IN VIVO STUDIES IN C4-DEFICIENT GUINEA PIGS

By LEONARD ELLMAN, M.D., IRA GREEN, M.D., FRANCIS JUDGE, D.V.M., and MICHAEL M. FRANK, M.D.

(From the Laboratory of Immunology and Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases; and the Laboratory Aids Branch, Division of Research Services, National Institutes of Health, Bethesda, Maryland 20014)

(Received for publication 2 March 1971)

The role of complement in a variety of immunological phenomenon has been studied by experimentally inducing complement depletion, or by the study of animals in which individual components of complement are absent due to genetic defects. The effects of genetically controlled total deficiencies of C5 and C6 have been studied in the mouse (1, 2) and the rabbit (3, 4), respectively. We have recently reported the discovery of guinea pigs with a total deficiency of the fourth component of complement (5). This defect is the first example of a total deficiency of one of the early reacting components of complement and is inherited as a simple, autosomal recessive trait. Animals heterozygous for C4 have intermediate levels of C4 by hemolytic assay and by gel diffusion analysis. We now wish to report our studies on in vivo immune responses in these guinea pigs.

Materials and Methods

Experimental Animals.—Normal "NIH multipurpose" guinea pigs, the strain from which the C4-deficient guinea pigs were derived, were obtained from the National Institutes of Health Animal Production Section and were mated with C4-deficient guinea pigs. The heterozygous offspring were mated with C4-deficient animals to produce litters containing approximately equal numbers of C4-deficient guinea pigs and guinea pigs heterozygous for C4. Screening of the animals' serum for the presence or absence of C4 was accomplished by Ouchterlony gel analysis with a specific guinea pig anti-C4 antiserum as previously described (5). Whenever possible, the immune response of C4-deficient animals was compared to their heterozygous littermates.

Hartley strain guinea pigs and New Zealand White rabbits, also obtained from the NIH Animal Production Section, were used for the production of various antisera. Strain 13 guinea pigs were used in experiments involving the reverse passive Arthus reaction and passive cutaneous anaphylaxis.

Materials.—Purified ovalbumin (OVA)[1] and human serum albumin (HSA) were obtained

[1] *Abbreviations used in this paper:* BGG, bovine gamma globulin; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; disodium-EDTA, disodium ethylenedinitrilotetraacetate; DNP, dinitrophenyl; HSA, human serum albumin; OCBC, *o*-chlorobenzyl chloride; OVA, ovalbumin; PBS, phosphate-buffered saline; PCA, passive cutaneous anaphylaxis; PPD, purified protein derivative; RPA, reverse passive Arthus.

from Nutritional Biochemical Corp., Cleveland, Ohio, and Behringwerke AG, Marburg-Lahn, West Germany, respectively. $DNP_{66}BGG$ and $DNP_{48}BSA$ were prepared by the reaction of 2,4-dinitrofluorobenzene with bovine gamma globulin (BGG) and bovine serum albumin (BSA) (6), both obtained from Armour Pharmaceutical Co., Chicago, Ill. The subscript refers to the average number of dinitrophenyl (DNP) groups per molecule. DNP-^{3}H–lysine was prepared as described by Katz et al. (7). Carrier-free Na-^{125}I purchased from New England Nuclear Corp., Boston, Mass., and HSA-^{125}I and BSA-^{125}I were prepared as described in ref. 8. *o*-Chlorobenzyl chloride (OCBC) was purchased from Eastman Organic Chemicals, Rochester, N. Y. Rabbit anti-guinea pig γ_1 globulin and rabbit anti-guinea pig γ_2 globulin were a gift of Dr. Victor Nussenzweig. Sheep anti-rabbit serum was purchased from Hyland Laboratories, Los Angeles, Calif. Complete Freund's adjuvant (CFA) containing *Mycobacterium butyricum* 0.5 mg/ml was obtained from Difco Laboratories, Detroit, Mich., or was prepared by adding killed *M. tuberculosis* ($H_{37}Rv$) to incomplete Freund's adjuvant in a final concentration of 2 mg/ml. Purified protein derivative (PPD) was obtained from Burroughs Wellcome & Co., Tuckahoe, N. Y. ^{51}Cr in the form of Na_2CrO_4, 185 Ci/μg was purchased from Amersham-Searle Corp., Chicago, Ill. Phosphate-buffered saline (PBS), 0.85% saline, pH 7.4, was used as a standard diluent. C4 assays were performed as previously described (5).

Immunization of Experimental Animals.—C4-deficient guinea pigs and their heterozygous littermates were immunized with 100 μg of $DNP_{66}BGG$ in CFA. Animals received 0.4 ml of an emulsion composed of equal parts of PBS containing the antigen and CFA, which was distributed into four footpads. C4-deficient guinea pigs and normal NIH guinea pigs matched for age and sex were also immunized with 100 μg of OVA, 1 μg of BSA, or 10 μg of BSA in CFA. A second set of C4-deficient guinea pigs and their heterozygous littermates were immunized with 0.4 mg of OCBC in the oil phase of an emulsion of CFA and PBS.

Antibody Production.—2 wk after immunization with $DNP_{66}BGG$ and 3 wk after immunization with OVA, experimental animals were bled from the retro-orbital sinus. Because of the known influence of complement on quantitative precipitin reactions (9), disodium ethylenedinitrilotetraacetate (disodium-EDTA) was added to the serum to a final concentration 0.01 M. Anti-DNP antibodies were measured by quantitative precipitation with DNP_{163}-fibrinogen as described by Eisen et al. (10).

