# Homozygous Human C3 Deficiency

## THE ROLE OF C3 IN ANTIBODY PRODUCTION, $C\overline{1s}$-INDUCED VASOPERMEABILITY, AND COBRA VENOM-INDUCED PASSIVE HEMOLYSIS

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ABSTRACT Studies of the family of a patient with marked deficiency of the third component of complement (C3) demonstrated that the patient was homozygous for a blank allele at the C3 locus, $C3^-$. Metabolic studies with purified radiolabeled C3 in the patient revealed a mildly elevated fractional catabolic rate and a markedly reduced synthesis rate, consistent with a lack of C3 synthesis as the patient's primary defect. There was also a mild increase in the rate of conversion of purified C3 added to her serum and incubated at 37°C in vitro. Major blood group-compatible erythrocytes from a patient with paroxysmal nocturnal hemoglobinuria had the same shortened survival in the C3-deficient patient as in a normal control. Although no leukocytosis developed in the patient in spontaneous infection by pyogenic organisms, there was a normal leukocytosis in response to the injection of typhoid vaccine. The intradermal injection of $C\overline{1s}$, which produces a marked increase in vasopermeability in the skin of normal subjects, produced no definite change in the patient, possibly implicating C3 or a protein in the alternative pathway as the normal mediator of this response. The patient's serum exhibited near-normal immune adherence activity, confirming the lack of requirement of C3 for this function. C5 inactivation and passive hemolysis of unsensitized guinea pig erythrocytes occurred normally in C3-deficient serum on incubation with cobra venom factor, indicating that C3 is not required for these reactions. The patient's humoral antibody response to both protein and carbohydrate antigens was entirely normal, making it unlikely that C3 is required for antigen processing.

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## INTRODUCTION

The discovery of a patient with genetically determined severe deficiency of C3 (1) and repeated infections provided the opportunity to investigate the genetic mechanism of the deficiency state and to examine the role of C3 in a variety of complement-dependent reactions. Her serum was grossly deficient in hemolytic complement and in opsonization for endotoxin particles. Furthermore, there was no cleavage of properdin factor B when her serum was incubated with zymosan. All of these abnormalities in vitro were corrected by the addition of purified C3 to her serum. There was no increase in peripheral blood polymorphonuclear leukocytes during the patient's episodes of severe systemic bacterial infection.

The nature of the inherited deficiency was suspected to be similar to that observed earlier only in heterozygotes (2), in whom it could be shown that a blank C3 gene ($C3^-$) was inherited as an allele of the common structural genes for C3, $C3^S$, and $C3^F$ (3). It was not possible at the time of the initial report of the present patient to demonstrate inheritance of a blank C3 gene in studies of the patient's immediate family; although her mother, father, and several siblings had half-normal levels of C3, in each instance it was C3 S.

The present report is concerned with further studies of this patient. Blood samples from over 100 of her relatives were analyzed to define the mode of inheritance of the deficiency state. The role of C3 in immune adherence, in the generation of vascular permeability induced by $C\bar{1}s$, in humoral antibody production, in cobra venom factor (CoF)[1] induced passive hemolysis of guinea pig erythrocytes, and in leukocyte mobilization was explored. The metabolism of C3 in homozygous C3 deficiency was investigated, as well as the behavior of red cells from a patient with paroxysmal nocturnal hemoglobinuria (PNH) in the C3-deficient subject's circulation.

## METHODS

*Genetic typing of C3.* C3 typing was performed in prolonged agarose gel electrophoresis as previously described (3).

*Quantitation of complement proteins.* Functional hemolytic titrations of complement components were carried out by standard methods (4), as was the determination of total hemolytic complement (5). Immunochemical estimations of complement protein concentration in serum were carried out by electroimmunoassay (6).

*Protein purification procedures.* C3 was prepared by the method of Nilsson and Müller-Eberhard (7) and labeled with $^{125}I$ by the iodine monochloride technique (8) of McFarlane. $C\bar{1}s$ was prepared by the method of Haines and Lepow (9). The starting plasma, the purified proteins, or both, were tested for Hb$_s$Ag by radioimmunoassay and were found to be negative.

*Radiolabeled C3 and PNH erythrocyte studies.* The metabolism of $^{125}I$-labeled C3 was studied in the patient and a healthy normal subject as described previously (10). The data were analyzed by the Matthews method (11). The conversion of $^{125}I$-labeled C3 in the patient's serum in vitro was assessed by agarose gel electrophoresis and radioautography as described previously (12).

