

Structural Differences between the Two Human Complement C4 Isotypes Affect the Humoral Immune Response

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Summary

An animal model has been used to address the question of the biological importance of the known structural difference between the two isotypes of human C4, i.e., C4A and C4B. Guinea pigs deficient in C4 were reconstituted transiently with either human C4A or C4B protein and immunized with the bacteriophage ϕ X174. Results from this study showed that C4A-reconstituted animals made a secondary response, i.e., switch from IgM to IgG; whereas the C4B-reconstituted animals did not.

MHC class I and class II proteins are highly polymorphic so as to increase the efficiency of binding to a wide range of peptides for antigen presentation (1–3). Of the class III proteins in humans, the fourth component of complement (C4) is also highly polymorphic, and >35 allotypes have been recognized (4–6). Null alleles of C4 are also common with a frequency of 0.1–0.3 in various populations as the result of gene deletions, gene conversions, and silent mutations (7–10). Comparison of the derived amino acid sequence of two common alleles of the C4A and C4B loci indicates that they are nearly identical with only 11 base substitutions, of which 10 are clustered within a small region of the α chain of C4, namely C4d (11, 12). Of these 10 base changes, six appear to be isotypic and the remaining allotypic based on serological determinants.

Porter (13) proposed that the polymorphism of C4 was important in binding to a wide range of antigens or antibodies. In a primary response, recognition of antigen by natural antibody is amplified by activation of the classical pathway of complement (14). An early step in this pathway is cleavage of C4 to C4b by the proteinase activity of C1. This exposes a highly reactive internal thioester, which is readily hydrolyzed by water unless a suitable acceptor is available; a covalent bond (either amide or ester) is then formed via a transacylation reaction (15). The efficiency of binding depends on the allotype of C4 and the chemical nature of the antigen or antibody. In general, allotypes of C4A bind preferentially to free amino groups of proteins, whereas those of C4B form ester bonds with carbohydrate or side chains of hydroxyl-containing amino acids, i.e., Ser, Thr, or Tyr (16, 17). This apparent selectivity appears to be the result of electrostatic

and steric interactions between the isotypic region (residues 1101–1106) in the α chain of C4 and the acceptor molecule as shown by site-directed mutagenesis (18).

To determine if the selectivity of the two isotypes would affect the biological function, C4-deficient guinea pigs (C4Def g.p.'s)¹ were reconstituted transiently with either human C4A or C4B protein and immunized with bacteriophage ϕ X174. Results from this study show that transient reconstitution with C4A but not C4B induced a secondary response characterized by IgM to IgG switching.

Materials and Methods

Plaque and Neutralization Assays. The neutralization assay described by Ochs et al. (19) was performed. In general, serum was diluted by 10 \times serial dilutions in phage buffer, and 100 μ l was added to an equal volume of phage (150 PFU) and incubated at 37°C for 60 min. As a control, test dilution is plated immediately after adding phage and *Escherichia coli*. Subsequently, test sera and phage are added to *E. coli* in soft agar and plated. PFU are counted after a 3-h incubation at 37°C. Results are evaluated by a reciprocal of the dilution required to neutralize 50% of PFU. IgM and IgG were distinguished based on the sensitivity of the former to mercaptoethanol. Test sera are incubated overnight in 0.1 M mercaptoethanol before assay.

Immunization Protocol. Male C4 Def/N g.p.'s (inbred strain originated from a National Institutes of Health colony) of \sim 750 g were used for this study. MHC-matched strain 13 g.p.'s (obtained from NIH) of equivalent weight were used as normal controls. For nega-

¹ Abbreviation used in this paper: C4Def g.p., C4-deficient guinea pig.

tive serum control, C4 Def/N g.p.'s were bled before immunization. g.p.'s were injected (intracardiac) with a dose of 2×10^9 PFU/kg for both the primary and secondary (4 wk later) immunizations. For transient reconstitution, antigen was mixed with 2.5 mg of human C4A or C4B in 1 ml of sterile PBS or 3 ml of normal g.p. serum (strain 13) before injection. Approximately 0.5 ml of blood was taken within 30 min of injection via puncture of the retro-orbital sinus to test for circulating phage and C4 activity.