Anti-DNP antibodies were also measured by a modification of the Farr technique in which the percentage binding of 10^{-8} M DNP-^{3}H–lysine by antisera was measured after precipitation with $(NH_4)_2SO_4$ (11–13). The percentage binding was determined for undiluted sera and $\frac{1}{10}$ and $\frac{1}{100}$ dilutions made in 20% normal guinea pig serum in PBS. Serum from unimmunized animals bound 0–7% of the antigen. The assay was performed in the presence or absence of disodium-EDTA. The amount of anti-OVA antibody was determined by quantitative precipitation with OVA (14).

Guinea pigs immunized with 1 μg or 10 μg of BSA were bled 2 and 4 wk after immunization of serum concentration of anti-BSA antibody was also assayed by the Farr technique as described above utilizing 0.7 μg of BSA-^{125}I as antigen. Serum from unimmunized animals bound 0–6% of the antigen.

Direct Arthus Reaction.—15 days after immunization experimental animals were skin tested with the immunizing antigen contained in 0.1 ml of PBS. Edema and hemorrhage at the site of intradermal injection were recorded at the peak of the reaction 90 min later. Representative Arthus reactions were excised and the histology of the skin examined.

Contact Sensitivity.—2 wk after immunization with OCBC the animals were skin tested by dropping 0.1 ml of a mixture of 1 part olive oil and 4 parts acetone containing 2% OCBC by volume on the shaved skin. The degree of erythema and induration was graded 24 hr later.

Delayed Hypersensitivity.—24 hr after measurement of the direct Arthus reaction elicited by $DNP_{66}BGG$ and OVA, the same skin reactions were again evaluated for induration as a

measure of delayed hypersensitivity to these antigens. Animals which had been immunized with CFA containing *M. tuberculosis* ($H_{37}Rv$) were simultaneously skin tested with PPD injected intradermally in 0.1 ml.

Reverse Passive Arthus (RPA) Reaction.—Hartley strain guinea pigs were immunized with 0.1 mg of HSA in CFA (Difco). Their serum was collected 2 wk later, and purified γ_1 and γ_2 guinea pig immunoglobulin fractions were prepared by the method of Oliveria et al. (15). The γ_1 fraction, although appearing pure on immunoelectrophoresis, was found to contain a small amount of γ_2 globulin by Ouchterlony analysis in gel with rabbit anti-guinea pig γ_2 antiserum. The γ_2 fraction was found to be completely free of γ_1 globulin by Ouchterlony analysis with rabbit anti-guinea pig γ_1 antiserum. Moreover, while the γ_1 fraction gave a passive cutaneous anaphylaxis reaction in strain 13 guinea pigs when 0.01 μg of anti-HSA antibody was injected intradermally, the γ_2 fraction failed to elicit a PCA reaction at a dose of 150 μg of anti-HSA antibody (see below for method of quantitation of anti-HSA antibody).

A New Zealand rabbit was immunized with 1 mg of HSA in CFA (Difco). 2 wk later it was bled and the serum was twice passaged through a diethylaminoethyl (DEAE) column equilibrated with phosphate buffer, 0.01 M, pH 8.6. The resulting fraction contained γG immunoglobulin and was found to be uncontaminated with other immunoglobulins on electrophoresis with a sheep anti-rabbit whole serum antiserum.

Because of the limited supply of γ_1 and γ_2 guinea pig anti-HSA fractions, the amount of antibody was determined by an indirect method. The amount of anti-HSA antibody in the sera of immunized guinea pigs was determined by quantitative precipitation with HSA (14). Anti-HSA antibody was also measured in these sera by the Farr technique in which the percentage binding of 0.1 ml of 10^{-5} M HSA-^{125}I by the globulin fraction of serum was determined. A standard curve relating percentage binding of HSA-^{125}I to actual amounts of anti-HSA antibody as measured by precipitin analysis was constructed. The percentage binding of the HSA-^{125}I by the γ_1 and γ_2 guinea pig anti-HSA fractions was then determined and the amount of anti-HSA antibody in milligrams per milliliter was extrapolated from the standard curve. It has been previously established that guinea pig γ_1 and γ_2 antibodies from individual guinea pigs have similar affinities (16).

Anti-HSA antibody in the purified rabbit γG fraction was determined by quantitative precipitation with HSA (14). The guinea pig γ_2 and the rabbit γG fractions were found to be entirely free of C4 by hemolytic assay. The guinea pig γ_1 fraction had a small amount of C4 activity which was fully inactivated by treatment with NH_4OH (14).

RPA reactions were elicited by the injection of known amounts of the guinea pig and rabbit anti-HSA antibodies intradermally in 0.1 or 0.15 ml of PBS. The animals immediately received an intravenous injection via the dorsal vein of the foot of 1 ml of PBS containing 1 mg of HSA. Edema and hemorrhage were recorded at the peak of the reaction 90 min later. Intradermal injection of the immunoglobulin fraction into control guinea pigs caused slight erythema, but no edema or hemorrhage.

Passive Cutaneous Anaphylaxis (PCA).—Purified rabbit and guinea pig anti-DNP antibody was prepared from anti-DNP_{48}BSA serum according to the method of Farah et al. (17). Purified rabbit anti-BGG antibody was prepared by acid elution of anti-BGG serum from a BGG Sepharose column (18). Amounts of antibody in the purified fractions were determined by quantitative precipitation (10, 14). The purified antibody fractions showed no C4 activity in hemolytic assay.

