IN VITRO STUDIES OF COMPLEMENT FUNCTION IN SERA OF C4-DEFICIENT GUINEA PIGS

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Complement-mediated cytolytic and bacteriocidal reactions are thought to require the sequential participation and activation of the complement component proteins in the order C1, C4, C2, C3, C5, C6, C7, C8, and C9 (1, 2). Many complement-mediated inflammatory responses, however, require the activation of only a portion of the complement sequence. Thus, activation need only proceed through C3 and C5 for generation of chemotactic and anaphylatoxic factors (3–7). It could therefore be anticipated that animals with a total deficiency of one of the later components in the complement sequence might have a defect in serum cytolytic and bacteriocidal functions, but remain healthy because of the existance of intact inflammatory functions. This is the case in strains of mice with a genetically controlled deficiency of C5 (8, 9) and in rabbits with a deficiency of C6 (10, 11).

We have recently reported development of a strain of guinea pigs with a total deficiency of hemolytically active C4, one of the components which acts early in the complement sequence (12). These animals also appear to be healthy and display normal complement-dependent inflammatory responses, including normal direct and reverse passive Arthus reactions and a normal exudative response to a foreign body (13). These findings suggest that the animals possess a bypass mechanism or alternate pathway into the complement sequence, which allows them to fix components beyond C4 and to generate biologically active fragments of complement in the absence of C4. This paper documents in vitro studies designed to demonstrate this postulated bypass mechanism. It attempts to further define the nature of the complement defect in these animals and to determine whether these deficient animals have deficiencies in other components of complement. In addition, the relationship of C4-deficient guinea pigs to an earlier described complement-deficient guinea pig strain (14) has also been investigated.

MATERIALS AND METHODS

Purified Complement Proteins and Complement Reagents.—Preparation of isotonic veronal-buffered saline containing 0.1% gelatin, 0.00015 M calcium, and 0.001 M magnesium (VBS),[1]

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[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; disodium-EDTA

0.065 μ isotonic dextrose–veronal-buffered saline containing 0.1% gelatin, 0.00015 M calcium, and 0.001 M magnesium (DVBS), isotonic veronal-buffered saline containing 0.01 M disodium ethylene-dinitrilotetraacetate and 0.1% gelatin (disodium-EDTA buffer), sources of antibody, preparation of washed optimally sensitized sheep erythrocytes (EA), preparation of complement–cell intermediates, and methods of complement component titration have all been described (15–17). Partially purified complement components were either prepared in our laboratory or obtained from Cordis Corp., Miami, Fla. Pooled guinea pig serum was obtained from Suburban Serum Laboratory, Silver Spring, Md., and was employed as the serum complement source for the preparation of cellular intermediates and in hemolytic analyses. Kinetic studies of the hemolytic activation of C1 were performed as described by Borsos et al. (18).

Materials Utilized For Immunization and Complement-Fixation Reactions.—Human serum albumin (HSA) was obtained from Behringwerke AG, Marburg-Lahn, West Germany. *Escherichia coli* lipopolysaccharide (endotoxin) strain 012:B8 and Freund's complete (CFA) and incomplete adjuvants were obtained from Difco Laboratories, Detroit, Mich. In studies of complement fixation in C4-deficient sera, normal serum was obtained from the same strain from which the C4-deficient animals were derived ("NIH multipurpose strain"). C4-deficient guinea pigs were mated with NIH multipurpose guinea pigs heterozygous for C4 (12). The serum of the C4-deficient offspring and the heterozygous littermates were utilized in the comparative studies presented in this report.