Purification of Human Complement C4. Human C4 was purified by using a two-step procedure of affinity chromatography (mAb specific for C4 β chain was a gift from Dr. Paul Levine, Washington University School of Medicine, St. Louis, MO) and fractionation of inactive from active C4 by FPLC mono-Q as described by Dodds and Law (20). The separate isotypes were purified from plasma of individuals typed as deficient in either C4A or C4B. The affinity columns were prepared by coupling protein A-purified antibody to affi-gel 10 (Bio-Rad Laboratories, Richmond, CA) using manufacturer's protocol. C4 was eluted at high pH (pH 10.5) as described (20). Eluted fractions were directly fractionated on a 1-ml mono-Q column (Pharmacia Fine Chemicals, Piscataway, NJ) using a FPLC apparatus and active C4 eluted using a gradient of 0.1–0.5 M NaCl in 20 mM Tris/Cl, pH 7.2. Pooled C4 was assayed for hemolytic activity, and aliquots were stored at -80°C . The relative amounts of active C4, i.e., 2.5 mg, were determined by calculating Z U/ml ($1 \text{ Z U} = -\ln [1 - \text{fractional lysis}]$) for each isotype and comparing with a standard plasma sample of C4A/C4B. Since C4B is about fourfold more active in this assay than C4A, the number of units was corrected for the difference. Thus, an equivalent number of units of C4A and C4B could be determined. As an independent assay, the purified C4 was treated with C1s, and the products were analyzed on SDS-PAGE gels. The level of activity could be determined as inactive C4 α chain is insensitive to cleavage with C1s as described (18). The hemolytic assay used was the same described previously (18).

Results

Reconstitution of C4Def g.p. with Human C4 Protein. Previous studies have shown that the impaired immune response of C4Def g.p.'s (21) would be restored by injection of 3 ml of normal g.p. serum at the time of primary immunization (19, 22). An equivalent amount of human C4 was determined to be 2.5 mg based on results from an hemolytic assay in vitro (data not shown). To determine if human C4 would be inactivated or cleared at a rate different from g.p. C4, C4Def g.p.'s were reconstituted with 2.5 mg of human C4A, C4B, or 3 ml of normal g.p. serum, and the level of C4 activity was determined over 48 h. The results in Fig. 1 show that the two isotypes of human C4 decayed at a similar rate, which was similar to that of g.p. C4. The lower hemolytic activity of C4A compared with C4B reflects the four- to fivefold difference in binding of C4A to the antibody-coated red cell surface (16, 17).

Clearance of Antigen after Primary and Secondary Immunization. C4Def g.p.'s were injected intravenously with a mixture of 2×10^9 PFU of bacteriophage $\phi\text{X} 174/\text{kg}$ and either 2.5 mg of human C4A, C4B, or 3 ml of normal g.p. serum. As controls, C4Def and strain 13 g.p. were administered antigen only (see diagram in Fig. 2). The presence of active C4 and bacteriophage in the circulation after the injection

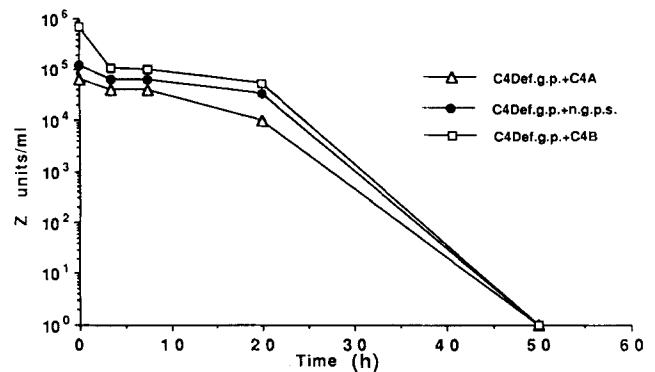


Figure 1. Comparison of human C4A and C4B with g.p. C4 protein for decay in hemolytic activity in vivo. C4-deficient g.p. were injected intravenously with either 2.5 mg of purified active human C4A or C4B or with 3 ml of fresh normal g.p. serum as a source of C4. Animals were bled at various time intervals and assayed for functional C4 activity.

was confirmed by analysis of blood samples, which were taken within 30 min postimmunization.

