Sera. Sera were obtained from three groups of patients seen in the hospitals and clinics of the University of Florida. Serum specimens were drawn from 29 patients, ages 4–16 yr (mean, 10.7 yr), early in the course of ARF. Only those patients clearly fulfilling the modified Jones Criteria (14) for the diagnosis of ARF were included. 16 of these children had cardiac mitral valvular involvement at the time of study. Sera from 16 children (4–17 yr; mean, 8.8 yr) with poststreptococcal AGN were obtained shortly after presentation to the hospital. Seven of the AGN patients had historical or clinical evident of antecedent impetigo without history of recent pharyngitis, four had recent pharyngitis only, while three patients had had both impetigo and pharyngitis. Of the remaining two patients, one had had a subcutaneous streptococcal abscess and the other had no clinical evidence of recent streptococcal infection. In addition, sera of 16 patients (2–23 yr; mean, 12.3 yr) with documented recent group A streptococcal infection uncomplicated by nonsuppurative sequelae were studied. This category included patients with streptococcal pharyngitis, cellulitis, or lymphadenitis; no patients with impetigo alone were included.

Sera of patients with streptococcal infection were selected in part on the basis of elevated A-antibody levels, since A-antibody binding studies require a significant degree of antibody activity. Sera were obtained 3–8 wk following streptococcal infection, so that the interval between infection and bleeding would approximate that of the ARF and AGN patients.

Sera were processed aseptically and stored at −10°C until studied. All assays were performed in groups including proportionate numbers of sera from each category.

Antibody determinations. Anti-streptolysin O (ASO) and anti-deoxyribonuclease B (anti-DNase B) titers were measured by the micromethods previously described (15, 16). A-antibody was assayed by the radioimmune precipitin technique, which was performed as detailed previously (8), with one modification: all sera which precipitated 70% or more of the ^{14}C-labeled A-carbohydrate antigen were reassayed after dilution to 1:4 with normal rabbit serum by using the same amount of antigen as in the original test. The fraction of [^{14}C]A-carbohydrate antigen precipitated at the 1:4 dilution of serum was then multiplied by 4 to yield the A-antibody value relative to whole serum. This modification obviated the limitation to a maximum value of 1.00 (or 100% precipitation) previously imposed on sera with high A-antibody content.

Measurement of A-antibody affinity. Antibody affinity, expressed as the K_0 value for the reaction between serum A-antibody and the univalent tritium-labeled α hapten, was measured as previously described (13). By this method, the concentration of free (unbound) α hapten is determined by measurement of the radioactivity in the supernatant fluid after precipitation of globulins with half-saturated ammonium sulfate. The concentration of specifically bound α hapten is determined from radioactivity counts in the washed precipitates, with subtraction of background hapten binding by normal rabbit serum. By using the method of Sips [as detailed by Karush (17)], K_0 values are calculated from plots of the free and specifically bound hapten concentrations obtained when varying concentrations of α hapten are reacted with constant amounts of serum. The majority of studies were performed in duplicate. Binding studies were performed with whole serum instead of with purified A-antibody in order to eliminate the possible selection of higher affinity antibody molecules during the isolation of A-antibody by immunoadsorbent techniques (18).

Quantitation of A-antibody binding sites. By using the α hapten, the molar concentration of antibody binding sites was determined from binding data as previously described (13). For each serum, increasing concentrations of hapten yielded specific hapten binding which approached a maximum level, reflecting saturation of antibody binding sites. Molarity of antibody binding sites was derived by extrapolation of the binding curve to infinite concentration of hapten added.

RESULTS

Anti-streptococcal antibody values. ASO and anti-DNase B titers, as well as A-antibody levels, were assayed on sera from the 29 patients with ARF, 16 with AGN, and 16 with uncomplicated streptococcal infection. As seen in Fig. 1, the ASO titers of the ARF patients were significantly higher ($P < 0.01$) than those of patients with AGN or those following uncomplicated streptococcal infection. No appreciable difference in ASO titers was apparent between the two nonrheumatic groups. By contrast, the anti-DNase B titers of both the ARF and the AGN patients were found to be significantly higher than those of the streptococcal infection category ($P < 0.001$ and $P < 0.01$, respectively). No significant difference in anti-DNase B was observed between the AGN and ARF groups. When the A-antibody levels of these three groups were compared, findings similar to those noted for the ASO titers were observed, in that significantly higher levels of A-antibody were found in the ARF group as compared with the AGN and infection groups ($P < 0.01$ and $P < 0.001$, respectively).