C4-deficient guinea pigs and normal NIH guinea pigs matched for age and sex received intradermal injections of known amounts of antibody contained in 0.1 ml of PBS. 4 hr later the guinea pigs received an intravenous injection of 1 ml of 0.5% Evans blue in PBS containing 250 μg DNP_{48}BSA. The diameter of the blue spot was measured after 30 min.

Exudative Response to a Nonimmunologic Stimulus.—Three C4-deficient guinea pigs and

three heterozygous littermates were anesthetized with ether and a small skin incision was made in the abdominal wall. A pocket was made in the subcutaneous tissue by blunt dissection. A sterile, chemically clean, round, glass cover slip was inserted in the pocket and the incision was sutured. 6 hr later the cover slip was retrieved, fixed in 95% ethanol, and stained with Giemsa stain. The number and type of white blood cell per high power field (× 400) was scored by two observers without knowledge of the animal from which the cover slip was obtained.

Immune Clearance.—NIH multipurpose strain guinea pig erythrocytes were obtained by heart puncture and washed three times with isotonic veronal buffer (14). 10^9 erythrocytes were labeled with 100 μCi of ^{51}Cr in 1 ml.

Anti-guinea pig erythrocyte serum was prepared by the injection of 0.2 ml of an emulsion of 5% erythrocytes in PBS with CFA (Difco) into each paw of a rabbit. The rabbit was bled 12 days later and the serum was found to have an agglutination titer against guinea pig erythrocytes of $\frac{1}{320}$.

Sensitization of guinea pig erythrocytes was achieved by incubation with dilutions of the rabbit antiserum at 37°C for 15 min in the presence of 0.01 M disodium-EDTA to prevent fixation of rabbit complement to the immune complexes. The sensitized erythrocytes were washed in disodium-EDTA buffer and then in isotonic veronal buffer to remove nonbound antibody. The cells were vigorously resuspended and no gross agglutinates were seen. Clearance studies were performed by injecting 2.5×10^8 labeled erythrocytes/100 g weight in a volume of 1 ml into a dorsal foot vein. At various times after injection 0.05 ml of blood was removed from the retro-orbital sinus with a calibrated, glass bleeding pipette and added to 1 ml of isoveronal buffer for counting in a well-type scintillation counter. The logarithm of the counts (C) per minute in the blood sample was plotted against time (T). The phagocytic indices

$$K = \frac{\log C_1 - \log C_2}{T_2 - T_1}$$

were calculated from the slope of the semilogarithmic curve. K values reported are the average of two or more clearances at each antibody dilution, and there was remarkably little variation between the values obtained at each antibody dilution.

15 min after the intravenous injection of sensitized erythrocytes, two normal NIH guinea pigs were sacrificed. A weighed portion of the organs was placed into test tubes and the radioactivity measured in a well-type scintillation counter.

RESULTS

Antibody Production in Normal, Heterozygous, and C4-Deficient Guinea Pigs.—C4-deficient and heterozygous guinea pigs immunized with $DNP_{66}BGG$ produced equivalent amounts of anti-DNP antibody 2 wk after immunization as determined by both quantitative precipitation and the Farr assay (Table I). C4-deficient guinea pigs immunized with OVA produced less anti-OVA antibody at 3 wk than normal NIH guinea pigs (Table I). Anti-BSA antibody in normal and C4-deficient guinea pigs was roughly equivalent after immunization with 10 μg of BSA (Table II). However, when 1 μg of BSA was used for immunization, none of the four C4-deficient guinea pigs produced detectable antibody at 2 wk while two of the three normal NIH guinea pigs produced substantial amounts of anti-BSA antibody as determined by the Farr assay (Table

II). At 4 wk all animals produced anti-BSA antibody, but the response of the C4-deficient guinea pigs continued to be less than that of the normal NIH animals.

The effect of complement on the degree of binding of ligands by antibody in the Farr assay was also investigated by performing the assays in the presence or absence of disodium-EDTA. While the degree of binding of a small ligand, DNP-^{3}H–lysine, was not influenced by the presence or absence of disodium-EDTA (Table I), the degree of binding of a large, multivalent ligand, BSA-^{125}I was considerably decreased in the presence of disodium-EDTA (Table II).

TABLE I

Antibody Production in Normal, C4-Deficient, and Heterozygous Guinea Pigs

	No. of animals	Immunizing antigen 0.1 mg	Quantitative precipitin determination performed in the presence of disodium-EDTA	Farr assay performed in the presence of disodium-EDTA; % binding of 10^{-8} M DNP-^{3}H-lysine by			Farr assay performed in the absence of disodium-EDTA; % binding of 10^{-8} M DNP-^{3}H lysine by		
				Undiluted serum	1/10 dilution	1/100 dilution	Undiluted serum	1/10 dilution	1/100 dilution
Anti-OVA antibody									
Normal NIH	4	OVA*	3.6 mg/ml‡ (3.4–4.2)	Not done					
C4 deficient	4		2.1 mg/ml (1.6–2.8)						
Anti-DNP antibody									
Heterozygous NIH	4	$DNP_{66}BGG$*	0.75 mg/ml (0.6–1.1)	89‡ (85–93)	62 (57–70)	42 (33–60)	90 (88–93)	66 (61–75)	43 (33–60)
C4 deficient	4		0.80 mg/ml (0.7–0.85)	89 (87–90)	65 (58–74)	46 (37–50)	92 (90–99)	68 (66–69)	47 (37–51)

* Animals immunized with the antigen in CFA containing *M. tuberculosis* ($H_{37}Rv$), 2 mg/ml.