Clearance of the bacteriophage after the primary and secondary immunization was followed by analyzing serum samples taken at intervals over the first 48 h. After the primary immunization, the phage were cleared to background by 48 h at a similar rate for all animals (Fig. 3 a). In contrast, a distinct difference in clearance among the groups was observed after the second immunization 4 wk later (Fig. 3 b). The level of circulating phage was <10 PFU/ml in strain 13 animals within the first 0.5 h of the injection of antigen. Comparison of the reconstituted C4Def g.p.'s at 0.5 h showed

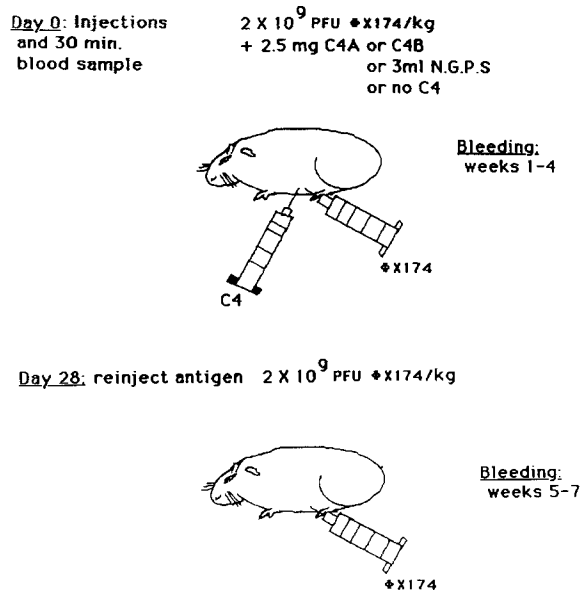


Figure 2. Diagram showing immunization protocol. For primary immunization, C4-deficient guinea pigs were administered antigen (2×10^9 PFU bacteriophage $\phi\text{X}174/\text{kg}$) alone or with 2.5 mg of purified human C4A or C4B protein or 3 ml of fresh normal g.p. serum. For secondary immunization, animals were given the same dose of antigen alone.

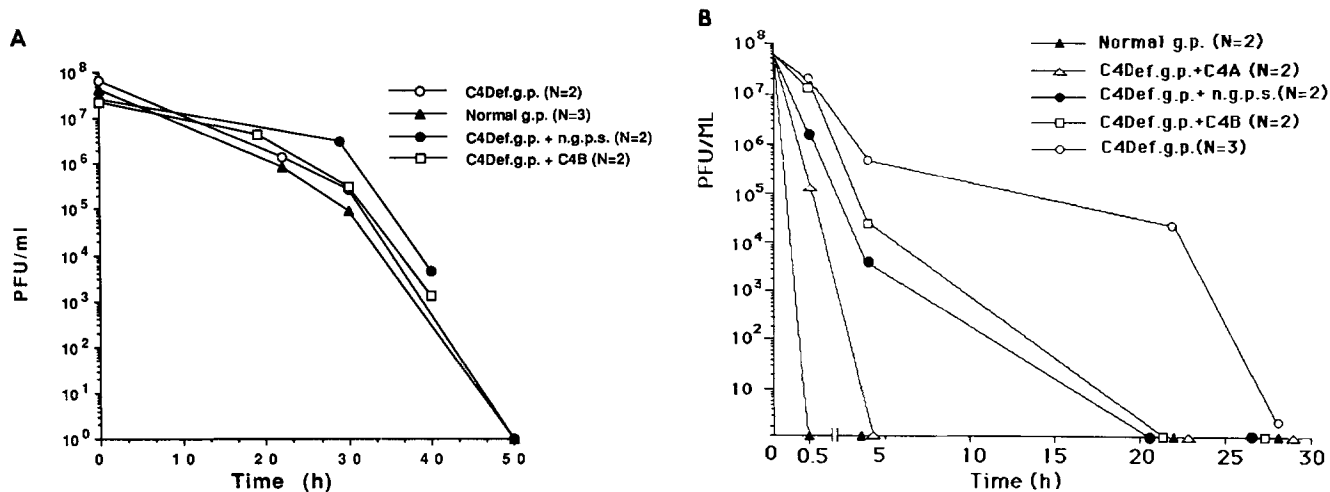


Figure 3. (a) Clearance of bacteriophage after primary immunization with bacteriophage ϕ X174. Note that results from reconstitution of C4-deficient g.p. reconstituted with human C4A are not included in the comparison. (b) Clearance of bacteriophage after secondary immunization. Animals were bled within 30 min of injection of phage and then at 5, 24, and 28 h.