Blood was obtained in acid citrate dextrose from a patient with PNH who had blood group B (as did the patient and the normal control). This blood was determined to be free of Hb$_s$Ag by radioimmunoassay. The erythrocytes were labeled with $^{51}Cr$ and the blood was stored for 4 days in cracked ice. 2 h before use, the erythrocytes were washed twice at 4°C in sterile physiologic saline containing 1% human serum albumin. 20 ml of washed $^{51}Cr$-labeled erythrocytes was injected intravenously into the patient and the normal control. The survival of these cells was determined by standard methods (13). When the patient's serum was acidified to pH 6.6–6.8 and incubated for 30 min at 37°C with erythrocytes from the patient with PNH in a Ham test (14), no lysis of these cells was observed, whereas lysis was obtained when the PNH cells were incubated with acidified normal AB serum or with the patient's serum to which purified C3 had been added.

*Immune adherence.* Immune adherence was tested using serial dilutions of patient and normal serum in a standard method (15).

*Intradermal injection of $C\bar{1}s$.* Purified $C\bar{1}s$ (9) was injected intradermally on the forearm of the patient and two normal subjects who had received one ampoule of Evans blue dye a few minutes earlier. The average diameter of bluing, the time of maximal bluing, and the extent of swelling were evaluated. The patient gave skin reactions identical to those of a normal control subject on the intradermal injection of 0.1 ml of $10^{-6}$ M histamine in saline and of 15 $\mu$g of morphine sulfate (a potent inducer of mast cell degranulation) in 0.1 ml saline.

*Leukocytosis in response to endotoxin.* An injection of 0.5 ml of typhoid vaccine was given intramuscularly and white cell counts and differential cell counts were performed at intervals for 6 h.

*Antibody production.* The patient's anti-blood group A agglutinating titer was determined by serial dilution. Her antibody response to diphtheria toxoid was evaluated by Dr. J. H. Mason of the serum-vaccine department of the South African Institute for Medical Research, Johannesburg, using a standard hemagglutination assay. The patient also received 50 $\mu$g of the polyribose phosphate antigen of *Haemophilus influenzae* (16) and her antibody response was measured by a radioligand binding assay (16).

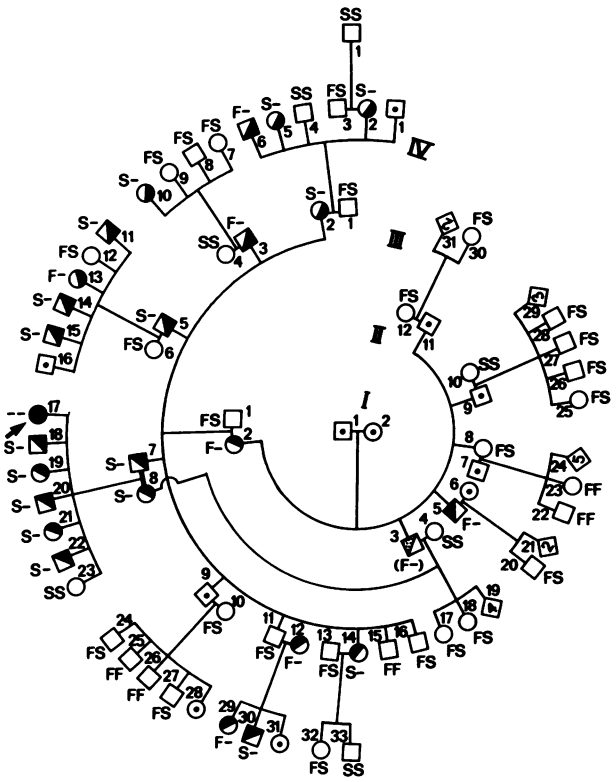

FIGURE 1 Partial pedigree of the kindred with C3 deficiency. Portions of the family tree in which the deficiency gene does not appear are not shown. Males are indicated by squares, females by circles. Heterozygotes are shown as half-blackened symbols and the proposita, who is homozygous, is shown as a fully-blackened circle and indicated by an arrow. C3 types are given adjacent to the symbols for each individual. Diamonds refer to untested siblings and their number is given within the symbols. The half-stippled square indicates that the individual II-3 is untested, but he could be presumed to be heterozygous deficient (F–) from the C3 types of his children and siblings. Symbols enclosing dots refer to untested individuals whose types could not be presumed.