‡ Average value (and range).

Contact Sensitivity and Delayed Hypersensitivity in C4-Deficient, Heterozygous, and Normal NIH Guinea Pigs.—Application of OCBC to the skin 2 wk after immunization led to marked erythema, swelling, and induration in all animals; no difference between C4-deficient and heterozygous guinea pigs was observed. As indicated in Table III, C4-deficient animals showed an equal degree of delayed hypersensitivity to PPD, $DNP_{66}BGG$, and OVA as compared to heterozygous and normal NIH guinea pigs.

Direct Arthus Reaction in C4-Deficient, Heterozygous, and Normal Guinea Pigs.—Table IV summarizes the result of direct Arthus reactions elicited by intradermal injection of OVA or $DNP_{66}BGG$ into animals immunized with these antigens. C4-deficient guinea pigs manifested a classical arthus reaction with marked central hemorrhage. These lesions were identical to those observed in

heterozygous and normal NIH animals. Histologic sections of these reactions revealed the typical neutrophil accumulation in venule walls with hemorrhage, edema, and neutrophil accumulation in the surrounding interstitial spaces of the skin.

Reverse Passive Arthus Reaction—Preliminary experiments with guinea pig γ_1 and γ_2 anti-HSA fractions were performed in strain 13 guinea pigs. The intradermal injection of 100 μg of γ_1 antibody (containing small amounts of γ_2 antibody) elicited only edema, while 200 μg of γ_2 antibody produced neither

TABLE II

*Anti-BSA Antibody Production in C4-Deficient and Normal NIH Guinea Pigs Immunized with Limiting Amounts of BSA**

	No. of animals	Time after immunization	Farr assay performed in the absence of disodium EDTA; % binding of BSA-^{125}I by			Farr assay performed in the presence of disodium-EDTA; % binding of BSA-^{125}I by		
			Undiluted serum	1/10 dilution	1/100 dilution	Undiluted serum	1/10 dilution	1/100 dilution
Immunization with 1 μg BSA								
C4 deficient	4	2 wk	0	0	0	0	0	0
Normal NIH	3		60 (0,90,91)‡	18 (0,20,35)	0	42 (0,49,77)	6 (0,6,13)	0
C4 deficient	4	4 wk	57§ (23-94)	12 (0-45)	0	Not done		
Normal NIH	3		75 (41-94)	28 (0-71)	3 (0-7)			
Immunization with 10 μg BSA								
C4 deficient	4	2 wk	94 (91-95)	61 (28-80)	8 (2-12)	83 (62-93)	34 (9-49)	2 (0-5)
Normal NIH	5		88 (68-95)	42 (3-70)	9 (2-11)	70 (25-92)	21 (6-37)	0
C4 deficient	4	4 wk	94 (92-96)	46 (37-63)	2 (0-10)	Not done		
Normal NIH	5		95 (94-96)	66 (42-84)	10 (0-17)			

* Animals immunized with the antigen in CFA containing *M. butyricum*, 0.5 mg/ml.

‡ Average value with individual values in parenthesis.

§ Average value (and range).

edema nor hemorrhage. However, when small amounts of γ_1 antibody (10–20 μg) were added to γ_2 antibody (50–100 μg) and injected at a single site, there was considerable edema and unequivocal hemorrhage.

Table V summarizes RPA reactions in two C4-deficient guinea pigs and a heterozygous control. As was observed with the direct Arthus reaction, C4-deficient guinea pigs showed no deficiency in their ability to have a normal RPA reaction, either with guinea pig antibody or rabbit γG antibody. Again, both guinea pig γ_1 and γ_2 antibodies were required for a classical Arthus reaction with a hemorrhagic center.

Passive Cutaneous Anaphylaxis in C4-Deficient and Normal Guinea Pigs.—As indicated in Table VI, C4-deficient guinea pigs showed a normal PCA reaction with both purified guinea pig and rabbit anti-DNP antibody when challenged

with an intravenous injection of $DNP_{66}BSA$. A control injection of rabbit anti-BGG antibody failed to elicit a PCA reaction in these animals.

Exudative Response to a Nonimmunologic Stimulus.—At the end of 6 hr a substantial cellular response was noted on the cover slip which had been removed from the abdominal wall. The average high power field showed 6–7 polymorphonuclear leukocytes and 1–2 mononuclear cells; as many as 30 polymorphonuclear leukocytes were occasionally seen in a single field. The exudative response in the C4-deficient guinea pigs appeared to be no different from their heterozygous littermates.

TABLE III

Delayed Hypersensitivity in Heterozygous, C4-Deficient, and Normal Guinea Pigs

Immunizing antigen		No. of animals	Average diameter of induration (and range) at 24 hr after i.d. injection of homologous antigen	
			10 μg PPD	*20 μg PPD*
M. tuberculosis ($H_{37}Rv$) 2 mg/ml*	Heterozygous NIH	8	14 mm (9–20)	18 mm (12–25)
	C4 deficient	8	16 mm (8–22)	18 mm (10–24)
			20 μg $DNP_{66}BGG$	*50 μg $DNP_{66}BGG$*
$DNP_{66}BGG$	Heterozygous NIH	4	5 mm (4–6)	8 mm (7–10)
	C4 deficient	4	6 mm (5–7)	8 mm (7–9)
			10 μg OVA	
OVA	Normal NIH	4	13 mm (9–16)	
	C4 deficient	4	11 mm (9–13)	

* Four animals in each group immunized with *M. tuberculosis* ($H_{37}Rv$) and OCBC, and four with *M. tuberculosis* ($H_{37}Rv$) and $DNP_{66}BGG$.