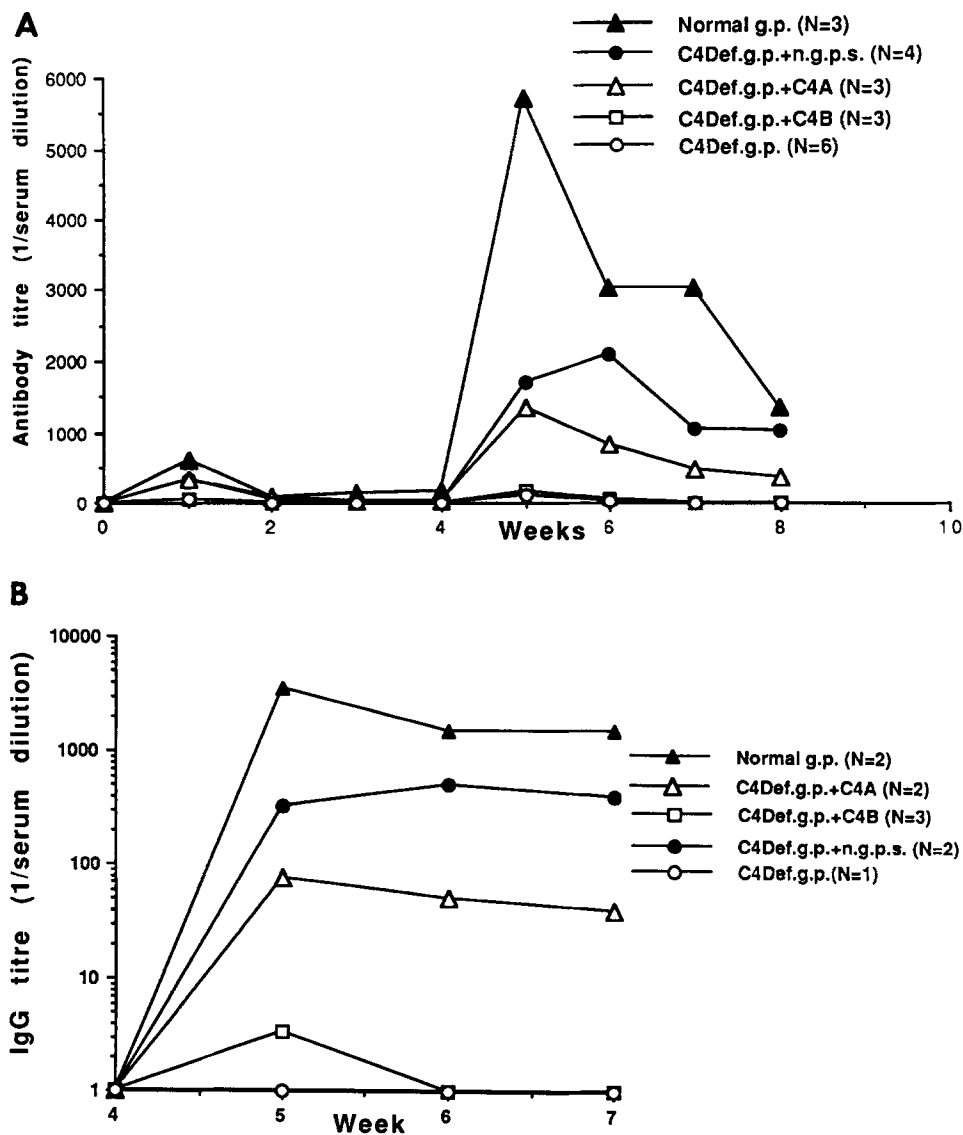


Figure 4. (a) Primary and secondary humoral immune response to ϕ X174. Animals were injected intravenously with 2×10^9 PFU/kg of body weight and with or without C4 (see inset for treatment) at day 0. Secondary immunization was administered with antigen only at week 4. Animals were bled at 1-wk intervals through week 8, and total antibody titer was determined using the plaque neutralization assay. (b) Secondary (IgG) immune response to ϕ X174. Animals were treated as above, except sera from weeks 4–7 were treated with 0.1 mM mercaptoethanol overnight before neutralization assay.

that the titer of phage circulating in the C4A animals was about two logs less than that of those treated with C4B. At 5 h postinjection, the level of phage was <10 PFU/ml in C4A, whereas the C4B had a titer of $\sim 10^4$ PFU/ml. Thus, a

significant difference in antigen clearance was observed after the secondary challenge with antigen.

