A MURINE C4 MOLECULE WITH REDUCED HEMOLYTIC EFFICIENCY*

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The serum substance (Ss) and sex-linked protein (Slp) molecules are mouse plasma proteins that exhibit genetic variations controlled by the S region of the H-2 gene complex (1). The function of these proteins remained unknown for over a decade until 1973 when Demant et al. (2) showed that total hemolytic complement levels were linked to H-2 and that antisera to Ss markedly decreased the whole complement titer. Subsequently, considerable evidence was developed to demonstrate antigenic (3), structural (4–6), and functional (6–10) homologies between murine Ss protein and human C4. It is now generally accepted that the Slp-negative subclass of Ss molecules is murine C4. However, Slp-positive Ss molecules, although structurally quite similar to Ss or C4 (5, 6), do not possess hemolytic activity (6). During studies designed to elucidate the function of the Slp-positive Ss molecules, a C4 molecule with reduced hemolytic efficiency was discovered in the H-2w7 haplotype.

Materials and Methods

The hemolytic assay for murine C4 has been described previously in abstract form (10) and will be reported in detail elsewhere. This sensitive, one-step hemolytic assay employs sheep erythrocytes (E) sensitized with a subagglutinating dilution (1/400) of rabbit IgG antiserum (Cordis Laboratories Inc., Miami, Fla.); C4-deficient guinea pig serum (C4DGPS) diluted 1/20, and the test sample. The concentrations of metals (0.015 mM Ca ++ and 1 mM Mg ++), the low ionic strength buffer utilized (isotonic dextrose mixed with equal portions of isotonic veronal-buffered saline, μ = 0.075) (DVBS), and the duration (2 h) and temperature of incubation (37°C) have been systematically evaluated and found to be similar to the assay described by Gaither et al. (11) for the determination of human or guinea pig C4. Critical differences permitting the measurement of murine C4 were the use of IgG, as opposed to IgM or hemolysin, to sensitize E, and relatively high concentrations of C4DGPS. Mouse antisera (predominantly IgG) raised against E also served as satisfactory sensitizing antibody in this assay system, but provided no advantages over rabbit IgG. Addition of 1 U of oxidized or 10 U of unoxidized human C2 (Cordis Laboratories Inc.) to the assay increased the titers approximately twofold, presumably helping to overcome a partial incompatibility between guinea pig C2 and mouse C4 (6, 12).

Murine C2 was measured with an identical assay except for the substitution of C2-deficient human serum for the C4DGPS and an incubation time of 1 h.

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Antisera to Ss and Slp were prepared by standard methods that have been recently described (3, 5, 13). Goat antiserum to human factor B that cross-reacts with murine factor B was purchased from Atlantic Antibodies, Westbrook, Maine (5). Rabbit antiserum to mouse C3 was a gift of Dr. Phillip Hoffsten, Washington University School of Medicine, St. Louis, Mo. and also purchased from N. L. Cappel Laboratories, Cochranville, Pa.

Mice, obtained from our colony, were bled from the retro-orbital venous plexus into microfuge tubes using capillary pipettes. Unless noted otherwise, blood was collected in EDTA to a final concentration of 5 mM. Reagents and dilutions were made in DVBS and kept at 4°C. Although long-term (up to at least 6 mo at −70°C) or short-term (2 h at 4°C) storage of mouse plasma (5 mM EDTA) does not result in the loss of hemolytic activity, for all of the experiments in this report, mouse plasmas, after procurement, were maintained at 4°C for no longer than 1 h before assay. Serum is an unacceptable test material for the determination of murine C4 (10) because of rapid depletion of murine C4 functional activity (even at 4°C).

For the anti-Ss and anti-Slp depletion experiments, Staphylococcus aureus, Cowan I strain (SACI) (gift of Dr. B. Schwartz, Washington University School of Medicine) or protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.) was washed once in phosphate-buffered saline, centrifuged at 1,800 g for 10 min at 4°C, and resuspended to a 10% suspension. EDTA mouse plasmas were incubated for 10 min at 4°C with a predetermined quantity of anti-Ss or anti-Slp (as determined by radial immunodiffusion) that was sufficient to bind the amounts of Ss or Slp antigen known to be present in a given strain. The mixture was then transferred to a pellet of SACI (10 volumes of a 10% suspension to 1 volume of antiserum) or protein A-Sepharose CL-4B (1 volume of swollen gel per 2 volumes of antiserum), resuspended and incubated for 10 min at 4°C. Plasma was diluted 1:3 with DVBS and the samples were centrifuged at 1,800 g for 10 min at 4°C. The supernate was then removed and one aliquot diluted as previously indicated for use in the hemolytic assay. Other aliquots were stored at −70°C for subsequent determination of Ss and Slp antigenic concentrations by radial immunodiffusion. Controls, to which the same quantities of SACI or protein A-Sepharose CL-4B but no antisera were added, were simultaneously evaluated for each test sample. Similar experiments were performed to deplete mouse C3 and factor B.