TABLE IV

Direct Arthus Reactions in Normal, C4-Deficient, and Heteroyzgous Guinea Pigs

	No. of animals	Immunizing antigen 0.1 mg	Average diameter (and range) of edema at 1 hr after i.d. injection of homologous antigen		Average diameter (and range) of hemorrhage at 1 hr after i.d. injection of homologous antigen	
			10 μg	*100 μg*	*10 μg*	*100 μg*
Normal NIH	4	OVA*	16 mm (14–21)	20 mm (15–24)	4 mm (0–7)	8 mm (5–11)
C4 deficient	4		17 mm (14–21)	18 mm (14–22)	5 mm (3–7)	8 mm (3–10)
			20 μg	*150 μg*	*20 μg*	*150 μg*
Heterozygous NIH	4	DNP_{66}-BGG*	8 mm (5–10)	11 mm (9–12)	3 mm (0–6)	6 mm (0–9)
C4 deficient	4		6 mm (0–10)	13 mm (10–15)	3 mm (0–6)	7 mm (5–9)

* Animals immunized with CFA containing *M. tuberculosis* ($H^{37}Rv$), 2 mg/ml.

Immune Clearance.—^{51}Cr-labeled guinea pig erythrocytes showed no reticuloendothelial clearance during the 2 hr period of observation. Other studies have shown these erythrocytes to have a half-life of 7.5 days.[2]

The clearance of erythrocytes coated with 1/100 dilution of rabbit anti-guinea pig erythrocyte serum was markedly accelerated in normal guinea pigs

TABLE V

Reverse Passive Arthus Reaction in C4-Deficient Guinea Pigs in Response to Intravenous Injection of 1 mg Human Serum Albumin

	No. of animals	25 μg guinea pig γ_1 anti-HSA antibody*		175 μg guinea pig, γ_2 anti-HSA antibody‡		25 μg guinea pig γ_1 and 175 μg guinea pig γ_2 anti-HSA antibodies‡		500 μg rabbit γG anti-HSA antibody*	
		Edema	Hemorrhage	Edema	Hemorrhage	Edema	Hemorrhage	Edema	Hemorrhage
C4 deficient	2	8 mm§	0	0	0	12.5mm (10–15)	5mm (4–6)	18.5 mm (17–22)	9.5 mm (8–11)
Heterozygous NIH	1	6 mm	0	0	0	17.0 mm	7 mm	20.0 mm	8.0 mm

* Antigen injected intradermally in 0.1 ml of physiologic saline.
‡ Antigen injected intradermally in 0.15 ml of physiologic saline.
§ Average diameter (and range) of reaction measured at 1 hr.

TABLE VI

*Passive Cutaneous Anaphylaxis Elicited by Intravenous Injection of DNP-BSA**

	No. of animals	Purified rabbit anti-DNP antibody‡		Purified guinea pig anti-DNP antibody		Purified rabbit anti-BGG antibody
		0.7 μg	*0.07 μg*	*2 μg*	*0.2 μg*	*2 μg*
Normal NIH	3	16.0 mm§ (15–17)	11 mm (10–12)	19 mm (18–20)	15 mm (10–20)	0
C4 deficient	3	16.5 mm (16–17)	8 mm (7–10)	18 mm (16–20)	14 mm (10–16)	0

* Animals injected with 1 ml of 0.5% Evans blue in physiologic saline containing 250 μg of $DNP_{68}BSA$.
‡ Antibody was injected intradermally in a volume of 0.1 ml of physiologic saline.
§ Average diameter of blue spot (and range).

(K = 0.100) (Fig. 1). In C4-deficient guinea pigs the rate of clearance was considerably slower at this dilution of antibody (K = 0.036). During the course of immune clearance in one C4-deficient pig, an injection of 0.5 ml of normal guinea pig serum as a source of C4 temporarily accelerated the clearance.

To study the effect of increasing amounts of opsonizing antibody, the eryth-

[2] Schreiber, A., and M. Frank. Unpublished observations.

rocytes were coated with twice the amount of rabbit antibody ($\frac{1}{50}$ dilution). Under these conditions there was a small increase in the rate of clearance in normal guinea pigs, and a marked increase in the C4-deficient guinea pigs which approached that of the normal guinea pigs. Two of the normal NIH guinea pigs were sacrificed at 15 min and a total of 70 and 75% of the injected radioactivity was recovered in the liver and spleen.

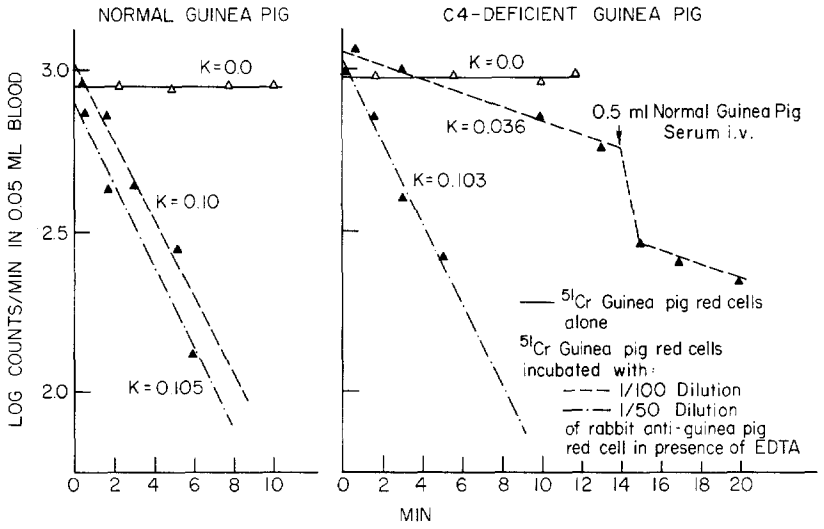

FIG. 1. Clearance of ^{51}Cr-labeled guinea pig red blood cells in normal and C4-deficient guinea pigs.