Ability of Zymosan-Absorbed Guinea Pig Serum to Restore the Hemolytic Capacity of C4-Deficient Sera.—Powdered zymosan (Nutritional Biochemical Corp., Cleveland, Ohio) was incubated with pooled guinea pig serum in concentrations of 24, 48, and 72 mg of zymosan/ml of guinea pig serum in an attempt to duplicate the conditions described by Hyde (19). After 2 hr incubation at 37°C the sera were centrifuged, the supernatants were decanted, and dilutions of 1:1000, 1:10,000, and 1:20,000 were prepared in cold DVBS. A volume of 0.25 ml of each of the various dilutions of zymosan-treated guinea pig serum was added to a mixture of 0.25 ml of EA containing 1.5×10^8 cells/ml of VBS and 0.25 ml of C4-deficient serum, diluted 1:50 in the same buffer. After incubation at 37°C for 90 min, 2.5 ml of disodium-EDTA buffer was added and the extent of hemolysis determined spectrophotometrically. Controls included EA incubated with either C4-deficient guinea pig serum alone, zymosan-treated normal guinea pig serum, or normal guinea pig serum diluted 1:20,000 in VBS.

Preparation of Guinea Pig and Rabbit Anti-C4.—Guinea pig anti-C4 antisera were prepared in C4-deficient guinea pigs by injection of 0.1 ml of an emulsion of pooled guinea pig serum in an equal volume of CFA into each footpad. Animals were bled 2 wk later.

To investigate the possibility of a C4-like molecule in the C4-deficient sera, an attempt was made to produce a guinea pig antiserum to the C4-deficient serum. Strain 13, Hartley strain, and normal NIH guinea pigs were injected with 0.1 ml of an emulsion of pooled C4-deficient serum in an equal volume of CFA into each footpad. Animals were bled 2 wk later.

Anti-C4 antiserum was also prepared in New Zealand albino rabbits by immunization with HSA complexed with rabbit anti-HSA and guinea pig C4. The complexes were prepared by reacting at equivalence purified HSA with anti-HSA serum (produced in rabbits by multiple intravenous injection of HSA) for 1 hr at 37°C in the presence of 0.01 M disodium-EDTA buffer to prevent fixation of rabbit complement. The precipitate was washed once with cold disodium-EDTA buffer, three times with cold saline, and resuspended in 0.25 ml of DVBS. It was then incubated with an excess of partially purified C1 for 15 min at 37°C. C4 was

buffer, isotonic veronal-buffered saline containing 0.01 M disodium ethylenedinitrilotetraacetate and 0.1% gelatin; DVBS, 0.065 μ isotonic dextrose–veronal-buffered saline containing 0.1% gelatin, 0.00015 M calcium, and 0.001 M magnesium; EA, washed optimally sensitized sheep erythrocytes; HSA, human serum albumin; VBS, isotonic veronal-buffered saline containing 0.1% gelatin, 0.0015 M calcium, and 0.001 M magensium.

added to the complex by the addition of whole guinea pig serum diluted 1:10 in disodium-EDTA buffer. During this procedure C1 is removed from the antigen–antibody complexes (16). The complexes were then thoroughly washed with disodium-EDTA buffer and DVBS, dissolved in saline, and emulsified with an equal volume of CFA. A volume of 0.25 ml of the emulsion was injected into the footpads and 0.5 ml into the neck of each rabbit. 2 wk later the animals received a booster injection of the complement–cell intermediate EAC4 (16) emulsified in incomplete Freund's adjuvant, and 2 wk after the booster injection the animals were bled.

Inhibition of SAC4 by Guinea Pig Anti-C4 Antiserum.—The ability of guinea pig anti-C4 to inhibit the activity of the cell–complement intermediate site SAC4 was examined in T_{max} studies (15) utilizing SAC4 which had been exposed to either guinea pig anti-C4 serum or C4-deficient serum (without antibody). 5 ml aliquots of SAC4 were incubated for 30 min at 37°C with either 0.1 ml of heat-inactivated anti-C4 serum or 0.1 ml of heat-inactivated C4-deficient serum. The cells were washed, resuspended in 60% dextrose buffer to their original concentration (1.5 × 10^8 cells/ml), and reacted with an excess of partially purified C1a to prepare SAC14. Kinetic analysis of the generation of SAC142 from SAC14 by the addition of C2 to SAC14 was then performed on each of the samples to determine the number and availability of SAC14.