[1] *Abbreviations used in this paper:* CoF, cobra venom factor; PNH, paroxysmal nocturnal hemoglobinuria.

TABLE I

*Functional Levels of Individual Complement Components in the Immediate Family of the C3-Deficient Patient*

| Subject | C1 | C4 | C2 | C3 | C5 | C6 | C7 | C8 | C9 |
|---|---|---|---|---|---|---|---|---|---|
| | *effective molecules* $\times 10^{-8}$*/ml* | | | | | | | | |
| Patient (IV-17) | 196 | 93 | 13 | 0.013 | 34 | 20 | 15 | 40 | 12 |
| Mother (III-8) | 287 | 179 | 34 | 7 | 20 | 32 | 26 | 60 | 10 |
| Father (III-7) | 135 | 317 | 30 | 11 | 26 | 21 | 26 | 57 | 11 |
| Sister 1 (IV-19) | 148 | 258 | 19 | 11 | 20 | 26 | 34 | 65 | 9 |
| Sister 2 (IV-21) | 127 | 186 | 19 | 8 | 18 | 26 | 34 | 72 | 7 |
| Sister 3 (IV-23) | 165 | 179 | 14 | 9 | 27 | 18 | 27 | 42 | 11 |
| Brother 1 (IV-18) | 173 | 339 | 19 | 8 | 20 | 25 | 34 | 65 | 6 |
| Brother 2 (IV-20) | 148 | 330 | 19 | 9 | 19 | 26 | 32 | 58 | 13 |
| Brother 3 (IV-22) | 230 | 143 | 18 | 18 | 34 | 34 | 19 | 37 | 12 |
| Normal range or mean | 100–300 | 200–600 | 15–30 | 15–30 | 15–30 | 10–35 | 10–30 | 30–90 | ~9 |

*Interaction of C3-deficient serum with CoF.* The anticomplementary protein in the venom of the Indian hooded cobra (*Naja naja*) was isolated by Sephadex G-200 gel filtration (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and preparative electrophoresis (17). The purified material produced a single band in agarose gel electrophoresis. This material was incubated with serum at a final concentration of 20 μg/ml for 60 min at 37°C. In some experiments, C3 and C5 destruction were measured by hemolytic titration (4) and in other experiments, guinea pig erythrocytes were added to the reaction mixture to measure passive lysis (18). In the passive lysis experiments, all sera were absorbed three times for 15 min each at 0°C with 2.5 $\times 10^8$ guinea pig erythrocytes/ml of serum to remove possible heterophile antibodies.

*Lymphocyte receptors for C3.* The patient's peripheral blood lymphocytes were tested for C3 receptors by a standard method (19). By this technique, the normal percentage of B lymphocytes is 17–25%.

## RESULTS

*Family studies.* The family tree of the patient with C3 deficiency is shown in Fig. 1. 24 heterozygotes for the deficiency state were detected in three generations. It is clear from the C3 types of the children of II-2, III-2, III-3, and III-5 that the deficiency state is determined by inheritance of a blank C3 gene ($C3^-$) that is an allele of the structural C3 genes, $C3^F$ and $C3^S$. There is no other explanation for a C3 S parent having a C3 F child or vice versa. Most remarkably, III-12, a woman not known to be related to the family of the proposita, married the proposita's unaffected uncle, III-11, and two of their children are heterozygous deficient. Investigation of this woman's family (not shown) indicated that her father and brother were also heterozygous for the deficiency state and the same kind of blank gene appears to be operative in this case. The mean C3 concentration in serum of all heterozygous deficient persons in the family was 68.8 mg/100 ml or 43% of the level of unaffected members.

Functional estimations of the nine components of complement in the proposita and her immediate family are given in Table I. It is evident that complement component levels except for C3 were normal in all family members. Hemolytic complement was slightly low in heterozygotes for C3 deficiency and undetectable in the proposita.

*Metabolic studies with $^{125}$I-labeled C3.* The results of the studies of the metabolism of radiolabeled C3 in the patient homozygous for C3 deficiency and a normal control subject are given in Table II. Although these studies demonstrate, as expected, that the patient's defect is accompanied by marked, if not complete, hyposynthesis, there was also an increased fractional cata-

TABLE II

*Metabolism of $^{125}$I-Labeled C3*

| Subject | C3 concentration | Fractional catabolic rate | Synthesis rate | Extravascular: plasma pool ratio |
|---|---|---|---|---|
| | *mg/100 ml* | *% plasma pool/h* | *mg/kg/h* | |
| C3-deficient patient | <0.1 | 4.44 | <0.002 | 0.91 |
| Normal control subject | 196 | 1.78 | 1.32 | 0.40 |
| Normal range* | 97–204 | 1.3–3.4 | 0.87–1.89 | 0.49–1.31 |

* Mean±2 SD of results in 11 normal individuals.

bolic rate of C3. The disappearance curve of $^{125}$I-labeled C3 in the C3-deficient patient is shown in Fig. 2.

