Immunology, 1970, **18**, 943.

Hereditary Deficiency of the Second Component of Complement (C2) in Man: Correlation of C2 Haemolytic Activity with Immunochemical Measurements of C2 Protein

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(*Received* 20*th November* 1969)

Summary. Measurements of the nine components of complement in the serums of 16 members of a kindred have established the diagnosis of hereditary deficiency of the second component of complement (C2). The autosomal recessive mode of inheritance resembles that of previously described families with C2 deficiency. Both C2 activity determinations with a stoichiometric haemolytic assay and C2 protein measurements with electroimmunodiffusion against antibody monospecific for C2 detect the heterozygous deficient state. Antigenic analysis, *in vitro* reconstitution experiments, and the constant ratio of C2 function to C2 protein indicate that the C2 synthesized by heterozygotes is indistinguishable from normal human C2. Studies of neonatal homozygous deficient serum and maternal heterozygous deficient serum show that transplacental passage of C2 does not occur. C2 deficiency in this family is not associated with clinical defects in host resistance.

INTRODUCTION

An inherited deficiency of the second component (C2)* of human complement was described in a single family by Klemperer, Woodworth, Rosen and Austen in 1966. In the sera of affected individuals the activities of the first (C1), fourth (C4) and the complex of the third component were within normal limits when measured by the reconstitution of systems made lacking in components by chemical treatment. C2 activities, measured by interaction with the intermediate cell EAC1^{gp}4^{gp}, were less than 5 per cent of normal. Studies of a second kindred confirmed an autosomal recessive mode of inheritance and further explored the biological implications of this deficiency state (Klemperer, Austen and Rosen, 1967; Gewurz, Pickering, Muschel, Mergenhagen and Good 1966). The selectivity of the defect for C2 in the probands of these two kindreds was confirmed by measurements

* The nomenclature used conforms to that agreed upon and published by the World Health Organization. Sheep erythrocytes (E), sensitized with rabbit antibody (A) react with the components of complement (C) in the sequence 1, 4, 2, 3, 5, 6, 7, 8 and 9. The activated form of a component is signified by a bar above the number, e.g., $\bar{C1}$. An additional convention is the use of the superscripts 'hu' and 'gp' to signify the species of origin, human or guinea pig, of the component, e.g., C1^{gp}.

of all nine components with specific haemolytic assays (Ruddy and Austen, 1967b). More recently, Cooper, Tenbensel and Kohler (1968) reported a third family with C2 deficiency. The very small amounts of C2 present in the sera of the homozygous deficient individuals were measured by the ability of the serum to promote the binding of radioactive C3 to the intermediate cell $EAC1^{hu}4^{hu}$. It was concluded that the serum of such homozygotes contained from 2 to 4 per cent of the normal amounts of C2.

The production of antibody (Polley, 1968; Klemperer, 1969) to highly purified human C2 has permitted the direct immunochemical quantification of C2 protein. No abnormal gene product has been found in the sera of homozygous deficient individuals, and sera from heterozygotes contain from 30 to 60 per cent of the normal amount of C2 protein (Klemperer, 1969).

In 1962, Kumate reported C2 deficiency in two brothers, one of whom had two episodes of bacterial meningitis following a skull fracture. Six other siblings and both parents had apparently normal levels of total haemolytic complement and C2. This kindred has now been studied more extensively and the results of these studies, contained in the present paper, demonstrate that: (1) the defect is selective for C2, as defined by measurements of all nine of the components of human complement by highly specific and sensitive haemolytic techniques; (2) the mode of inheritance in this kindred conforms to that of those previously described, a heterozygous state being detectable with either a functional stoichiometric assay or an immunochemical assay of protein specific for human C2; (3) the C2 synthesized by members of this kindred is normal with respect to units of C2 activity per μg of C2 protein; and (4) C2 deficiency in this family is not associated with an abnormal defect in host resistance as manifested by recurrent infections.