DISCUSSION

Most of the immunologic parameters studied were found to be normal in C4-deficient guinea pigs; these included contract and delayed hypersensitivity, passive cutaneous anaphylaxis, Arthus and reverse passive Arthus reactions, and foreign body-induced exudative response. An unexpected finding was the entirely normal direct and RPA reactions. Tissue destruction of the Arthus type is thought to be dependent upon activation of late-acting complement components with generation of neutrophil chemotactic factors and invasion of blood vessel walls by neutrophils (19, 20). Cochrane et al. have shown that rats depleted of neutrophils or C3 (by treatment with cobra venom) failed to have a hemorrhagic reaction in the RPA reaction (21). Guinea pigs depleted of complement by cobra venom treatment also showed absent hemorrhage and a decrease in edema in both the direct and RPA reactions. Serum or isolated C3 restored the Arthus reaction when injected locally with the antigen (22). The direct and RPA reaction studied in C6-deficient rabbits showed a marked de-

crease in gross hemorrhage (3, 23). Since the C4-deficient guinea pigs appear to have a total absence of C4, we would suggest that their ability to have a normal Arthus reaction involves the use of an alternate pathway to activate the late complement components with normal generation of neutrophil chemotactic factors. In vitro experiments by other investigators have suggested that such pathways exist (24–26). It appears that the presence of normal Arthus reactions in C4-deficient guinea pigs is the first in vivo demonstration of an alternate pathway in the complement sequence. Confirmation of the existence of this in vivo bypass mechanism will involve the demonstration of C3 fixation at the site of the Arthus reactions; these studies are being performed in collaboration with Dr. Charles Cochrane.

Our studies with the RPA reaction in which the antibody is injected intradermally are in accord with the recent findings of Maillard and Voisin (27) that guinea pig γ_2 injected intravenously does not elicit any lesion by itself and that γ_1 and γ_2 antibodies are necessary to make up a complete, classical Arthus reaction.

Although the generation of complement-derived chemotactic factors is required for the attraction of neutrophils to the site of deposition of antigen–antibody complexes both in vitro and in vivo (19, 20), the role of complement in the development of neutrophil exudation induced by nonimmunological means is not clear (28). An individual with hereditary (partial) C2 deficiency showed a depression of neutrophil exudation in a Rebuck skin window, and his serum gave a diminished chemotactic response in a Boyden chamber (29). Thus, there is some suggestion that participation of complement may be required in the exudative response to nonimmunologic stimuli such as trauma or a foreign body. The normal pattern of cellular exudation in response to a cover slip implanted subcutaneously in the C4-deficient guinea pigs may therefore represent another example of their ability to bypass their complement deficiency.

The PCA reaction elicited by either guinea pig γ_1 or rabbit γG antibody was normal in C4-deficient guinea pigs. It cannot be established from these studies whether this observation reflects utilization of an alternate pathway by the C4-deficient animals or lack of dependence of PCA on complement activity (30). Recent studies demonstrating normal PCA in guinea pigs decomplemented with cobra venom also suggests that complement does not play a role in the PCA reaction (21, 22).

The immune clearance of bacteria or erythrocytes depends upon their removal from the blood by fixed macrophages of the reticuloendothelial system, principally in the liver and spleen (31, 32). Both specific antibody and complement are required for immune clearance (31–34). Since guinea pigs have "natural" antibodies against a variety of bacteria, it seemed desirable to investigate the immune clearance of an antigen against which there are no

natural antibodies. It was therefore decided to study the immune clearance of isologous erythrocytes coated in vitro with specific rabbit antibody. The clearance of these cells in the C4-deficient guinea pigs was found to be impaired and the intravenous administration of a small amount of normal guinea pig serum as a source of C4 led to a transient acceleration of the clearance. Recent in vivo studies of the immune clearance of erythrocytes (35) support the earlier in vitro findings of Nelson and Gigli (36) that C3 attachment plays an important role in the opsonization of red blood cells. Our results suggest that in vivo opsonization of erythrocytes by fixed, active C3 was defective in the C4-deficient guinea pigs, but this defect could be overcome by increased amounts of specific antibody. Studies by Spiegelberg et al. (32) of the immune clearance of *Escherichia coli* in complement depleted mice also demonstrated that excess antibody could correct for the slow clearance induced by complement deficiency.

Little attention has been given to the possible role of complement in antibody synthesis. Recently, Dukor et al. (37) and Bianco et al. (38) have described the existence of a specific immunoglobulin-C3 binding site on bone marrow-derived lymphoid cells. These authors suggested that these complement receptors may be intimately related to the cellular interactions which are necessary for antibody production. Our studies failed to show a simple relationship between C4 deficiency and antibody production. Although C4-deficient animals and their heterozygous littermates immunized with DNP-BGG made equivalent amounts of anti-DNP antibody, C4-deficient animals clearly made less antibody than normal NIH animals when immunized with OVA. Moreover, when immunized with limiting amounts of BSA, all C4-deficient animals failed to make detectable amounts of antibody at 2 wk while two of the three normal animals produced substantial amounts of antibody. At 4 wk, the response of the C4-deficient animals was still depressed in comparison to normal NIH guinea pigs. Although these studies involve a limited number of animals, there is a suggestion that the antibody response of C4-deficient animals may be impaired under certain circumstances. Whether these findings of impaired antibody production are directly related to the deficiency of C4 is currently being investigated.