Studies of A-antibody affinity. Binding studies with α hapten and patient sera yielded K_0 values shown in Fig. 2. Mean K_0 values ± standard deviations, as well as mean A-antibody levels and A-antibody binding site concentrations for the three study groups, are recorded in Table I. When K_0 values determined for sera of ARF patients (mean $= 4.13 \times 10^5$ liters/M) were compared to the values for sera of AGN patients (mean $= 5.97 \times 10^5$ liters/M), significant differences were obtained ($P < 0.02$). Likewise, comparison of ARF values with those of the streptococcal infection group (mean $= 7.64 \times 10^5$ liters/M) revealed highly significant differences ($P < 0.001$). No significant difference was found between K_0s of the AGN and streptococcal infection sera. Thus, serum A-antibody of ARF patients is of lower affinity than the A-antibody present in the serum of patients with

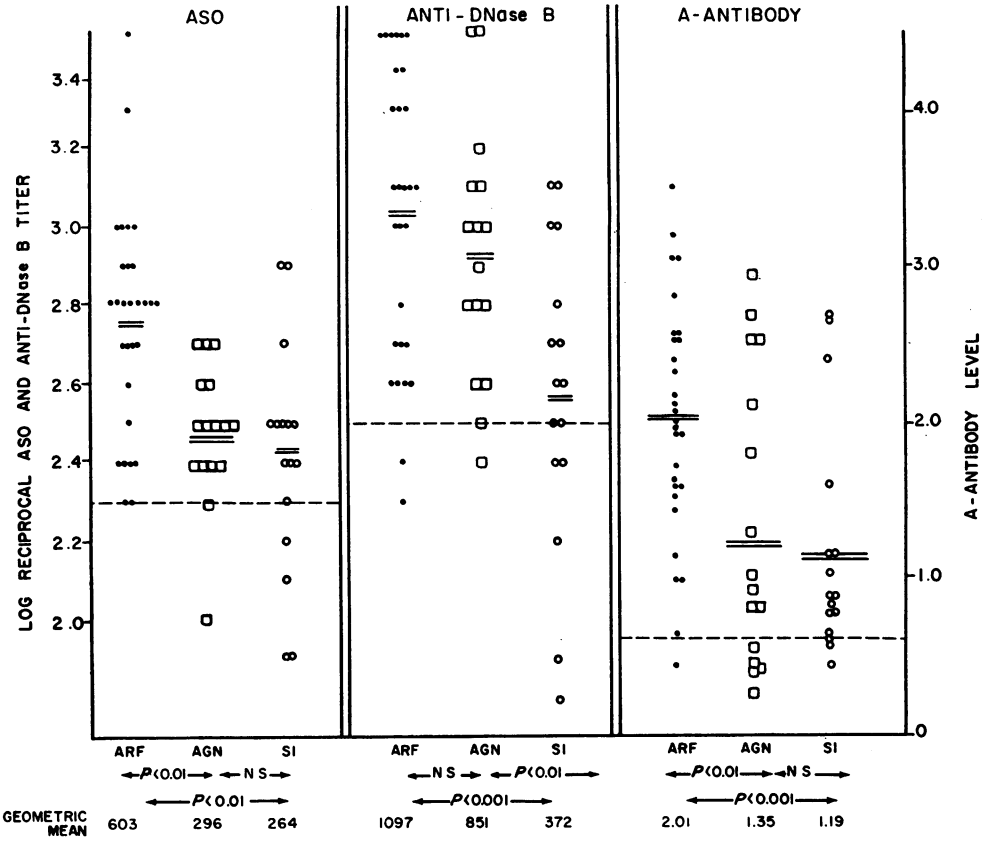

FIGURE 1 ASO, anti-DNase B, and A-antibody levels among 29 patients with ARF, 16 with AGN, and 16 following uncomplicated streptococcal infection (SI). The double bars represent the geometric mean values for each group, while the dashed lines signify the upper limits of normal for each antibody. Levels of statistical significance and geometric means are indicated at the bottom.