MATERIALS AND METHODS

Serum was collected from fresh blood specimens which had been allowed to clot at room temperature for 30–60 minutes and then centrifuged at 4°. The samples were frozen immediately and stored on dry ice in tightly stoppered vials. At the time of the first experiment, specimens were thawed, divided into small portions, and refrozen for use in subsequent experiments.

The preparation of veronal buffered saline (GVB) and dextrose-veronal buffered saline (DGVB) containing calcium, magnesium and 0·1 per cent gelatin has been described (Ruddy and Austen, 1967a), along with the preparation of the veronal buffered saline with gelatin containing either 0·01 or 0·04 M disodium ethylenediaminetetraacetic acid (EDTA-GVB). Total haemolytic complement activity was determined as described by Klemperer *et al.* (1966)

Stoichiometric determinations of the activities of C1, C4, C3, C2, C8, and C9

The technique of Borsos and Rapp (1963) was used to measure C1. The serum sample was tested for its ability to convert the cellular intermediate $EAC4^{hu}$ to the state $EAC1^{hu}4^{hu}$, and the lytic reaction completed by the sequential addition of excess $C2^{gp}$ (50 effective molecules per cell) and excesses of C3, C5, C6, C7, C8, and C9 in the form of guinea pig serum diluted 1/15 in 0·04 M EDTA-GVB ($C\ EDTA^{gp}$). For C4, the technique of Ruddy and Austen (1967a) was used. The reaction sequence was identical to that for C1 except that $EAC1^{gp}$ cells were substituted for $EAC4^{hu}$ cells, and the serum used as a source of C4 for the conversion of these $EAC1^{gp}$ cells to the state $EAC1^{gp}4^{hu}$. $C2^{hu}$ activities were measured by interacting the test sera both with $EAC1^{gp}4^{hu}$ cells prepared by the technique

of Borsos and Rapp (1967) and with $EAC1^{gp}4^{hu}$ cells prepared by treating $EAC1^{gp}$ with 500 effective molecules per cell of purified $C4^{hu}$. After incubation of the serum dilutions and the $EAC1^{gp}4^{hu}$ cells at 30° for the t_{max}, the EAC142 thus generated were lysed by the addition of C $EDTA^{gp}$. It should be noted that the use of $EAC1^{gp}4^{hu}$ cells resulted in an approximate five-fold increase in sensitivity for $C2^{hu}$ over the $EAC1^{gp}4^{gp}$ assay; the standard serum was, therefore, assigned a value of 3000 units/ml. Human C3 was determined by a modification (Ruddy and Austen, 1969) of the assay described by Nelson, Jensen, Gigli and Tamura (1966) for $C3^{gp}$. The test serum was used to convert $EAC1^{gp}4^{hu}2^{gp}$ cells to the state EAC1423 in the presence of relative excesses of functionally pure $C5^{hu}$, $C6^{hu}$, and $C7^{gp}$; cells converted by this reaction to the state EAC1–7 were lysed by the addition of excesses of $C8^{gp}$ and $C9^{gp}$. $C8^{hu}$ and $C9^{hu}$ were measured by the ability of the test serum to lyse $EAC1–7^{hu}$ cells in the presence of excesses of either $C9^{gp}$, in the case of the $C8^{hu}$ measurements, or $C8^{gp}$ in the case of $C9^{hu}$. The $EAC1–7^{hu}$ were generated with a chromatographic fraction of human serum devoid of the activities of C8 and C9 (Schultz and Zarco, 1969).

