Brief Definitive Report

EVIDENCE FOR THE INVOLVEMENT OF THE Ss PROTEIN OF THE MOUSE IN THE HEMOLYTIC COMPLEMENT SYSTEM*

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The *Ss* locus in the mouse was originally defined by quantitative variation (Ss-H, high; Ss-L, low) in a specific serum protein that was detected with a heteroimmune antiserum (1). It was subsequently shown that *Ss* maps within the *H-2* complex between the *K* and *D* regions, which code for the major transplantation antigens (1). An allotypic variant of the Ss protein, the sex-limited protein (Slp), was also described (1). Slp-positive strains are denoted Slp^a and Slp-negative strains are denoted Slp^o.

Demant et al. have shown that *H-2* haplotype influences the serum levels of hemolytic complement (C) (2). Through use of intra-*H-2* recombinant lines, the C determinant was shown to map in the same chromosomal region as the *Ss* locus (2). Male mice of strains that are phenotypically Ss-H, Slp^a were found to have higher levels of C than strains that are Ss-H, Slp^o, which in turn have higher levels than strains that are phenotypically Ss-L.

The experiments described in this communication were designed to determine: (*a*) Whether the *Ss* locus itself or a closely linked gene is responsible for quantitative differences in C activity; and (*b*) what effect the removal of Ss protein from serum has on hemolytic C activity. A preliminary attempt to answer the second question by reacting Ss-H serum with anti-Ss antibodies was reported (2). However, because of the technique used, it was not possible to determine whether the observed removal of C activity was the result of secondary C fixation to antigen-antibody complexes or of the removal of a necessary C component (3). Our results now indicate that the Ss protein itself is a component of the C system.

Materials and Methods

Preparation of Anti-Ss and Anti-Bovine Serum Albumin (BSA). Rabbit anti-Ss was prepared by immunizing with partially purified Ss protein derived from a pool of Ss-H serum. The immunization schedule was as follows: day 0, 1 mg protein in 0.5 ml phosphate-buffered saline (PBS) intraperitoneally (i.p.) with 0.5 ml Freund's complete adjuvant; day 7, 0.5 mg protein in 0.5 ml PBS i.p. with 0.5 ml Freund's complete adjuvant; day 14, 0.25 mg protein in 0.5 ml intravenously; days 21, 23, and 25, bleedings were taken. The immune serum was absorbed with Ss-L serum at an approximate ratio of 1:10 (1 part Ss-L serum:10 parts immune serum) to remove all contaminating antibodies detectable by immunodiffusion and immunoelectrophoresis. Rabbit anti-BSA was prepared following the same immunization schedule described above.

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IgG Separation and Enzymic Digestion. Immune serum (either anti-Ss or anti-BSA) was first separated on a DEAE Sephadex A-50 column equilibrated with 0.1 M ethyline diamine, 0.05 M sodium acetate, pH 8. Antibody-containing fractions were pooled, concentrated, and dialyzed against 0.1 M sodium phosphate buffer, pH 8. The sample was then fractionated on a 0.1 M phosphate-buffered Sephadex G-200 column. Reactive fractions were pooled and concentrated to about 100 mg/ml. This IgG preparation was then dialyzed against several changes of 0.07 M sodium acetate, 0.05 M sodium chloride, pH 4, for 24 h. For $F(ab')_2$ preparation, a portion of this sample was incubated with pepsin in a water bath at 37°C for 18 hr at a ratio of 3 mg enzyme/100 mg protein. At the end of the digestion period, the sample was adjusted to pH 8 with NaOH and then dialyzed against phosphate buffer, pH 8. The $F(ab')_2$ fragments were purified by passage through Sephadex G-100 equilibrated with 0.1 M phosphate buffer, pH 8. The control IgG preparation went through the same sequence as the $F(ab')_2$ except that no pepsin was added and the G-100 passage was omitted. The IgG and $F(ab')_2$ preparations were concentrated to give levels of antibody reactivity equivalent to the original antiserum and then dialyzed against 0.1 M tris, 0.15 M NaCl buffer, pH 8. They were then spun down at 30,000 *g* for 2 h, aliquoted, and stored at -80°C.

Mouse Sera. For the Ss protein-C level correlation experiments, mice were bled from an incision in the ventral artery of the tail after the mice had been warmed for about 5 min in a glass jar placed under a 100 W lamp. For the Ss-depletion experiments, the mice were bled from the retro-orbital sinus. In both instances, the blood was allowed to clot for 30 min at room temperature. After the clot was rimmed, the blood was stored at 4°C for 60 min and then centrifuged at 1,000 *g* for 10 min at 4°C. The serum was collected and kept on ice until 2 h within use.

C Fixation Test (4). C fixation buffer (Oxoid, London, England) was used throughout the test. The following three reactants were combined and incubated for 45 min at 37°C in a Dubnoff shaker-incubator (Dubnoff Precision Scientific, Chicago, Ill.) with 5% CO_2: (*a*) 0.1 ml of ^{51}Cr-labeled sheep erythrocytes (3×10^8 cells/ml); (*b*) 0.1 ml of rabbit antisheep hemolysin, 1:100 (Difco Laboratories, Detroit, Mich.); and (*c*) 0.1 ml of the reaction mixture to be tested. The test was stopped by adding cold saline after which the samples were spun down (1, 200 *g* for 10 min) and the supernates counted.