Complement Consumption by Immune Complexes and Endotoxin.—Immune complexes of HSA–rabbit anti-HSA were prepared and washed as described above, and the final protein concentration determined by Kjeldahl nitrogen analysis. Studies of fixation of C' components by immune complexes were performed with pooled sera from normal, heterozygous, and homozygous C4-deficient guinea pigs. The immune complexes (575 or 57 μg) were incubated with 1 ml of freshly obtained, pooled serum. To eliminate dilution of the guinea pig serum, samples were added directly to washed antigen–antibody precipitates. After mixing, the samples were incubated at 37°C for 1 hr. They were centrifuged for 10 min at 48,000 g at 0°C and clear supernatant sera were decanted. These were titrated for C1, C4, C2, and the classical C3–9 complex. In additional experiments immune complexes were prepared with rabbit antisera which were preheated to 56°C for 20 min and to which disodium-EDTA buffer was added to a final concentration of 0.04 M. Both the disodium-EDTA buffer and the heating exclude the possibility of participation of rabbit C4 in the formation of these complexes.

The ability of *E. coli* endotoxin to deplete C4-deficient serum of the late-acting complement components was also explored. Dried samples of endotoxin (120 or 30 μg) were incubated with 1 ml of freshly obtained serum from homozygous C4-deficient, heterozygous, or normal multipurpose guinea pigs. The serum was incubated as described above and component titrations performed.

RESULTS

Effect of Zymosan-Absorbed Guinea Pig Serum on the Hemolytic Capacity of C4-Deficient Sera.—Pooled guinea pig serum treated with 76 mg/ml of zymosan and subsequently diluted 1:20,000 restored the hemolytic capacity of the C4-deficient sera. This quantity of zymosan reduced whole complement activity of the serum by 98.3%. Controls consisting of EA with the zymosan-absorbed sera and EA with C4-deficient serum showed no hemolysis (Table I). Thus, there was no evidence that zymosan removed the factor that enables normal guinea pig serum to restore the hemolytic capacity of the C4-deficient serum.

Properties of Guinea Pig and Rabbit Anti-C4 Serum.—Guinea pig anti-C4 gave a strong precipitin line against normal guinea pig serum in Ouchterlony

analysis in gel (2). It failed to react with rabbit, mouse, rat, or goat serum, but one antiserum gave a weak precipitin line against human serum. The anti-C4 serum agglutinated EAC4 and failed to agglutinate EAC1 or EA. EAC4 incubated with guinea pig anti-C4 showed no lysis on addition of C2 and the late components of complement (Fig. 1). This compares to the normal hemolytic curve (T_{max}) generated after these cells were incubated with C4-deficient sera containing no anti-C4 antibody. Thus, anti-C4 can efficiently block the active, cell bound, C4 site.

Strain 13, Hartley strain, and normal NIH guinea pigs immunized with C4-deficient serum failed to produce detectable antibody by Ouchterlony analysis against C4-deficient serum. These animals were immunized in a manner identical to the immunization of C4-deficient guinea pigs with normal guinea pig

TABLE I

Ability of Zymosan-Treated Normal Serum to Restore Hemolytic Activity of
C4-Deficient Serum

Per cent lysis of optimally sensitized sheep erythrocytes incubated for 90 min in the presence of	
	%
C4-deficient serum 1:50	0.6
Zymosan-treated normal guinea pig serum or normal guinea pig serum 1:20,000	0.3
C4-deficient serum 1:50 plus 1:20,000 normal guinea pig serum	93.5
C4-deficient serum 1:50 plus 1:20,000 zymosan-treated normal guinea pig serum	96.5

serum, which yielded precipitating antibody against a protein in normal guinea pig serum. Thus, the possibility of an altered C4 molecule in the C4-deficient serum is unlikely.

Fig. 2 illustrates that rabbit anti-C4 and guinea pig anti-C4 both show a line of precipitation in gel against normal guinea pig serum. These lines fuse indicating partial identity. The rabbit anti-C4 and the guinea pig anti-C4 fail to show a precipitin line against C4-deficient serum.