The reaction mixtures for all of the stoichiometric component titrations contained 0·5 ml of serum dilution and 0·5 ml of DGVB in which were suspended $0{\cdot}5 \times 10^8$ of the appropriate cellular intermediate. At the completion of the reaction, the proportion of cells lysed (y) was calculated from measurements of oxyhaemoglobin in the supernatant fluid, and the average number ($\mathcal{Z}$) of reactive sites generated at each dilution of the test serum calculated from an application of the Poisson distribution ($\mathcal{Z} = -\ln(1-y)$) (Borsos, Rapp and Mayer, 1961). Plots of $\mathcal{Z}$ versus amounts of serum assayed were linear for $\mathcal{Z}$ values of up to 1·5. The number of units of a given component was defined as the reciprocal of the dilution required to generate an average of 1·0 sites per cell ($\mathcal{Z} = 1{\cdot}0$). Variations in sensitivity from batch to batch of cellular intermediates were corrected for by adjusting the results of determinations of any given day by a factor derived from the observed value of a standard reference control. For C1, C4, and C2, the standard reference was a single normal human serum. This serum contained 3000 units of C2 per ml, a value which represents the mean of nine determinations with separate batches of EAC14 over the course of six months. For C3, C8, and C9, purified components were used as reference standards. Controls for lysis in the absence of added test serum were included with each set of determinations, and did not exceed 2·5 per cent for C1, C4, and C2; 5 per cent for C8 and C9; and 10 per cent for C3. In addition, the specificity of each assay was checked by its ability to measure the activity of functionally pure preparations of $C1^{gp}$, $C4^{hu}$, $C2^{hu}$, $C3^{hu}$, $C8^{hu}$, or $C9^{hu}$.

Determination of C5, C6, and C7

Microtitre plate assays were used for these components according to the method of Nelson *et al.* (1966). To one drop (0·025 ml) of doubling dilutions of the test serum were added: one drop of $C2^{gp}$ (100 effective molecules per cell), one drop of a reagent containing 50 effective molecules each per cell of either $C3^{hu}$, $C5^{hu}$, $C7^{gp}$; or $C3^{hu}$, $C6^{gp}$, $C7^{gp}$; or $C3^{hu}$, $C5^{hu}$, $C6^{gp}$; and one drop of $EAC1^{gp}4^{hu}$ cells, 1×10^8/ml. After 30 minutes incubation with shaking at 30°, one drop of a mixture of $C8^{gp}$ and $C9^{gp}$ (10 units each) was added, and incubation completed at 37° for 1 hour. The microtitre plates were then centrifuged and 50 per cent haemolysis endpoints estimated visually. Variations in sensitivity of the $EAC1^{gp}4^{hu}$ cells were adjusted for by reference to a standard preparation of $C5^{hu}$, $C6^{hu}$ or $C7^{gp}$ titrated in the same plate.

Purification of complement components for haemolytic assays

C1, C2, C6, C7, C8, and C9 were isolated from guinea pig serum in a functionally pure state, i.e., devoid of the other components of complement, by the methods of Nelson *et al.* (1966). $C4^{hu}$ and $C2^{hu}$ for use in haemolytic assays were purified from human serum by previously described (Ruddy and Austen 1967a) modifications of the technique of Vroon and Nelson (1966). Human C3 and C5 were isolated by the method of Nilsson and Müller-Eberhard (1965). A chromatographic fraction containing the activities of human C4, C2, C3, C5, C6, and C7 was kindly supplied by Dr D. Schultz, Cordis Laboratories, Miami, Florida; this material was used to generate EAC1–7 cells used in the assays for C8 and C9.

Immunochemical determination of serum C2 protein concentration

Rabbit antibody to human C2 was prepared as described by Klemperer (1969). Serum C2 concentration was determined immunochemically employing the electroimmunochemical technique of Laurell (1966).

Immunochemical determinations of C4 and C3

Radial immunodiffusion in agar containing anti-C4 or anti-C3 was used to measure C4 and C3. Monospecific rabbit antibody prepared against purified preparations of these proteins were incorporated into 1·5 per cent agar gel in veronal buffered saline ($\mu = 0{\cdot}05$, pH 8·4). Serum samples were placed in 1 mm holes punched in the gel containing antibody, and the size of the precipitin rings surrounding the holes after 24 hours diffusion at 25° measured to the nearest 0·1 mm with a calibrated magnifier. Protein concentrations were read from a curve obtained with dilutions of a previously calibrated serum standard which had diffused in the same gel.