Primary and Secondary Humoral Immune Response. Animals were bled each week after the primary and secondary immu-

Table 1. *Primary and Secondary Humoral Immune Response*

Guinea pig	Primary response* (week 1)	Secondary response* (week 5)	Standard error†		<i>p</i> value‡ C4A vs. C4B	
			week 1	week 5	week 1	week 5
C4Def g.p.						
173	40	100				
168	100	200				
169	100	100				
121	10	40				
112	10	200				
113	10	10				
Mean	45	180	18	33		
C4Def g.p. + C4A						
119	400	1,000				
171	500	1,000				
143	200	ND				
144	200	2,000				
Mean	325	1,333	75	340	<i>p</i> < 0.02	<i>p</i> < 0.03
C4Def g.p. + C4B						
153	50	200				
154	50	ND				
126	100	200				
128	50	100				
Mean	62	167	12.5	33.5		
C4Def g.p. + n.g.p.s.						
151	200	400				
152	200	ND				
170	400	200				
163	400	6,000				
164	400	200				
Mean	320	1,700	50	1,430		
Normal g.p.						
125	200	2,000				
115	800	5,000				
172	800	10,000				
Mean	600	5,667	200	2,350		

* Antibody titer is the reciprocal of serum dilution resulting in 50% neutralization of phage.

† SEM of titer at weeks 1 and 5.

‡ Comparison of the standard errors of C4A and C4B using student's *t* test.

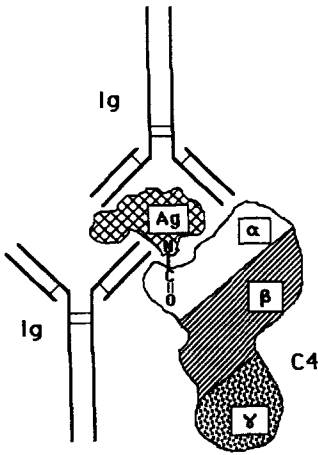


Figure 5. Model of binding of nascent C4b to antigen in the primary immune response after activation by C1. Efficiency of covalent binding of C4b is determined by steric and electrostatic interactions between the polymorphic region of C4 and the antigen.

nization, and their serum was assayed for specific antibody using a phage neutralization assay (titer = reciprocal of the serum dilution resulting in 50% neutralization). As shown in Fig. 4 *a* and Table 1, a significant ($p < 0.02$) difference was observed in the primary immune response of the C4A and C4B g.p.'s. C4Def g.p.'s reconstituted with either C4A or normal g.p. serum gave a greater response than did those reconstituted with C4B or those untreated. By the second week, the titer of all C4Def g.p.'s dropped to background; whereas the titer of strain 13 g.p.'s was still detectable and increased slightly over the next 2 wk. A dramatically different pattern was observed after the second injection at week 5. The mean titer of the C4A ($1,333 \pm 340$ SEM) was about eightfold greater than that of C4B (167 ± 33.5 SEM), which was significant ($p < 0.03$). Over the next 2 wk the mean titer of C4B had dropped to near background; whereas the mean titer of C4A was ~ 800 . As reported by others, reconstitution of C4Def g.p.'s with normal g.p. serum also restored their immune response (see Table 1).

To confirm that the apparent secondary response of C4A animals was a memory response, i.e., switch from IgM to IgG, aliquots of serum were treated with 2-ME before the phage neutralization assay. The IgM but not the IgG fraction of specific antibody should be sensitive to the treatment and no longer neutralize the phage (19). Although the mean titer of the reconstituted g.p.'s was lower, the pattern observed was similar to the untreated sera (Fig. 4 *b*). At week 5, the mean titer of C4A-reconstituted g.p.'s was about eightfold greater than those reconstituted with C4B, and it dropped only slightly over the next 2 wk as is characteristic of a secondary response. For the same time period, the mean titer of C4B animals dropped to background as seen in the primary immune response. As expected, the C4Def g.p.'s reconstituted with normal g.p. serum also switched from IgM to IgG; whereas the untreated C4Def g.p.'s failed to secrete a detectable level of IgG.

Discussion

Assays *in vitro* had shown that the two isotypes of human C4 protein differed dramatically in binding to hydroxyl (car-

bohydrate) and amino groups (protein) (16, 17). However, it was not clear if this difference would affect the role of C4 in the immune response *in vivo*. To address this question, C4Def g.p.'s were reconstituted with either human C4A or C4B, and their humoral immune response to bacteriophage antigen was examined.