The conversion rate of $^{125}$I-labeled C3 in the C3-deficient patient's serum after 1 h of incubation at 37°C was greater than that observed in normal serum, as shown in Fig. 3. This modestly accelerated conversion was less than that observed in serum from a patient (12) with partial lipodystrophy and nephritic factor (C3ase). Furthermore, the conversion of C3 in the C3-deficient patient's serum was not accelerated by admixture with normal human serum (Fig. 3) or purified C3 to normal serum concentration (not shown), as occurred in the serum with nephritic factor.

*Antibody response.* The patient, who has blood group B, had an anti-A agglutinating titer of 1:128 at room temperature. Before injection of 1 ml of diphtheria-tetanus toxoid, the patient's serum contained 0.1-0.2 U/ml of antitoxin to diphtheria toxoid, unchanged 2 days after injection. By 8 days, however, her antitoxin level rose to 7.0 U/ml, constituting a normal anamnestic response. 2 wk after the injection of 50 μg of polyribose phosphate antigen from *H. influenzae*, the patient's serum contained approximately 30 ng antibody/ml, constituting a normal response.

*In vivo survival of $^{51}$Cr-labeled PNH red cells.* The survival of radiolabeled erythrocytes from a patient with PNH was shortened in both the C3-deficient and normal subjects with no significant difference between the disappearance curves. The $t_{\frac{1}{2}}$ in the normal subject was 17 days, whereas in the C3-deficient patient it was 16 days.

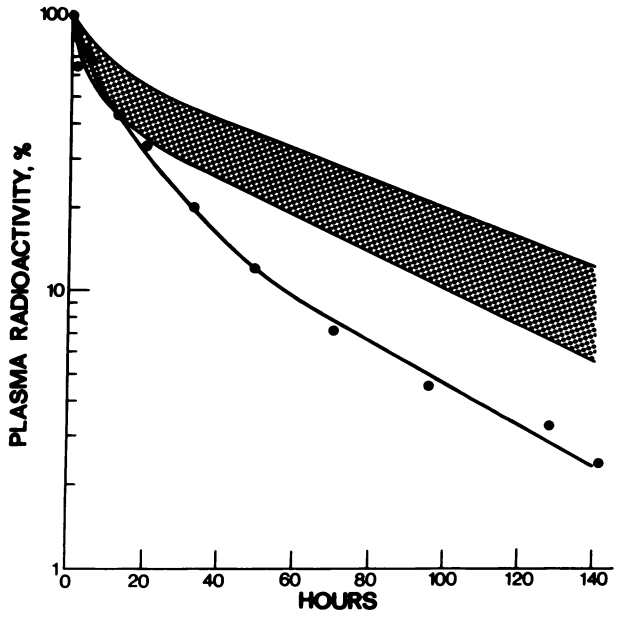


FIGURE 2 The plasma disappearance curve of $^{125}$I-labeled C3 in the patient with homozygous C3 deficiency. The stippled area is the range of disappearance curve in 11 normal subjects.

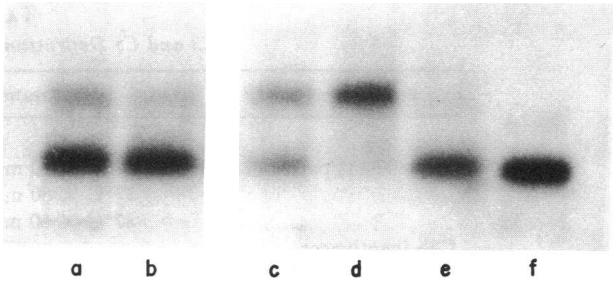


FIGURE 3 The conversion in vitro of $^{125}$I-labeled C3 in the homozygous C3-deficient patient's serum. Labeled C3 was added to serum or serum mixtures and incubated for 1 h at 37°C. Samples were then chilled and subjected to electrophoresis in agarose gel at pH 8.6 in barbital buffer ($\Gamma/2 = 0.05$) for approximately 45 min. After protein fixation, washing, and drying, radioautographs were made on Kodak No-screen X-ray film (Eastman Kodak Co., Rochester, N. Y.). Samples were (*a*) patient's serum, (*b*) a mixture of equal parts of patient's serum and normal serum, (*c*) serum from a patient with partial lipodystrophy and nephritic factor, (*d*) the serum with nephritic factor mixed with an equal volume of normal human serum, (*e*) normal serum, and (*f*) buffer alone. All the patterns shown are from the same electrophoresis plate.