AGN and in serum of patients recovering from uncomplicated streptococcal infection.

A-antibody binding site concentration. The mean A-antibody binding site concentration for each of the patient categories is recorded in Table I. It is apparent that the antibody site concentrations are significantly higher for the ARF group when compared with those of the AGN and streptococcal infection groups ($P < 0.01$ and $P < 0.001$, respectively).

Relative contributions of antibody binding site concentration and affinity to radioimmune precipitin A-antibody level. Comparison of the mean radioimmune pre-

TABLE I

*A-Antibody K_0, A-Antibody Levels, and Binding Site Concentrations for Patients with ARF, AGN, and Following Streptococcal Infection**

	ARF (n = 29)	AGN (n = 16)	Streptococcal infection (n = 16)
K_0, $\times 10^5$ liters/M	4.13±1.84	$\leftarrow P < 0.02 \rightarrow$ 5.97±3.23 $\leftarrow$ NS $\rightarrow$	7.64±2.25
		$\longleftarrow P < 0.001 \longrightarrow$	
A-antibody level	2.01±0.75	$\leftarrow P < 0.01 \rightarrow$ 1.35±0.92 $\leftarrow$ NS $\rightarrow$	1.19±0.73
		$\longleftarrow P < 0.001 \longrightarrow$	
Binding site concentration, $\times 10^{-6}$ M	1.76±0.84	$\leftarrow P < 0.01 \rightarrow$ 0.98±0.80 $\leftarrow$ NS $\rightarrow$	0.81±0.66
		$\longleftarrow P < 0.001 \longrightarrow$	

* All values are mean±SD.

cipitin A-antibody levels of the various patient groups (Table I) with the group means of A-antibody binding site concentrations yielded a highly positive correlation ($r = +0.99$; $P < 0.001$). Further analysis was performed to determine whether the same high degree of correlation yielded by the mean values would be obtained with the individual values. The relationship between these two parameters for individual serum specimens is shown in Fig. 3, in which the radioimmune precipitin A-antibody level is plotted against antibody site concentration. As can be seen, there is a strong positive correlation between these measurements, with $r = +0.91$ ($P < 0.001$). This positive correlation was apparent for each of the categories examined. The possible influence of A-antibody K_0 upon the radioimmune precipitin A-antibody level was also evaluated by plotting A-antibody levels against the Sips K_0 values determined for individual serum samples (Fig. 4). As can be seen, little correlation between these functions was found, with $r = -0.25$.

Influence of quantity of A-antibody upon K_0. To determine any effect of A-antibody binding site concentration per se upon K_0, a comparison of K_0 values of matched patient sera from the ARF and streptococcal infection groups was made. Nine pairs of sera, each pair representing one ARF and one streptococcal infection serum, could be closely matched for A-antibody binding site concentration. When the K_0s of these pairs were compared (Table II), it was evident that significant differences in K_0 values were found among the paired groups of sera ($P < 0.001$). These data support the concept that the differences in K_0 values observed among the patient groups are not directly influenced by quantitative differences in A-antibody, as measured by A-antibody binding site concentration or by radioimmune precipitin A-antibody level. It appears that the observed differences in K_0 in fact do reflect qualitative differences in the serum A-antibody of these groups of patients.

DISCUSSION

The data presented demonstrate qualitative differences in serum A-antibody from rheumatic and nonrheumatic individuals, as reflected by differences in antibody affinity. Significantly lower K_0 values were found among ARF patients when compared with K_0 values determined on sera from patients with AGN or uncomplicated streptococcal infection. The K_0 values for the AGN patients were intermediate with respect to the ARF and streptococcal infection groups.