The role of complement in delayed hypersensitivity reactions is not clear. Although some investigators have found that in vivo decomplementation was associated with a depression of delayed sensitivity (39, 40), this has not been confirmed by others (21, 22). In studies with C6-deficient rabbits by Rother et al. a decrease in delayed hypersensitivity response to PPD (41) and a delayed rejection of skin grafts in some animals was observed (42). However, the delayed hypersensitivity and allograft reactions were normal in another colony of C6-deficient rabbits described by Biro et al. (23). In the C4-deficient guinea pigs both contact and delayed sensitivity to a variety of antigens appeared to be entirely normal.

We also wish to note that C4-deficient guinea pigs were found to have normal hematocrit, white blood cell count and differential, and sedimentation rate. The growth rate and sex ratio of offspring were also found to be normal. The C4-deficient animals do not appear to have an increased incidence of infection in the controlled environment of our animal room.

SUMMARY

Guinea pigs with a genetically determined total deficiency of the fourth component of complement have been studied for various in vivo immunological functions. Passive cutaneous anaphylaxsis, contact and delayed hypersensitivity, and the cellular exudative response to a foreign body were normal. These animals also have normal direct and reverse passive Arthus reactions which suggest that they possess a mechanism to bypass C4 and directly activate late-acting complement components. This would appear to be an unequivocal demonstration of an alternate pathway in the complement sequence. Immune clearance of guinea pig erythrocytes sensitized with rabbit antibody was impaired in the deficient animals. Antibody production in C4-deficient animals was impaired for two of the three antigens studied.

The authors wish to express their appreciation to Dr. Baruj Benacerraf for his encouragement, help, and advice during the course of these studies. Expert technical assistance was provided by Mrs. Clara Horton and Miss Thelma Gaither.

BIBLIOGRAPHY

1. Rosenberg, L. T., and D. K. Tachibana. 1962. Activity of mouse complement. *J. Immunol.* **89**:861.
2. Cinader, B., S. Dubiski, and A. C. Wardlaw. 1964. Distribution, inheritance, and properties of an antigen, Mu**B**1, and its relation to hemolytic complement. *J. Exp. Med.* **120**:897.
3. Rother, K., M. Rother, and F. Schindera. 1964. Passive Arthus-Reaktion bei komplement defekten Kanenchen. *Z. Immunitaetsforch. Allergie Klin. Immunol.* **126**:473.
4. Biro, C. E., and G. Garcia. 1965. The antigenicity of aggregated and aggregate-free human gamma-globulin for rabbits. *Immunology.* **8**:411.
5. Ellman, L., I. Green, and M. Frank. 1970. Genetically controlled total deficiency of the fourth component of complement in the guinea pig. *Science* (*Washington*). **170**:74.
6. Benacerraf, B., and B. B. Levine. 1962. Immunological specificity of delayed and immediate hypersensitivity reactions. *J. Exp. Med.* **115**:1023.
7. Katz, D. H., W. E. Paul, E. A. Goidl and B. Benacerraf. 1970. Carrier function in anti-hapten responses. *J. Exp. Med.* **132**:261.
8. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**:185.
9. Weigle, W. O., and P. H. Maurer. 1957. The effect of complement on soluble antigen–antibody complexes. *J. Immunol.* **79**:211.