Reconstitution of C2 deficient serum with functionally pure human C2

To one 13·5 ml aliquot of a 1/100 dilution in GVB of C2 deficient serum was added 0·81 ml of functionally pure human C2 containing 480 units of this component, and to another aliquot was added 0·81 ml of GVB. The final concentration of $C2^{hu}$ in the 1/100 dilution was, therefore, 27·2 units per ml. This approximates the number of units of C2 found in a 1/100 dilution of normal serum. Each of the 1/100 dilutions was then titrated for total haemolytic complement activity by the Mayer 7·5 ml technique (Mayer, 1961). A normal human serum containing 3,500 units of C2/ml was also diluted 1/100 and titrated at the same time.

Titration of functionally pure human C2 in the presence of C2 deficient sera

A set of doubling dilutions of $C2^{hu}$ ranging from 1/200 to 1/3200 was prepared in DGVB. To one 0·25 ml aliquot of each dilution was added an equal volume of C2 deficient serum diluted 1/50 and to another aliquot was added an equal volume of DGVB. Both sets of aliquots were then titrated for $C2^{hu}$ activity by reacting them at 30° with 0·5 ml DGVB containing $0{\cdot}5 \times 10^8$ $EAC1^{gp}4^{hu}$ cells for the t_{max} period (1·5 min), and then adding 1·5 ml C $EDTA^{gp}$. After incubation at 37° for 60 minutes, the average number of sites in the state SAC142 was determined.

RESULTS

The pedigree for Family D. is given in Fig. 1, together with the serum levels of C2 activity and C2 protein for 16 members of the kindred. The normal mean ± 2 S.D. for the

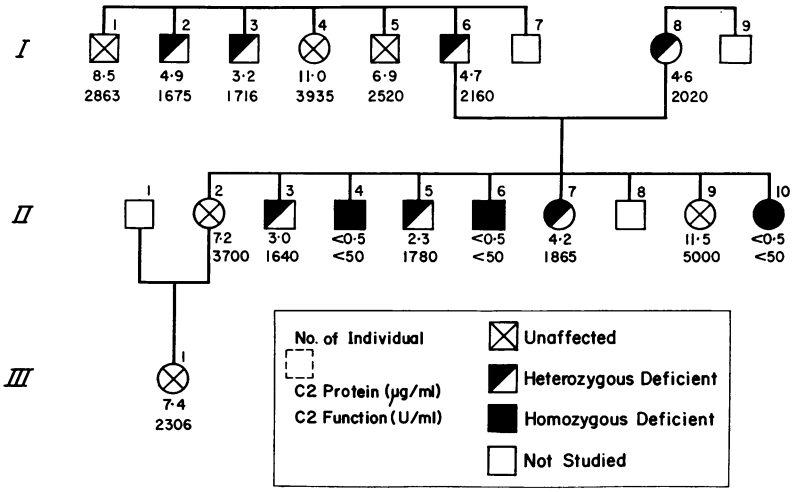

FIG. 1. Pedigree of Family D.

functional C2 assay is 2900±900 units per ml, and the corresponding values for the immunochemical determination of C2 are 10±2 μg/ml. The experimental error of either method is ±10 per cent. The proband and two of his siblings have levels of C2 which are less than 2 per cent of the normal mean. Three other siblings, both parents, and two paternal uncles have C2 activities which range from 57 to 74 per cent of the normal mean and C2 protein concentrations which range from 23 to 49 per cent of the normal mean. The remaining members of the kindred have C2 activities and protein concentrations which are either normal or, in two instances, elevated. The pattern of inheritance is consistent with an autosomal recessive trait, heterozygous individuals being recognizable by the presence of approximately 1/2 the normal amounts of C2 in their serum. From the static determinations of serum C2 levels given in Fig. 1, it is not possible to conclude whether the deficiency state arises from decreased synthesis of C2 or from increased catabolism of this protein.