Among the factors which might influence the observed differences in A-antibody K_0 among the several patient groups are the interval between antigenic stimulation (infection) and time of bleeding, and the quantity of A-antibody. The former effect is related to the phenomenon of increasing antibody affinity with time after immunization, an association which has been described in a number of animal studies (19). To control this variable, careful attention was given to the interval between streptococcal infection and bleeding. Sera from the streptococcal infection patients were obtained 3–8 wk

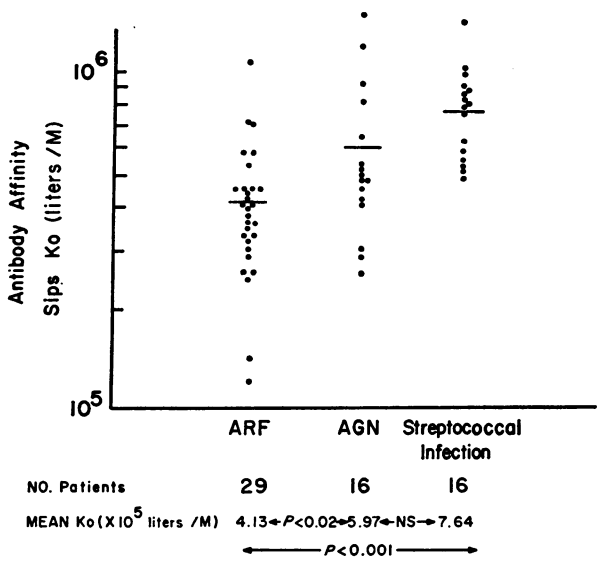

FIGURE 2 A-antibody K_0 obtained in studies with α hapten and sera of patients with ARF, AGN, and streptococcal infection. The mean K_0 for each patient group is indicated by the solid line and levels of statistical significance are noted at the bottom of this figure.

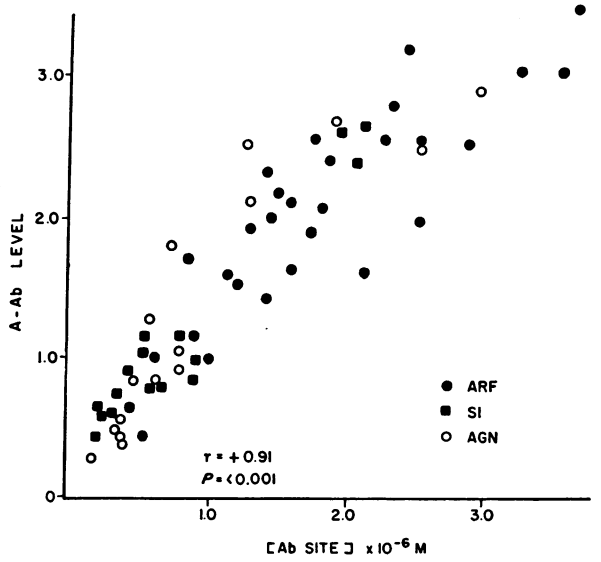

FIGURE 3 Radioimmune precipitin assay A-antibody (A-Ab) levels plotted against A-antibody binding site concentration for individual sera from patients with ARF (closed circles), streptococcal infection (boxes), and AGN (open circles). The highly positive correlation is apparent.

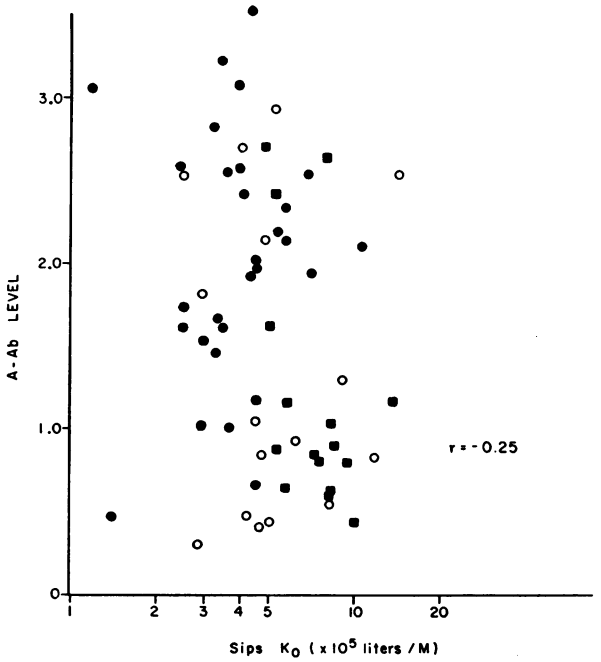

FIGURE 4 Radioimmune precipitin assay A-antibody (A-Ab) levels plotted against K_0 obtained for individual sera from patients with ARF (closed circles), streptococcal infection (boxes), and AGN (open circles). The correlation coefficient r indicates little correlation between these functions.

following streptococcal infection to correspond approximately to the presumed interval between streptococcal infection and bleeding among the patients with ARF and AGN, from whom sera in general were obtained 1–3 wk following the onset of the poststreptococcal sequelae.