Comparison of animals reconstituted transiently with either C4A or C4B showed a dramatic difference in both their clearance of antigen and their humoral immune response after the secondary challenge with antigen. The C4A-reconstituted animals not only cleared the phage antigen quicker, they also produced a memory response, whereas C4B-reconstituted animals did not. The most probable explanation for the difference in secondary response is that reconstitution with C4A resulted in a more efficient activation of B lymphocytes in the primary immune response. It seems unlikely that enhancement of the immune response was due to a differential interaction between the isotypes and the g.p. immune system; because the C4A and C4B proteins decay at a similar rate in the C4Def g.p.'s (Fig. 1) and they interact similarly with g.p. components of classical pathway as demonstrated by the reconstitution of C4Def g.p. serum.

In light of the selective difference in binding between the two C4 isotypes, the phage capsid surface would provide a more suitable acceptor surface for C4A than C4B. Its icosahedral capsid is thought to be assembled from repeating units of the F protein (48 kD) and three smaller spike proteins, and it does not appear to bear carbohydrate (23). The diagram in Fig. 5 provides a model illustrating the putative interaction of nascent C4b (activated C4) with antigen and natural antibody (IgM) in a primary immune response. In this model, binding of nascent C4b to antigen depends on steric and electrostatic interactions between the C4 polymorphic region and the antigen (18). Allotypic variation, which occurs in the same region (11, 12), may also be important in the binding reaction.

It is interesting that after the secondary challenge the strain 13 animals cleared the antigen to near background within 30 min (earliest time point) (Fig. 3 *b*). The pronounced difference in clearance between the strain 13 and C4A-reconstituted g.p.'s reflects, in addition to a difference in phage-antibody titer, the importance of immune adherence (24, 25), which is mediated by the classical pathway. In the transiently reconstituted animals, clearance was probably mediated by antibody and cells bearing Fc receptors since the human C4 was no longer active at week 4. However, a role for the alternative pathway of complement, which is intact and can be activated by immune complexes (26), could not be ruled out. Since the clearance of phage was based on a biological plaque assay, it is possible that circulating phage were present in the test serum but were not active. However, this possibility seems unlikely at least in the C4Def g.p.'s in light of the low titer of antiphage antibody measured before the secondary challenge with antigen. The finding that the ratio of IgG to IgM was much lower in the C4A animals than strain 13 also suggests the importance of an intact classical pathway for a normal memory response. As observed by others (19, 22), the C4Def

g.p.'s reconstituted with normal g.p. serum also developed a secondary response.

Individuals homozygous for deficiency in C4A or C4B are more at risk to certain diseases than those expressing both isotypes (27). For example, susceptibility to infection with encapsulated bacteria segregates with homozygous deficiency in C4B rather than with C4A (28, 29). However, homozygous deficiency in C4A is a major risk factor for systemic lupus erythematosus (30, 31). According to the hypothesis of Porter (13), these associations reflect the distinct binding preferences of the two isotypes. The functional difference found in this study provides the first support in vivo for this hypothesis.

Rescue of the memory response in C4Def g.p.'s by transient reconstitution with human C4A protein emphasizes the importance of an intact classical pathway in the early stages of a primary immune response. Attachment of the C3b/C4b ligands to complexes of natural antibody and antigen in the first 48 h of the primary response appears to be critical for efficient immune clearance and priming of the B cell memory response (32-35), although this effect can be overridden by using nonphysiologic doses of antigen or adjuvant (19, 22). It will be important to extend these studies with different variants of C4 and chemically different antigens.

We thank Drs. H. Ochs and S. Heller for providing the bacteriophage and procedures for plaque and neutralization assays; Dr. J. Moulds for providing Chido- and Rodgers-deficient plasma and the C4A-specific mAb; Dr. P. Levine for the C4 β chain-specific monoclonal; J. Shohet and J. Laning for help with the figures; and Drs. B. Benacerraf, J. Cerny, M. Dorf, D. E. Isenman, G. K. Kelsoe, and L. Steiner for helpful discussions.

This work was supported in part by a grant from the National Institutes of Health (AI-22537) and the PEW Scholars Program in the Biomedical Sciences (M. C. Carroll).

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Received for publication 16 September 1991.

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