In Fig. 2, the results of the stoichiometric haemolytic determinations of C2 activity are plotted against the results of the electroimmunodiffusion measurements of C2 protein. The distinction between unaffected members of the D. family, heterozygous deficients, and homozygotes is clear. The equation for the regression of C2 protein on C2 activity is: C2 Protein = 0·0024×C2 Activity+0·047. The correlation coefficient for the two sets of results is 0·95. The slope of 0·0024 indicates that 1 μg of C2 contained in serum is equivalent to 397 haemolytic units as measured in the present assay system. The molecular weight of human C2, as determined by Polley and Müller-Eberhard (1969) is 117,000; 1 μg, therefore, contains $5{\cdot}15\times10^{12}$ molecules. One haemolytic unit of C2 is defined as the amount required to generate an average of one effective site in the state SAC142 per erythrocyte. One unit, therefore, contains 10^8 effective molecules of C2, and one μg of C2 contains 397×10^8 effective molecules. Under the conditions of the assay systems employed then, approximately 130 molecules of C2 as determined on a weight basis are required to produce a single 'effective molecule' measured haemolytically.

The regression line in Fig. 2 intercepts the ordinate at the value of 0·047 μg of C2 protein. If significant amounts of antigenically intact but haemolytically inactive C2

protein were present in the serums studied, this would have been manifested by a higher intercept on the ordinate. Within the limits of the two techniques employed, no such inactive protein can be detected.

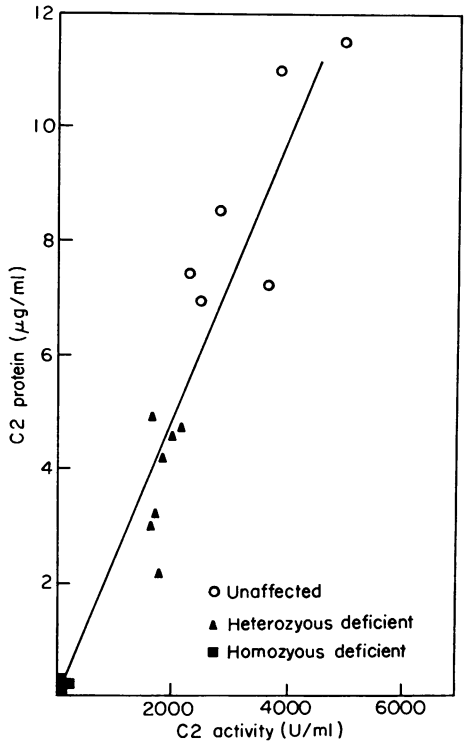

FIG. 2. Relationship of C2 protein to C2 activity in the serums of unaffected, heterozygous deficient, and homozygous deficient members of Family D. The equation for the regression line is: C2 protein = 0·0024 × C2 activity + 0·047. The correlation coefficient is 0·95.

MEASUREMENT OF COMPLEMENT COMPONENTS OTHER THAN C2

The results of activity measurements for C1, C4, C3, C5, C6, C7, C8, and C9 and radial immunodiffusion determinations of C4 and C3 are given in Table 1 for the propositus, seven of his siblings, and his parents. Normal levels for adults are also given; the broad ranges reflect variation in the normal population, not experimental error, which is ± 10 per cent for the stoichiometric titrations. C1 activities are within the range of normal in all 10 individuals studied. By contrast, C4 levels are elevated in six of the ten cases, and these elevations are confirmed by immunodiffusion determinations of serum C4 protein concentrations. The activities of C3, C5, C6, C7, C8, and C9 are also within normal limits. The deficiency is selective for C2.

ABSENCE OF TRANSPLACENTAL PASSAGE OF C2

The third homozygous deficient individual (II-10) was born on January 21, 1968. A sample of the infant's serum was obtained at the time of delivery, and a sample was simultaneously obtained from her mother (I-8). Specimens of the infant's and mother's serum