Results

In >50 experiments with 13 different strains (including 9 recombinants) that possess the S region of the H-2k haplotype (denoted S* region) on three backgrounds (B10, C3H, and A), the hemolytic C4 activity for an S* strain was always <15% of that for 48 other strains (including 30 independent and 18 recombinant haplotypes) not possessing the S* region. The only exceptions were strains with the S~ region, in which a large percentage of the Ss molecules contain the Slp antigenic marker. Data for the standard haplotypes on a B10 background are shown in Table I. Except for the S~w7 strain, the C4 hemolytic activity of the non-S* strains was ~10 times that of B10.K. B10.WR (S~w7) hemolytic titers were two to three times those of B10.K and one-third to one-fourth those of the other strains. One possible explanation for these data would be that a large amount (50-60%) of the total Ss in this strain consists of Slp-positive Ss molecules which have been shown to lack classical C4 functional activity (6). However, calculation of the residual Ss (C4) antigenic concentrations after correction for the Slp-positive Ss molecules suggested that the hemolytic titers of S~w7 mice should still be severalfold higher than those obtained.

To analyze this observation further, C4 hemolytic activity was measured in selected strains after depletion of Ss or Slp molecules (Table II). EDTA plasmas from mice of the standard haplotypes on B10 background were incubated with anti-Ss serum (which removes both Slp-positive and Slp-negative Ss molecules) and cleared as described in Materials and Methods. These samples contained no detectable functional C4 or antigenic Ss or Slp activity after such treatment, but retained full C2 hemolytic activity and normal antigenic concentrations of factor B and C3. When the
Table I

| Strain | H-2 | Ss or L | Sslt | % U/ml | C4 |
|--------|-----|---------|------|--------|----|
|        |     |         | Concentration | + or - |    |
| B10 | b | H | 0.72 | - | 0 | 3,380 ± 758 |
| B10.D2 | d | H | 1.00 | + | 20-30 | 4,313 ± 846 |
| B10.M | f | H | 1.01 | - | 0 | 4,887 ± 1,104 |
| B10.K | k | L | 0.07 | - | 0 | 410 ± 79 |
| B10.Q | q | H | 0.81 | - | 0 | 4,529 ± 1,434 |
| B10.S | s | H | 0.93 | + | 5-10 | 4,085 ± 1,785 |
| B10.WR | u7 | H | 1.24 | + | 50-60 | 1,158 ± 212 |

* These data represent the mean ± SEM for 5-27 mice between 6 and 12 wk of age for each strain. Human C2 was not added in these assays, although, except for the approximately twofold increase in titers, the results were identical in three similar experiments. 

† Expressed in arbitrary units: 1.0 equals the level in a pool of B10.D2 male mice. Taken from published data (2).

Table II

| Strain | SACI | SACI + anti-Slp | CA/Ss† |
|--------|------|----------------|--------|
|        | Ss (level) | C4 | Ss (level) | C4 |
| B10.WR | 1.97 ± 0.03 | 50-65 | 1,921 ± 221 | 1.47 ± 0.03 | 963 ± 53 | 0.70 ± 0.04 | 979 ± 0.88 | 1,399 |
| B10.A | 1.16 ± 0.07 | 20-30 | 5,166 ± 210 | 0.88 ± 0.07 | 4,676 ± 190 | 0.67 ± 0.04 | 4,123 ± 274 | 6,134 |
| B10.K | 1.48 ± 0.31 | 4-10 | 6,592 ± 274 | 1.33 ± 0.30 | 3,011 ± 351 | 1.14 ± 0.40 | 6,246 ± 1,039 | 4,784 |
| B10.M | 1.55 ± 0.22 | 0 | 6,905 ± 549 | 1.44 ± 0.26 | 5,247 ± 338 | 1.34 ± 0.24 | 5,712 ± 569 | 4,263 |

* These data represent the mean ± SEM for three 6-9-wk-old males for each strain. Similar results were obtained with B10.WR females and C3H.WSlp males and females. 