The fact that the observed differences in K_0 between the ARF and streptococcal infection groups are independent of A-antibody concentration is demonstrated by the data in Table II. These data show that when K_0s of sera closely matched in terms of A-antibody concentration were compared, highly significant differences in K_0 among rheumatic and nonrheumatic individuals were found. Since hapten binding studies require the use of serum containing high levels of antibody, the streptococcal infection sera selected for this study contained elevated A-antibody levels. Despite this selection bias, the lowest titers of all three antistreptococcal antibodies measured in the present study were found among the streptococcal infection group, probably reflecting the influence of antibiotic therapy (20, 21).

The quantitative difference in mean ASO titer observed among the ARF patients and the nonrheumatic groups ($P < 0.01$) conforms with previous studies which also demonstrated higher mean ASO titers among patients developing ARF than among patients in whom nonsuppurative sequelae did not develop after streptococcal infection (1, 2, 7, 22, 23). The higher mean ASO titer previously observed among AGN patients as compared to an ARF group (4) may reflect the fact that all AGN patients in the previous study had had pharyngitis as the antecedent streptococcal infection. This circumstance contrasts with the present study in which about one-half of the AGN patients had postimpetigo nephritis, which has been shown to be less frequently associated with ASO elevation (24). The anti-DNase B titers in the present study are comparable to those previously reported in that the highest mean titer was found in ARF patients, the next highest in the AGN group, and the lowest mean titer in patients following uncomplicated streptococcal infection (4, 8).

In addition to these quantitative differences in antibodies to streptococcal extracellular products, A-antibody levels were higher among the ARF patients in the present study than in the AGN and streptococcal infection patient groups. The discrepancy between the present findings and those from previous studies may be explained by variations in technique. Using the radioimmune precipitin technique, Dudding and Ayoub (8) found no significant difference in A-antibody values among patients with ARF and AGN. The finding of higher A-antibody values among the ARF group than among the AGN patients in the present study may reflect the modification of the A-antibody assay used, by which the dilution of serum enabled differences in high levels of A-antibody to become apparent.

The degree of influence of antibody affinity upon the radioimmune precipitin assay employing [^{14}C]A-carbo-

TABLE II

Comparison of Sips K_0 Values of Patients with Uncomplicated Streptococcal Infection and with ARF Matched for A-Antibody Binding Site Concentration

ARF		Streptococcal infection	
A-antibody binding site concentration	K_0	A-antibody binding site concentration	K_0
$\times 10^{-6}$ *M*	$\times 10^5$ *liters/M*	$\times 10^{-6}$ *M*	$\times 10^5$ *liters/M*
0.46	1.40	0.46	10.00
0.66	4.52	0.65	5.80
1.00	2.96	0.89	8.70
1.00	3.72	1.04	8.40
1.16	4.47	1.16	5.87
1.60	2.52	1.60	5.03
2.40	4.08	2.40	5.38
2.56	2.48	2.64	8.00
2.80	3.17	2.68	4.93
Mean 1.52±0.86	3.26×10^5 ±1.03	1.50±0.86	6.91×10^5 ±1.87

A-antibody binding site concentration: $P > 0.9$ (NS)
K_0 values: $P < 0.001$

hydrate for the determination of serum A-antibody levels was also investigated in the present study. The data showed that by the technique employed A-antibody levels correlated directly with the concentration of antibody binding sites as determined by α hapten binding studies. In addition, it was demonstrated that there was little influence of antibody K_a upon the radioimmune precipitin assay. This finding would appear to provide definitive documentation supporting the impression that the radioimmune precipitin procedure, which involves primary interaction of antigen and antibody, predominantly reflects concentration of antibody binding sites and, within the range of antibody affinity encountered, is independent of K_a. This independence contrasts with the results of secondary and tertiary antibody assays in which qualitative parameters of antibody such as affinity assume great importance (25). It should be noted in this respect that workers using a hemagglutination technique reported that A-antibody titers were elevated to a comparable degree in ARF and AGN patients (6), while another group using the same technique found A-antibody levels to be elevated among AGN but not among ARF patients (26). The direct comparison of an antibody assay reflecting primary antigen-antigen binding, such as the radioimmune precipitin reaction, with one dependent upon secondary or tertiary phenomena, such as hemagglutination, may be misleading (25).