Serum C Assay. Hemolytic titers of serum samples in terms of CH_{50} units were measured according to Terry et al. (5). In cases where serum samples were contaminated with hemoglobin, the OD values contributed by the contaminant were subtracted from experimental values.

Immunodiffusion Techniques. Radial immunodiffusion plates were set up according to a technique described earlier (6).

Results and Discussion

Male mice of two inbred strains were individually bled and their serum tested for hemolytic C activity by the total serum C assay and for Ss reactivity by radial immunodiffusion. In a group of 11 C3H.OH ♂ mice (Ss-H, Slp^a), a significant positive correlation (0.82) was found. In a group of nine C.SW ♂ mice (Ss-H, Slp^o) a significant positive correlation (0.87) was also found. These significant correlations found within strains tend to implicate the Ss protein itself rather than the product of another gene linked to *Ss* in the *H-2*-associated quantitative variation in hemolytic C levels. The positive correlation could conceivably be the result of joint regulation, by an *Ss*-region gene product, of both the Ss protein and a limiting C component. However, if this is so, the high correlation suggests a very precise and direct regulatory mechanism for the two proteins.

To further explore this relationship, the Ss serum protein was specifically removed from mouse serum with a pepsin digest of the IgG fraction of anti-Ss. This anti-Ss [$F(ab')_2$] was employed to avoid any nonspecific C binding to Ss-anti-Ss complexes. The result of absorption of a constant amount of mouse serum with varying dilutions of anti-Ss [$F(ab')_2$], as compared with intact anti-Ss (IgG) is shown in Fig. 1. The two absorbants were used at concentrations that gave equal antibody activity as assayed by an inhibition test on radial

immunodiffusion. The anti-Ss [$F(ab')_2$] removed C activity concomitantly with the removal of Ss reactivity. The anti-Ss (IgG) was somewhat more effective in removing C activity than the anti-Ss [$F(ab')_2$], presumably because the anti-Ss (IgG) when complexed with Ss protein can fix C. Four other experiments which were carried out analogously to the one shown in Fig. 1, gave the identical results.

To demonstrate that the anti-Ss [$F(ab')_2$] did not contain residual, C-binding IgG molecules, the experiment shown in Fig. 2 was performed. This experiment demonstrated that the anti-Ss [$F(ab')_2$] complexed with antigen did not fix rabbit C, in contrast to the anti-Ss (IgG), which fixed considerable amounts. In this experiment a constant amount of Ss partially purified by G-200 gel filtration was mixed with varying dilutions of antibody, either anti-Ss (IgG) or anti-Ss [$F(ab')_2$], and rabbit serum. The same results were obtained in two additional, identical experiments.

It has been reported that, under certain experimental conditions, precipitates of $F(ab')_2$ and antigen can fix significant amounts of guinea pig C, via the alternate pathway (7). To determine whether $F(ab')_2$-antigen complexes could fix mouse C in our system, the experiment shown in Fig. 3 was done. In this experiment a constant amount of BSA was mixed with varying dilutions of

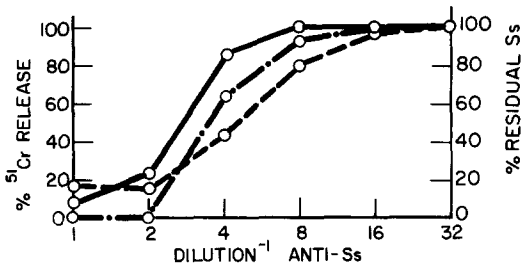

FIG. 1. Absorption of a constant amount of mouse serum with varying dilutions of anti-Ss [$F(ab')_2$] or anti-Ss (IgG). Each sample contained 33 μl of fresh B10.A(5R) mouse serum (8 CH_{50} units) and 33 μl of antibody solution and 33 μl buffer. The samples were kept on ice for 1.5 h before they were added to sensitized sheep erythrocytes. (—), Anti-Ss [$F(ab')_2$] + mouse serum; (---), anti-Ss (IgG) plus mouse serum; and (-·-), concentration of Ss (after absorption with either $F(ab')_2$ or IgG anti-Ss.

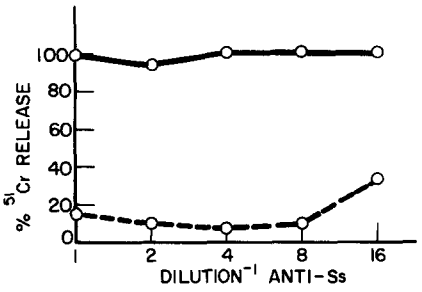

FIG. 2. Fixation of rabbit C by partially purified Ss complexed with either anti-Ss [$F(ab')_2$] or anti-Ss (IgG). Each sample contained 33 μl partially purified Ss, 33 μl of serially diluted antibody solution, and 33 μl of rabbit C solution (2 CH_{50} units). After incubation for 40 min at 37°C, the residual C activity was tested on ^{51}Cr-labeled, sensitized sheep erythrocytes. (—), Anti-Ss [$F(ab')_2$] plus Ss plus rabbit serum; and (---) anti-Ss (IgG) plus Ss plus rabbit serum.