† Expressed in arbitrary units: 1.0 = level in a B10.D2 male standard. The Ss percentage refers to the percent of Ss molecules that possess the Slp marker. Values were determined by radial immunodiffusion. 

‡ These numbers were obtained by dividing (after Slp depletion) the C4 hemolytic activity by the Ss antigenic level. B10.WR females and C3H.WSlp males and females, strains with the u7 haplotype at the S region and therefore possessing large quantities of Slp, also had ratios between 500 and 1,500.

Slp-positive Ss molecules were depleted, there was no reduction in C4 hemolytic activity, confirming the observations of Ferreira et al. (6) that the Slp-positive Ss molecules have no detectable C4 activity. Based on the residual level of Ss antigenic activity after Slp depletion, the level of hemolytic activity in B10.WR plasma was lower than expected. For example, although the residual Ss antigen concentrations for B10.WR and B10.A plasmas after depletion (Table II) were almost identical, B10.A plasma had approximately four times as much hemolytic activity. Further evidence in this regard was obtained by comparing hemolytic C4 titers and antigenic concentrations of Ss and Slp between S\textsuperscript{w7} strains and PL/J. Because PL/J has approximately the same total Ss and Slp levels as the S\textsuperscript{w7} strain, it would be expected to have equal quantities of C4 yet the hemolytic C4 titers of PL/J mice are severalfold higher than those of S\textsuperscript{w7} strains (Table III). Another strain, FM, also has similar total Ss and Slp levels and a hemolytic C4 level comparable to that of the PL/J. These data strongly suggest that the C4 molecules from the S\textsuperscript{w7} B10.WR and C3H.WSlp strains have a lower hemolytic efficiency.
TABLE III
Comparison of Hemolytic C4 Titers between B10.WR and PL/J*

|        | S‡ (level) | Slp‡ (level) | C4      |
|--------|------------|--------------|---------|
| B10.WR (6) | 1.96 ± 0.16 | 4.32 ± 0.38  | 1,805 ± 257 |
| PL/J (5)   | 1.93 ± 0.07 | 4.69 ± 0.17  | 6,161 ± 878 |

* These data represent the mean ± SEM for 6- to 12-wk-old males. These samples were analyzed in parallel.
‡ Expressed in arbitrary units relative to a standard reference serum.

TABLE IV
Effect of Mixing w7 and k Haplotype Plasmas*

| Haplotype (percentage of each in test sample) | Hemolytic U/sample |
|----------------------------------------------|-------------------|
| k  | w7 | Experimental | Calculated |
|---|---|-------------|-----------|
| 100 | 0 | 676 | — |
| 75 | 25 | 830 | 789 |
| 50 | 50 | 947 | 902 |
| 25 | 72 | 1,130 | 1,015 |
| 0  | 100 | 1,128 | — |

* This experiment is representative of four similar experiments in which, before C4 functional assay, plasmas from B10.WR mice were mixed in the above proportions with B10.M(f), B10.A(d), and B10.HTT(k) plasmas. The data for the k haplotype are shown because low C4 titers of this haplotype provide the optimal opportunity to observe inhibition by S w7 plasma.

The possibilities that these results could be accounted for by inhibitors in S w7 plasma, by increased lability of the S w7 C4 molecule, or by the Slp molecules functioning as an inefficient or false C4 were evaluated. The last consideration seems unlikely because Slp-positive molecules had been depleted from the plasma at the time of the C4 functional assay in some of these experiments (Table II), because PL/J mice have Slp levels as high as B10.WR but possess the expected C4 hemolytic activity (Table III), and because of the data of others (6) demonstrating that C15 does not cleave the α-chain of Slp-positive molecules. To evaluate the first hypothesis, four experiments were performed in which EDTA plasmas from several different strains, with S regions from the d, f, and k haplotypes, were mixed in varying proportions with plasmas from mice of the w7 haplotype (Table IV). In each case, the resultant hemolytic titers were equal to the calculated sum of the contributions of each strain determined separately. To examine the second possibility, EDTA plasma and serum samples from S w7 strains were compared to S k or S d strains with regard to the lability of the C4 hemolytic activity. There was a progressive loss of C4 hemolytic activity in sera maintained at 4, 22, or 37°C and the rate in S w7 strains was similar to that in four other standard strains (B10.M, B10.S, B10.D2, and B10.P). C4 activity in EDTA plasmas remained unchanged in both S w7 and the standard haplotypes for up to 90 min at 4, 22, or 37°C. Taken together, these data suggest that the reduced functional efficiency is not a result of the presence of an inhibitor or of the altered stability of the C4 molecule.