The finding of serum A-antibody of low affinity among rheumatic patients may represent an isolated immunologic phenomenon involving the A-carbohydrate-A-antibody system. This possibility may be supported by the finding of in vitro differences in cell-mediated immunity to group A carbohydrate but not to group C carbohydrate or to group A mucopeptide among acute rheumatic patients.[3] Such an isolated phenomenon could result from the primary synthesis of low-affinity A-antibody by rheumatic individuals or secondarily from the removal of higher affinity A-antibody molecules in the form of immune complexes, resulting in serum antibody molecules of relatively low average association constant. The primary synthesis of low-affinity A-antibody might occur as the result of a defect in antigenic processing mechanisms, or could represent the influence of antigenic dose or state upon the quality of antibody synthesized. For example, it has been demonstrated in experimental animals that lower affinity antibody is induced by the administration of higher doses of antigen and that soluble antigens have the capacity to render high-affinity antibody-producing cells selectively unresponsive (19). Alternatively, the low-affinity antibody observed among rheumatic individuals in the present study may be a manifestation of a generalized immunochemical difference in the nature of antibody synthesized by these patients. Such a generalized defect might occur as the result of a subtle disturbance in antigenic processing and/or reflect poorly understood genetic factors.

Comparison of the mean A-antibody K_a values of the 16 ARF patients with apparent cardiac involvement and the 13 patients without obvious heart disease showed a higher mean K_a in patients with carditis ($P > 0.02$). However, exclusion of two extremely low K_a values obtained for determinations on two patients without heart disease yielded means which were not significantly different ($P = 0.07$). This comparison is of particular interest in view of the suggested relationship between valvular glycoprotein and antibody to the group A carbohydrate (27), a relationship which raises the possibility that high affinity antibody might be removed by adsorption to this cross-reactive antigen. Although the present data do not support this hypothesis, the limited number of patients involved in this comparison, as well as the difficulty in differentiating primary valvulitis from valvular insufficiency secondary to dilatation of the annulus during the acute stage of rheumatic fever, do not allow for any conclusions. Long-term follow-up studies should enable elucidation of any such differences in antibody affinity between patients with or without primary rheumatic valvulitis.

Assessment of A-antibody affinity was undertaken because of the increasing awareness of the role of antibody affinity in the humoral immune response. As pointed out by Siskind and Benacerraf (19), antibody affinity may well be of greater relevance than quantity of antibody since there exists a wider range of affinities than of antibody quantity and since most biological phenomena involving antigen-antibody reactions in vivo occur at low antigen concentrations. That differences in antibody affinity may have biological significance was first established by the demonstration that the guinea pig passive cutaneous anaphylaxis (PCA) response could be shown to depend upon antibody affinity (28, 29). Thus, high-affinity antibody was found to be significantly more effective than low-affinity antibody in producing PCA reactions. Similarly, the recent work of Soothill, Steward, Alpers, and Petty (30–32) has demonstrated a relationship between affinity of antibody synthesized by mice of various strains and the relative susceptibility of these strains to the immune complex nephritis of chronic lymphocytic choriomeningitis (LCM) virus infection. These workers showed that the mouse strains with the highest incidence of LCM nephritis were the very strains which produced the lowest affinity antibody and which could be shown to demonstrate the least efficient immune elimination. While the biologic significance of the differences in K_a values among the several patient groups in the present study has not been established, these differences are comparable in magnitude to those observed in both the guinea pig and the mouse experiments, in which

[3] M. L. Schulkind and E. M. Ayoub. 1974. Cell-mediated immunity in rheumatic disease. Submitted for publication.

as little as a twofold change in antibody K_a was shown to have functional implications. It seems likely, therefore, that the differences in K_a observed in the present work may be of biologic significance.

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