Level of Complement Components in C4-Deficient Guinea Pigs.—Table II lists the mean titers and range of C1, C4, C2, and C3-9 complex in normal, heterozygous C4-deficient, and homozygous C4-deficient guinea pig sera. Serum titers of C2 in the C4-deficient animals were consistently about one-half the C2 titers of the heterozygous and normal animals. The titer of C2 in the C4-deficient guinea pigs, although less than heterozygous or normal NIH multipurpose animals, is in the same range as that obtained utilizing normal pooled Hartley strain guinea pig serum. Analysis of C1 titers indicated that the C4-deficient animals fell into two distinct groups. In five of eight homozygous deficient ani-

mals studied, C1 was consistently found to be present in very low titers. Three animals had titers of C1 which were roughly in the normal range (Table II). In an effort to determine whether a low C1 titer indicated an absolute decrease in the C1 level or resulted from abnormal activation of C1 or the presence of a C1 inhibitor in these sera, kinetic studies of the activation of C1 were performed. As can be seen in Fig. 3, C1 activation in C4-deficient sera appeared to be normal although the quantity of C1 was reduced. The presence of C1 inhibitor
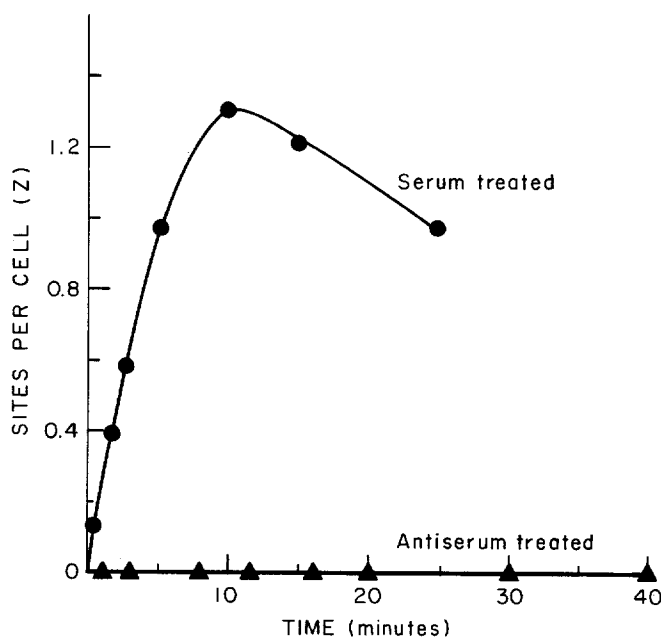

FIG. 1. Ability of guinea pig anti-C4 to inhibit the functional activity of the cellular site SAC4.

or impaired activation of C1 seems unlikely. The titers of C3–9 complex were normal in the C4-deficient animals.

Complement Consumption in the C4-Deficient Sera by Immune Complexes and Endotoxin.—Incubation of immune complexes with normal, heterozygous, and homozygous deficient sera led to substantial consumption of both the early and late components (C3–9 complex) of complement (Fig. 4). The per cent consumption of components beyond C4 was significantly reduced in the sera of C4-deficient animals. However, C2 and the late components are unequivocally consumed.

The effect of endotoxin on the consumption profile of complement components is quite different from that obtained with antigen–antibody complexes

(20). While antigen–antibody complexes fixed both early and late complement components, incubation of normal NIH guinea pig serum with endotoxin led to considerable fixation of the late components (C3–9) with only minimal fixation of C1, C4, and C2 (See Fig. 5). In a similar manner, incubation of C4-deficient sera with endotoxin led to substantial fixation of the late components with
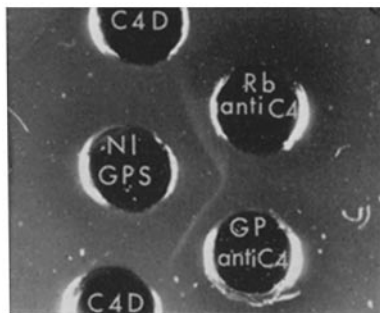

F FIG. 2. Ouchterlony analysis demonstrating absence of C4 protein in C4-deficient guinea pig serum. C4D, C4-deficient serum; Nl GPS, normal guinea pig serum; Rb anti-C4, rabbit anti-C4; GP anti-C4, guinea pig anti-C4 prepared in a C4-deficient guinea pig.