TABLE 1

COMPLEMENT COMPONENT LEVELS OF PROPOSITUS, SIBLINGS AND PARENTS

Individual	CH_{50} (U/ml)	C1 (U/ml)	C4 (U/ml)	C4 Protein (μg/ml)	C3 (U/ml)	C3 Protein (μg/ml)	C5* (U/ml)	C6* (U/ml)	C7* (U/ml)	C8 (U/ml)	C9 (U/ml)
I–6	32	126,000	67,000	1,145	66,000	1,430	1,200	6,400	36,000	64,000	29,000
I–8	43	146,000	102,000	890	92,000	1,620	1,600	9,600	29,000	72.000	19,000
II–2	32	169,000	73,000	772	69,000	1,380	800	6,400	19,200	66,000	24,600
II–3	30	104,000	102,000	1,112	60,000	1,390	800	6,400	14,400	87,000	32,000
II–4	< 1	162,000	87,000	805	77,000	1,600	800	9,600	9,600	66,000	38,200
II–5	37	98,000	38,500	675	44,000	1,090	600	3,200	9,600	69,500	36,500
II–6	< 1	134,000	41,000	383	59,000	1,600	1,200	6,400	9,600	87,000	35,500
II–7	34	176,000	53,000	748	42,600	1,510	800	3,200	9,600	80,500	16,000
II–9	48	118,500	76,000	928	48,000	1,510	1,200	6,400	19,200	70 400	33,500
II–10	< 1	104,000	46,500	443	66,500	1,350	1,200	3,200	14,400	66,000	20,000
Normals	32–45	96,600–176,000	23,000–57,000	208–636	48,000–87,000	940–1,820	600–2,400	3,000–12,000	6,400–51,200	60,000–85,000	Not available

* Microtitre plate determinations

TABLE 2

C2 ACTIVITIES IN NEONATAL AND MATERNAL SERUM

Source of sample	C2 activity (U/ml)	
	Date of sample	
	1-21-68	2-8-68
Mother (I-8)	1,980	2,020
Infant (II-10)	< 50	< 50

were also obtained on February 8, 1968, when the infant was 18 days old. The results of C2 activity determinations performed on these serums are given in Table 2. The mother (I-8) was heterozygous for C2 deficiency, but none of the 1980 units/ml C2 activity present in her serum was found in her infant's blood at the time of delivery.

DETERMINATION OF TOTAL HAEMOLYTIC COMPLEMENT ACTIVITY (CH50)

Also given in Table 1 are the results of CH50 measurements on representative sera from the D. kindred. This assay reflects the resultant of the interaction of all nine components. All three homozygous deficient individuals tested had essentially no haemolytic complement activity. It is noteworthy, however, that only one of the five heterozygous individuals had a CH50 level which was clearly subnormal.

ANALYSIS OF GENE PRODUCT IN HETEROZYGOUS INDIVIDUALS

Fig. 3 shows an immunodiffusion study of a normal serum and three serums from individuals heterozygous for C2 deficiency. The centrally-placed antibody to C2 yields a line of complete fusion between the C2 contained in a normal serum and that of the heterozygous C2 deficients from two previously described kindreds (Family W. and Family P.) and the present one (Family D.). Therefore, immunodiffusion analysis does not reveal any differences between the C2 protein contained in the serum of heterozygous C2 deficients of family D., the C2 protein contained in normal human serum, and the C2 protein contained in heterozygous C2 deficients from two previously reported kindreds (Klemperer *et al.*, 1966, 1967).

RECONSTITUTION OF C2 DEFICIENT SERUM WITH PURIFIED HUMAN C2

An amount of purified human C2 calculated to replace completely the deficiency in the serum of patient II-4 was added to one aliquot of this serum, and to another aliquot buffer was added. CH50 determinations were performed on both aliquots and normal control serum. The control serum contained 55·6 CH50/ml, and the reconstituted C2 deficient serum contained 47·2 CH50/ml.

TITRATION OF PURIFIED HUMAN C2 IN THE PRESENCE OF C2 DEFICIENT SERA

A preparation of purified C2 was titrated by interaction with $EAC1^{gp}4^{hu}$ in the presence and in the absence of a 1/50 dilution of serum from patient II-4. The titration curves were superimposable: the activity of the purified C2 was 265 units per ml in the absence of added C2 deficient serum and 256 units per ml in its presence.