Discussion
In a previous report (5), we demonstrated that plasma C4 molecules from strains...
possessing the $S^{w7}$ region have a slightly smaller molecular weight than plasma C4 molecules from all other strains tested. These data were obtained by immunoprecipitation of the C4 molecules and characterization of the $\alpha$, $\beta$, and $\gamma$-chains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These analyses revealed that the $\alpha$-chain of the $S^{w7}$ haplotype has an apparent 93,000 mol wt, whereas the $\alpha$-chains from other inbred strains examined had apparent 95,000 mol wt (5). Molecular weight differences between $S^{w7}$ and other haplotypes were also documented for C4 molecules produced by macrophages in short-term, in vitro cultures. These differences suggest that the $S^{w7}$ haplotype molecule is synthesized at a different molecular weight, is differentially susceptible to an immediate postsynthetic proteolytic degradation step, or is differently glycosylated. In any case, a primary structural difference in the $S^{w7}$ C4 $\alpha$-chain versus the C4 $\alpha$-chains of other haplotypes is indicated.

In this study, this C4 product of the $S^{w7}$ haplotype has also been shown to be functionally inefficient. For a given quantity of antigenic C4, the $S^{w7}$ haplotype C4 molecules produce one-third to one-fourth as much lysis as C4 molecules from other strains. Reduced stability of this $S^{w7}$ C4 molecule in plasma or serum or the possibility of inhibitors in $S^{w7}$ plasma were shown to be unlikely explanations. Moreover, the C4 molecule from $S^{w7}$ mice was at least as stable as the C4 molecules derived from mice of other haplotypes. In view of the previously described molecular weight differences, it seems likely that the structural change in the $\alpha$-chain of $S^{w7}$ C4 molecules makes it hemolytically less efficient in a standard C4 hemolytic assay. Further studies will be necessary to determine whether the reason for this observation relates to reduced activation by C1S or inefficient formation and/or function of the C3 and C5 convertases.

Although there are examples in certain enzyme systems and in hemoglobins where amino acid substitution(s) or altered peptide chain lengths have important effects on the specific function of the molecule, this is the first report of a complement molecule partially deficient in hemolytic activity. Allotypes of C3 and factor B have been carefully evaluated for functional activity and these variants have been demonstrated to possess similar hemolytic efficiency (14, 15). There is one report suggesting differences in opsonic activity among C3 allotypes (16). In the uncommon variant of hereditary angioedema, normal or elevated concentrations of a nonfunctional C1 esterase inhibitor molecule are present (16, 17) and this molecule does not inhibit the ability of C1S to split its natural substrates. One difference between these previously described variations in the complement system and that of the $S^{w7}$ C4 molecule is that the former presumably represent single amino acid substitutions, whereas the latter is an apparent difference of ~2,000 daltons. The availability of methodologies that permit a careful dissection of structural (5) and functional (6, 10; and this paper) differences in the C4 and Slp molecules and their genetically controlled variants should make it possible to ask additional important questions with regard to the immunogenetics of these proteins.

**Summary**

C4 functional activity and antigenic levels were determined in H-2-congenic mouse strains. In strains with the $H-2^{w7}$ haplotype, the C4 hemolytic activity per unit of residual Ss antigenic activity, after depletion of the nonfunctional Slp-positive molecules was 25–33% that found with other H-2 haplotypes. This reduced hemolytic efficiency was not the result of either a more labile C4 molecule or of the presence of
inhibitors. Moreover, other strains with comparable antigenic concentrations of Ss (C4) and Slp had three- to fourfold higher levels of C4 hemolytic activity. Based on these data and previously reported structural differences between C4 molecules from the $H_{-2}^{\alpha7}$ haplotype compared with other standard $H-2$ haplotypes, the reduced hemolytic efficiency of this molecule is probably secondary to alterations in the structure of its $\alpha$-chain.

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