TABLE II

Titers of Complement Components in C4-Deficient, Heterozygous Deficient, and Normal Guinea Pigs

	C1	C4	C2	C3-9
Normal	52,338* (4)	73,230 (4)	13,094 (6)	1,654 (6)
	39,420–58,828‡	40,000–125,000	6,138–18,867	917–3,300
Heterozygous	40,734 (4)	15,713 (4)	10,161 (6)	753 (4)
	30,303–52,631	5,556–22,727	8,333–13,699	500–1,190
Homozygous	(A) 34,027 (3)	0 (15)	4,402 (7)	1,654 (5)
	29,411–40,000		2,632–7,672	658–3,225
	(B) 7,815 (5)			
	4,545–12,195			

* Mean (number of animals).
‡ Range.

negligible consumption of C1 and C2. The degree of fixation of the late components was roughly equivalent for C4-deficient, heterozygous, and normal NIH guinea pig serum.

DISCUSSION

Previous studies utilizing an extremely sensitive hemolytic assay have shown a total absence of functional C4 molecules in the serum of C4-deficient guinea

pigs (12). In this study, an attempt was made to determine whether sera of these animals possess a C4-like molecule without hemolytic activity. The ability of C4-deficient animals to produce large amounts of anti-C4 when immunized with normal guinea pig serum, suggests that the C4-deficient guinea pigs either entirely lack C4 or possess a nonfunctional variant of the molecule which differs from the normal C4 molecule by multiple antigenic determinants.
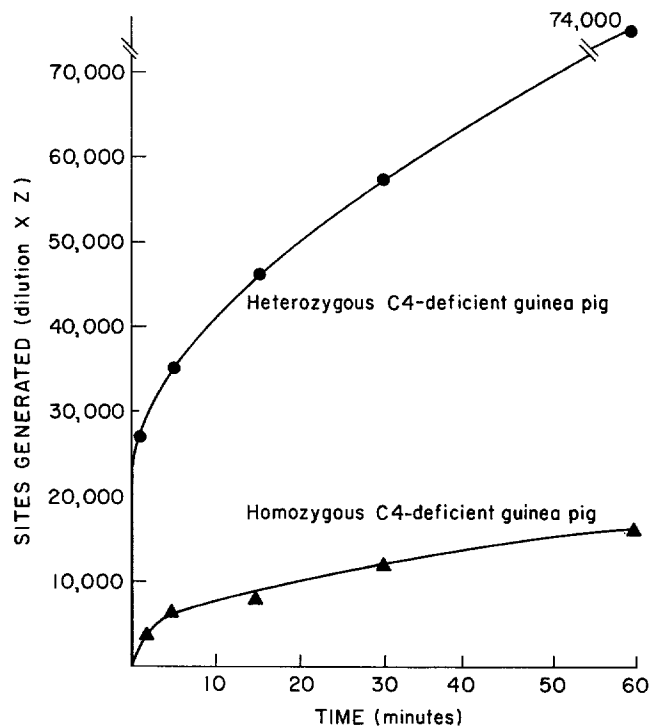

FIG. 3. Kinetics of activation of C1 to C$\overline{1}$ in the hemolytic system. Sites generated equal to $-\ln (1 - Y) \times$ dilution.

Since strain 13, Hartley strain, and normal NIH guinea pigs immunized with C4-deficient serum fail to make antibody to any component in the C4-deficient serum, the presence of an allotypic variant or an aberrant C4 molecule in the serum of the C4-deficient guinea pigs is unlikely. Moreover, antibody to normal C4 produced in an unrelated species (the rabbit) recognizes nothing in the serum of the C4-deficient guinea pigs. The evidence thus strongly suggests that C4-deficient guinea pigs are entirely lacking in C4.