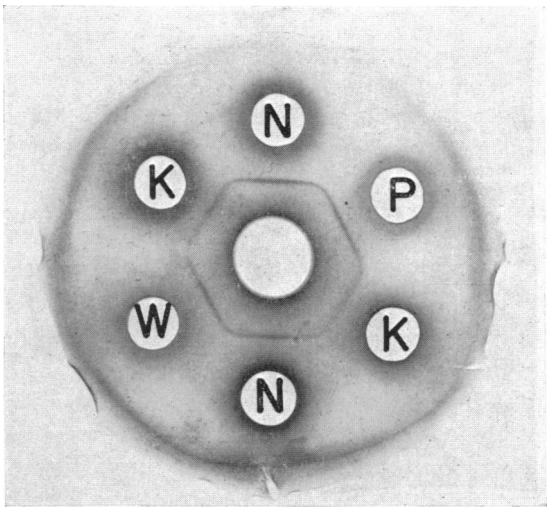

FIG. 3. Immunodiffusion analysis of normal human serum (N) and sera from heterozygous deficient members of Families D., (K), W., and P. The centre hole contained monospecific rabbit antibody to human C2.

DISCUSSION

Although this is the fourth kindred with C2 deficiency to be completely studied, the D. family was the subject of a previous report by one of us (Kumate, 1962). Two of the brothers (II-4 and II-6) were found to have undetectable total haemolytic complement activities, and a deficiency of C2 was found when the sera were analysed for their ability to convert the intermediate $EAC1^{gp}4^{gp}$ to the state $EAC1^{gp}4^{gp}2^{hu}$. Neither the CH50 determination nor the C2 assay using $EAC1^{gp}4^{gp}$ cells served to distinguish the heterozygous deficiency state in either of the proband's two parents or six other siblings. Because of this inability to identify heterozygosity, and because only males appeared to be affected, a mode of inheritance differing from that of the previously reported kindreds was considered (Klemperer *et al.*, 1966).

Analysis of the sera from the D. family for all nine components of complement (Table 1) and the determinations of C2 both by activity in the conversion of $EAC1^{gp}4^{hu}$ cells and by electroimmunodiffusion (Fig. 1), clearly identify the heterozygous deficiency state in three of the proband's nine siblings, both parents, and two paternal uncles. In addition, a female homozygous deficient sibling has been born since the original report (II-10). The inheritance of C2 deficiency in family D. thus conforms to the autosomal recessive mode in the three previously studied C2 deficient kindreds (Klemperer *et al.*, 1966, 1967; Cooper *et al.*, 1968). In addition, family D. is the first instance in which both parents of a C2 deficient proband were available for study. As expected both parents of the homozygous C2 deficient individuals were heterozygous for the deficiency state.

The failure of the total haemolytic complement measurements to distinguish the heterozygous state may be explained by the markedly elevated C4 levels present in the sera of

certain members of the kindred (Table 1). It seems likely that the availability of large amounts of C4 in the CH50 assay may lead to the generation of relatively increased numbers of sensitized erythrocytes in the state EAC14, with consequent increased efficiency of utilization of the subnormal amounts of C2 present in the sera of heterozygous individuals.

An explanation is also available for the failure of the C2 activity measurements performed with EAC1 $^{gp}4^{gp}$cells to detect heterozygosity for C2 deficiency. Subsequent to the initial development of this assay (Austen and Beer, 1964), it has become clear that the cellular intermediate EAC1$^{gp}4^{gp}$ is incapable of reacting directly with human C2, but that sites on these erythrocytes in the state SAC1^{gp}, not combined with C4^{gp}, must first interact with the C4^{hu} contained in the test serum to generate EAC1$^{gp}4^{hu}$ cells which then, in turn, react with the C2 in the serum (Austen and Russell, 1967). The EAC1$^{gp}4^{gp}$ assay for serum C2 activity, therefore, is a function of *both* the C4^{hu} and C2^{hu} content of the serum being tested. As in the case of the CH50 determination, the large amounts of C4 contained in these sera probably served to increase the sensitivity of the assay for the C2 activities of the same sera, producing results which were spuriously normal. When the intermediate EAC1$^{gp}4^{hu}$, which is capable of reacting directly with human C2, is used to measure the C2 activity, individuals who are heterozygous for C2 deficiency have from 56 to 74 per cent of the normal mean.