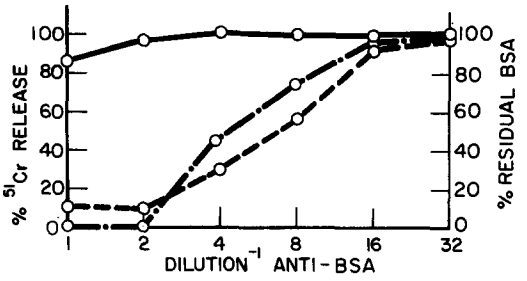

FIG. 3. Fixation of mouse C by anti-BSA [$F(ab')_2$ or IgG] complexed with BSA. Each sample contained 33 μl BSA solution, 33 μl of a solution of serially diluted antibody, and 33 μl of a solution of fresh mouse serum (2 CH_{50} units). The samples were incubated at 37°C for 40 min and then the residual C activity was tested using ^{51}Cr-labeled, sensitized sheep erythrocytes. (—), Anti-BSA [$F(ab')_2$] plus BSA plus mouse serum; (---), anti-BSA (IgG) plus BSA plus mouse serum; and (-·-), concentration of BSA (after absorption with either $F(ab')_2$ or IgG anti-BSA.

anti-BSA [$F(ab')_2$] or anti-BSA (IgG) and a constant amount of fresh mouse serum. The concentration of BSA used in this experiment was equivalent to the estimated concentration of Ss in an equal vol of whole serum (50 μg/ml). The anti-BSA [$F(ab')_2$]-BSA complexes fixed a negligible amount of mouse C relative to the anti-BSA (IgG)-BSA complexes in two independent experiments.

Because the anti-Ss heteroimmune serum was prepared by immunization with an Ss preparation which was only partially purified, then made specific by absorption with Ss-L serum, it was possible that there could be a contaminating antibody to a C component in the anti-Ss which was used for these experiments. No such antibody could be detected by immunodiffusion or immunoelectrophoresis, but to rule out this possibility, an experiment similar to that shown in Fig. 1 was carried out, but with addition of highly purified Ss protein to the anti-Ss [$F(ab')_2$] before addition to the mouse serum. The highly purified Ss protein (kindly provided by T. Krasteff, Department of Human Genetics, University of Michigan, Ann Arbor, Mich., prepared by a method to be reported later) completely neutralized the complement-depleting effect of anti-Ss [$F(ab')_2$]. Therefore it appears extremely unlikely that contaminating antibodies were responsible for the results reported above.

Summary

A significant within-strain correlation has been demonstrated between the levels of Ss and hemolytic complement (C) activity in two Ss-high strains. Mouse serum specifically depleted of Ss by absorption with $F(ab')_2$ fragments of anti-Ss had negligible C activity. In control experiments, Ss-specific antigen-antibody complexes formed with $F(ab')_2$ fragments did not fix rabbit C, and bovine serum albumin-specific antigen-antibody complexes formed with $F(ab')_2$ fragments did not fix mouse C. Therefore the removal of C activity by anti-Ss [$F(ab')_2$] was apparently not due to C fixation. These results suggest that the Ss protein is a necessary component of the C system.

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References

1. Shreffler, D. C., and H. C. Passmore. 1971. Genetics of the H-2 associated Ss-Slp trait. *In* Immunogenetics of the *H-2* System. A Lengerova and M. Vojtiskova, editors. S. Karger AG, Basel, Switzerland. 85.
2. Demant, P., J. Capkoka, E. Hinzova, and B. Voracova. 1973. The role of the histocompatibility-2-linked Ss-Slp region in the control of mouse complement. *Proc. Natl. Acad. Sci. U. S. A.* **70:**863.
3. Capkova, J., and P. Demant. 1974. Genetic studies of the *H-2*-associated complement gene. *Folia Biol. (Prague).* **20:**101.
4. Rosenberg, L. T., and D. K. Tachibana. 1962. Activity of mouse complement. *J. Immunol.* **89:**861.
5. Terry, W. D., T. Borsos, and H. Rapp. 1964. Differences in serum complement activity among inbred strains of mice. *J. Immunol.* **92:**576.
6. Hansen, T. H., T. N. Krasteff, and D. C. Shreffler. 1974. Quantitative variations in the expression of the mouse serum antigen Ss and its sex-limited allotype Slp. *Biochem. Genet.* **12:**281.
7. Sandberg, A. L., B. Oliveria, and A. G. Osler. 1971. Two complement interaction sites in guinea pig immunoglobulins. *J. Immunol.* **106:**282.