Because of their absolute deficiency in this early component in the complement sequence, C4-deficient guinea pigs offer a unique opportunity to study

alternate methods of activation of complement. Several studies of in vitro complement function have suggested that there may be alternate pathways of activation for the components of complement which act late in the complement sequence. Gewurz et al. showed that incubation of fresh guinea pig serum with
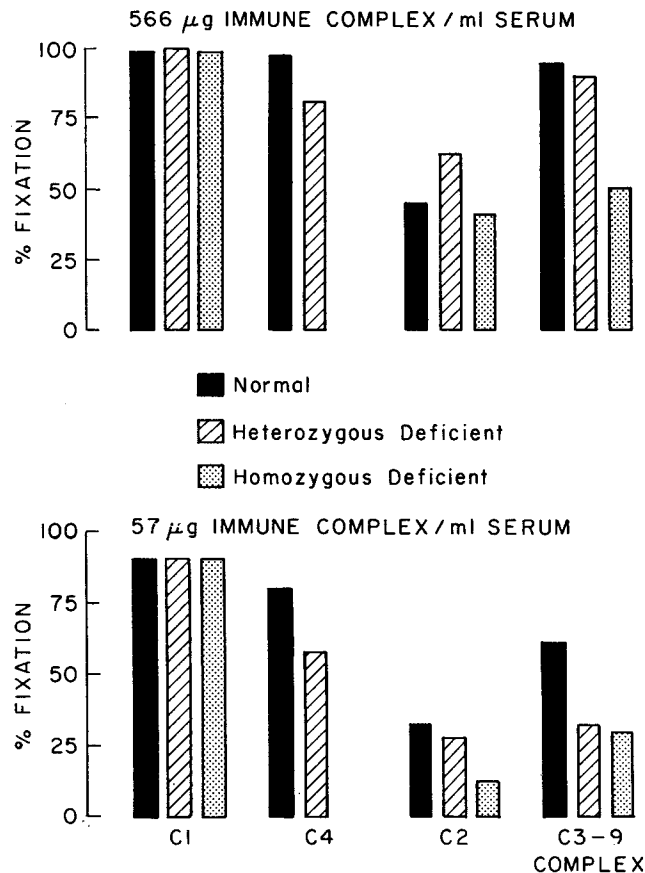

FIG. 4. Fixation of complement components by antigen–antibody complexes formed at equivalence. Comparison of pattern of component fixation in normal guinea pig serum, heterozygous C4-deficient, and C4-deficient serum.

endotoxin selectively removed the late components of complement with minimal consumption of C1, C4, and C2 (20). Our studies with endotoxin and antigen–antibody complexes confirm these findings. Sandberg et al. reported that guinea pig γ_1 antibody, classically considered to be a noncomplement fixing immunolgobulin (21), when assayed in the form of immune aggregates fixed late-acting complement components in a pattern similar to that seen with

endotoxin (22). Marcus et al. found that rabbit anti-guinea pig C2 antibody could prevent immune hemolysis but was unable to inhibit complement fixation by endotoxin (23). These studies all suggest the existance of an alternate pathway, but suffer from the defect that it is impossible to rule out the presence of antibodies which utilize minute amounts of C1, C4, and C2 with extreme efficiency and activate the late components by the normal pathway. Studies of
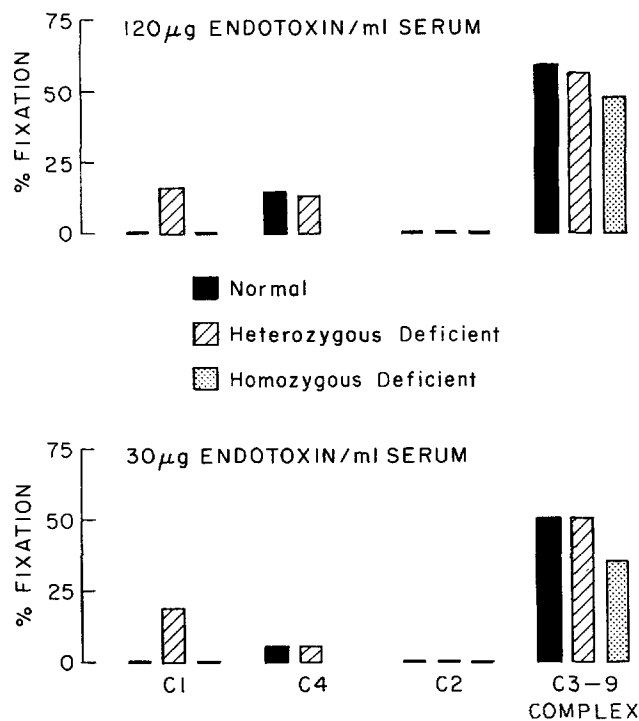