10. Eisen, H. N., M. E. Carsten, and S. Belman. 1954. Studies on hypersensitivity to low molecular weight substances. III. The 2,4-dinitrophenyl group as a determinant in the precipitin reaction. *J. Immunol.* **73**:296.
11. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I* BSA and antibody. *J. Infec. Dis.* **103**:239.
12. Green, I., B. Benacerraf, and S. H. Stone. 1969. The effect of the amount of mycobacterial adjuvants on the immune response of strain 2, strain 13 and Hartley strain guinea pigs to DNP-PLL and DNP-GL. *J. Immunol.* **103**:403.
13. Green, I., J. K. Inman, and B. Benacerraf. 1970. Genetic control of the immune response of guinea pigs to limiting doses of bovine serum albumin: relationship of the poly-L-lysine gene. *Proc. Nat. Acad. Sci. U.S.A.* **66**:1267.
14. Kabat, E. A., and M. M. Mayer. 1964. Experimental Immunochemistry. Charles C Thomas Publisher, Springfield, Ill. 2nd edition.
15. Oliveira, B., A. G. Osler, R. P. Siraganian, and A. L. Sandberg. 1970. The biologic activities of guinea pig antibodies. I. Separation of γ_1 and γ_2 immunoglobulins and their participation in allergic reactions of the immediate type. *J. Immunol.* **104**:320.
16. Siskind, G. W., W. E. Paul, and B. Benacerraf. 1966. Studies on the effect of the carrier molecule on antihapten antibody synthesis. *J. Exp. Med.* **123**:673.
17. Farah, F. S., M. Kerm, and H. N. Eisen. 1960. The preparation and some properties of purified antibodies specific for the 2,4-dinitrophenyl group. *J. Exp. Med.* **112**:1195.
18. Wofsy, L., and B. Burr. 1969. The use of affinity chromatography for the specific purification of antibodies and antigens. *J. Immunol.* **103**:380.
19. Müller-Eberhard, H. J. 1968. Chemistry and reaction mechanisms of complement. *Advan. Immunol.* **8**:1.
20. Cochrane, C. G. 1968. Immunologic tissue injury mediated by neutrophilic leucocytes. *Advan. Immunol.* **9**:97.
21. Cochrane, C. G., H. J. Müller-Eberhard, and B. S. Aiken. 1970. Depletion of plasma complement *in vivo* by a protein of cobra venom: its effect on various immunologic reactions. *J. Immunol.* **105**:55.
22. Maillard, J. L., and R. M. Zarco. 1968. Decomplementation par un facteur extrait du venim de cobra. Effet sur plusieurs reactiones immunes du cobaye et du rat. *Ann. Inst. Pasteur (Paris).* **114**:766.
23. Biro, C. E. 1966. The role of the 6th component of complement in some types of hypersensitivity. *Immunology.* **10**:563.
24. Osler, A. G., B. Oliveira, H. S. Shin, and A. L. Sandberg. 1969. The fixation of guinea pig complement by γ_1 and γ_2 immunoglobulins. *J. Immunol.* **102**:269.
25. Sandberg, A. L., A. G. Osler, H. S. Shin, and B. Oliveira. 1970. The biological activities of guinea pig antibodies: modes of complement interaction with γ_1 and γ_2 immunoglobulins. *J. Immunol.* **104**:329.
26. Gewurz, H., H. S. Shin, and S. E. Mergenhagen. 1968. Interactions of the complement system with endotoxic lipopolysaccharide: consumption of the six terminal complement components. *J. Exp. Med.* **128**:1049.
27. Maillard, J. L., and G. A. Voisin. 1969. Elicitation of Arthus reactions in guinea pigs by homologous γ_1 and γ_2 immunoglobulins. *Proc. Soc. Exp. Biol. Med.* **133**:1188.

28. Gewurz, H., A. R. Page, R. J. Pickering, and R. A. Good. 1967. Complement activity and inflammatory neutrophil exudation in man: Studies on patients with glomerulonephritis, essential hypocomplementemia and agammaglobulinemia. *Int. Arch. Allergy Appl. Immunol.* **32**:64.
29. Gewurz, H., R. J. Pickering, L. H. Muschel, S. E. Merganhagen, and R. A. Good. 1966. Complement-dependent biological functions in complement deficiency in man. *Lancet.* **2**:356.
30. Ovary, Z., B. Benacerraf, and K. J. Bloch. 1963. Properties of guinea pig 7S antibodies involved in passive cutaneous and systemic anaphylaxis. *J. Exp. Med.* **117**:951.
31. Benacerraf, B., M. M. Sebestyen, and S. Schlossman. 1959. A quantitative study of the kinetics of blood clearance of P^{32}-labelled *Escherichia coli* and staphylococci by the reticuloendothelial system. *J. Exp. Med.* **110**:27.
32. Spiegelberg, H. L., P. A. Miescher, and B. Benacerraf. 1963. Studies on the role of complement in the immune clearance of *E. coli* and rat erythrocytes by the reticuloendothelial system in mice. *J. Immunol.* **90**:751.
33. Biozzi, G., J. G. Howard, B. N. Halpern, C. Stiffel, and D. Mouton. 1961. Measurement of the opsonic effect of normal and immune sera on the phagocytosis of *S. typhi* by the reticuloendothelial system. *J. Immunol.* **87**:296.
34. Biozzi, G., and C. Stiffel. 1962. Role of normal and immune opsonins in the phagocytosis of bacteria and erythrocytes by the reticuloendothelial system. *In* Mechanism of Cell and Tissue Damage Produced by Immune Reactions. *Immunopathol. Int. Symp. 2nd.* 249.
35. Brown, D. L., P. J. Lachmann, and J. V. Dacie. 1970. The *in vivo* behavior of complement-coated red cells: studies in C_6 deficient, C_3-depleted and normal rabbits. *Clin. Exp. Immunol.* **7**:401.
36. Gigli, I., and R. A. Nelson. 1968. Complement dependent immune phagocytosis. I. Requirements for C1, C4, C2 and C3. *Exp. Cell Res.* **51**:45.
37. Dukor, P., C. Bianco, and V. Nussenzweig. 1970. Tissue localization of lymphocytes bearing a membrane receptor for antigen–antibody–complement complexes. *Proc. Nat. Acad. Sci. U.S.A.* **67**:991.
38. Bianco, C., R. Patrick, and V. Nussenzweig. 1970. A population of lymphocytes bearing a membrane receptor for antigen–antibody–complement complexes. I. Separation and characterization. *J. Exp. Med.* **132**:702.
39. Nevu, T., and G. Biozzi. 1965. The effect of decomplementation on delayed type hypersensitive reactions to a conjugated antigen in rats. *Immunology.* **9**:303.
40. Willoughby, D. A., E. Coote, and J. L. Turk. 1969. Complement in acute inflammation. *J. Pathol. Bacteriol.* **97**:296.
41. Rother, K. R., R. T. McClusky, and M. Rother. 1967. Tuberculin sensitivity in C_6 deficient rabbits. *Fed. Proc.* **26**:787.
42. Rother, M., D. L. Ballantyne, Jr., C. Cohen, and K. Rother. 1967. Allograft rejection in C'6 defective rabbits. *J. Exp. Med.* **126**:565.