Static determinations of serum C2 activities such as presented in this report do not identify the mechanism of the apparent deficiency. Measurements of the catabolic rates for C2 in affected members of the kindred will be required to distinguish between decreased synthesis and increased catabolism as the cause of this deficiency state. From the *in vitro* experiments presented, however, it is clear that the sera of homozygous deficient individuals do not contain materials which block the reactivity of purified human C2 with EAC1$^{gp}4^{hu}$ cells, and that the total haemolytic complement activity of a deficient serum can be restored by the addition of appropriate amounts of purified human C2. The normal levels of C4 exclude the diagnosis of subclinical hereditary angioedema. In addition, the latter disease is rarely associated with a total absence of haemolytically detectable C2, even at the height of an attack of oedema.

By all available criteria, the C2 synthesized by heterozygous deficient individuals is indistinguishable from normal human C2. Antigenic analysis by immunodiffusion fails to delineate differences in antigenicity from the normal. Most importantly, the ratio of C2 activity to weight of C2 protein contained in the heterozygous serums is precisely that which would be expected. The correlation ($r = 0{\cdot}95$) between C2 activity and C2 protein is remarkably good, considering the experimental error of the two assays. Approximately 130 molecules of C2 protein contained in whole serum were required to generate a single haemolytically effective molecule. The corresponding figure found by Polley and Müller-Eberhard (1969), based on a measurement of $1{\cdot}9 \times 10^{11}$ effective molecules and an estimate of 20 μg of C2 protein per ml of serum, was 572 molecules of C2 protein per single haemolytically effective molecule. The difference between these two estimates of the efficiency of human C2 reflects the facts that: (1) the concentration of 20 μg of C2 protein/ml estimated by these authors was twice that observed in the present study, and (2) the efficiency of the haemolytic assay for C2 used by Polley and Müller-Eberhard was approximately one-half that used in the present study. They used the EAC1$^{hu}4^{hu}$ intermediate and C EDTA^{hu} as a converting reagent, which probably provides less efficient utilization of C2^{hu} than does the EAC1$^{gp}4^{hu}$ cell and C EDTA^{gp}. It should be added that the sensitivity of the latter assay

for $C2^{hu}$ was uninfluenced by the presence, in the reaction mixture, of a 1/50 dilution of C2 deficient serum.

The intercept of 0·047 μg of C2 protein for the regression line in Fig.2 indicates that significant amounts of immunoreactive, but functionally inactive, C2 protein are not present in the sera of members of Family D.

The biological implications of C2 deficiency for Family D. resemble those for the three previously described kindreds. Although the proband was originally investigated because of recurrent bacterial meningitis, these infections proved to be related to a skull fracture. So far as can be determined, then, Family D. exhibits no defects in host resistance associated with deficiency of C2. Previous studies on the immune adherence reactivity, phagocytosis-enhancing ability, and bactericidal activity of sera from C2 deficient individuals have demonstrated that the amount of C2 present, even in the homozygous state, is sufficient to promote these phenomena to the extent required by the serum's own natural antibody against the particular organism used in the test system. Only when organisms sensitized with an excess of antibody are used in the immune adherence, phagocytosis, or bactericidal reactions does the deficiency of C2 become apparent (Klemperer *et al.*, 1967).

ACKNOWLEDGMENTS

This work was supported by grants AI-07722 and AI-05877 from the National Institutes of Health. Dr Ruddy is an Investigator of the Howard Hughes Medical Institute, and Dr Rosen is the recipient of a Career Development Award (1-K3-Am19, 650) from the United States Public Health Service.

We are grateful for the able technical assistance of Miss Mary Graves, Miss Barbara Moyer, and Miss Lillian Watson.

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