FIG. 5. Fixation of complement components by *E. coli* endotoxin. Comparison of pattern of component fixation in normal guinea pig serum, heterozygous C4-deficient, and C4-deficient serum.

immunoglobulin function have suggested that different classes of antibody utilize complement components with different efficiencies (24, 25). Our studies indicate that endotoxin and antigen–antibody complexes are able to utilize the late components of complement in the sera of C4-deficient guinea pigs. Therefore, an alternate pathway for the activation of the late components must exist. In experiments to be reported in detail elsewhere, in vitro polymorphonuclear neutrophilic chemotactic factor generation in C4-deficient sera, requiring activation of late-acting complement components, was also found to be normal.[2] These results also indicate operation of a C4 bypass mechanism in

[2] Clark, R., H. Kimball, L. Ellman, and M. M. Frank. Unpublished observations.

these sera. The demonstration of this bypass mechanism in vitro is in complete agreement with the in vivo demonstration of normal complement-dependent inflammatory responses in C4-deficient animals.

Titers of C1 and C2 may also be decreased in C4-deficient guinea pigs. Although levels of C2 in the deficient animals are within the normal range of our laboratory of pooled, commercially available, Hartley strain sera, they are about one-half those of either their heterozygous littermates or normals of the same strain. We do not know whether this represents decreased synthesis of C2, reflecting a feedback control of C2 synthesis, or in increased catabolism.

The C1 titer is not uniform in the group of C4-deficient animals. Three of eight animals tested had a normal C1 titer; the remaining five animals had titers of C1 which were 10–20% of normal. The titers were not low because of a gross defect in activation of C1 (Fig. 3) and the explanation for this variation is unknown.

We have attempted to establish whether these complement-deficient animals might have the same defect as those reported by Moore in 1919 (14) and subsequently described in detail by Hyde (19, 26). This earlier strain was thought to have a deficiency of the "classical" third component of complement, but since C4 was not discovered until 1926 (27), sera from the earlier strain were not tested for the presence of C4 activity. This earlier strain has subsequently been lost, and since the origin of the NIH multipurpose strain is unknown, it was of interest to determine whether the C4-deficient guinea pigs have the same defect as the earlier strain. In both cases the complement component missing from the serum was heat stable. Yeast (zymosan)-treated serum, now known to be specifically depleted of C3, was unable to restore the serum hemolytic activity in the earlier described strain. In contrast, the serum hemolytic activity of the C4-deficient strain was restored with zymosan-treated normal guinea pig serum. Therefore, C4-deficient guinea pigs appear to be a new strain of animals unrelated to those discovered in 1919. These animals offer unique opportunities for studies of complement function and pathways of complement activation.

SUMMARY

In vitro studies were performed utilizing sera from a strain of guinea pigs with a total absence of hemolytically active C4. Previous studies in these animals have demonstrated normal complement-dependent inflammatory reactions, suggesting that they are able to bypass their deficiency of C4. In vitro studies with C4-deficient serum also indicate normal activation of late-acting C components. Thus, endotoxin was capable of fixing normal amounts of the late components of complement (C3–9) in these sera, but did not fix C1 and C2. Antigen-antibody complexes fixed both early and late components of complement, although components beyond C4 were fixed less efficiently than in normal sera. Therefore, both in vivo and in vitro evidence indicates

that the C4-deficient guinea pigs possess an alternate pathway for activation of late-acting complement components.

Antigenic analysis of C4-deficient serum utilizing both guinea pig anti-C4 antibody and rabbit anti-C4 antibody suggests an absolute deficiency of C4-like molecules. Sera from animals with C4-deficiency were found to have one-half the normal level of C2. Sera from five of eight animals tested had 10–20% normal C1 activity. C3–9 assayed as a complex was normal.

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