*Immune adherence.* Immune adherence was about one third reduced in C3-deficient serum compared with normal controls. The titer in C3 deficient serum was 1/800 compared with a normal control of 1/1,200.

*Skin reaction to $C\bar{1}s$.* The results of the intradermal injection of $C\bar{1}s$ in the C3-deficient and normal subjects are given in Table III. No reaction was observed in the C3-deficient patient's skin on the injection of an amount of $C\bar{1}s$ that produced marked bluing in the normal subjects' skin.

*Leukocyte response to typhoid vaccine.* A normal leukocytosis occurred in the C3-deficient patient after the intramuscular injection of typhoid vaccine. The patient's white blood cells rose from 7,600/mm$^3$ before injection to 12,700 by 6 h after injection, with neutro-

TABLE III
*Vasopermeability Induced by $C\bar{1}s$**

| Subject | Maximum reaction time | Lesion size |
|---|---|---|
| | *min* | *mm* |
| C3-deficient patient | 8 | 2 × 2 |
| Normal subject 1 | 8 | 10 × 12 |
| Normal subject 2 | 8 | 10 × 10 |

* 2 U of $C\bar{1}s$ were injected in 0.1 ml of sterile saline intradermally.

TABLE IV

*C3 and C5 Destruction in Serum Induced by CoF*

| Serum source | Treatment | C3 concentration | C5 concentration |
|---|---|---|---|
| | | *effective molecules* $\times 10^{-3}$*/ml* | |
| C3-deficient patient | 4°C × 60 min + buffer | — | 42.7 |
| | 37°C × 60 min + buffer | — | 42.7 |
| | 37°C × 60 min + CoF | — | 0.6 |
| C3b inactivator-deficient patient | None | 0.27 | 7.9 |
| | 37°C × 60 min + buffer | 0.15 | 3.2 |
| | 37°C × 60 min + CoF | 0.22 | 4.6 |
| Normal subject | None | 60.9 | 19.7 |
| | 37°C × 60 min + buffer | 64.8 | 18.8 |
| | 37°C × 60 min + CoF | 0.13 | 0.13 |

phils rising from 3,600 to 10,500 in the same period. The normal control subject's white blood cells rose from 6,600 to 9,300 and his neutrophils from 4,300 to 6,100/mm³ under the same conditions.

*CoF-induced passive hemolysis.* When the C3-deficient patient's serum was incubated with purified CoF in the absence and presence of guinea pig erythrocytes, normal C5 destruction (Table IV) and normal lysis of guinea pig erythrocytes occurred (Table V). That this lysis was complement-mediated is seen from the lack of lysis when this experiment was performed with C3b inactivator-deficient or C5-deficient human serum. These observations indicate that CoF-induced passive hemolysis requires C5 and a protein or proteins of the alternative pathway other than C3 but not C3 itself.

The patient had a normal number of circulating B lymphocytes as defined by the presence of C3 receptors. Of her lymphocytes, 18% had such receptors.

## DISCUSSION

The inheritance pattern of C3 deficiency in the present family is identical to that observed earlier in a family with heterozygotes only (2) in which affected persons had inherited a gene, *C3⁻*, that produced no detectable product but was allelic to the structural C3 genes. In the present family, of 29 offspring of known or presumed heterozygotes, 16 were heterozygous deficient. Thus, *C3⁻* is inherited as an autosomal codominant trait, and the proposita is homozygous for this trait. This is consistent with the autosomal recessive mode of inheritance of marked C3 deficiency.

Since a heterozygote for C3 deficiency by chance married a first-degree relative of the proposita, it appears that *C3⁻* has a rather high frequency among Afrikaners in South Africa. This impression is strengthened by the establishment of the diagnosis of hereditary C3 deficiency in an Afrikaner child in Durban, South Africa.[3] That child died recently at the age of 7 and had a history of recurrent infections.

The metabolic studies with $^{125}$I-labeled C3 confirm that the C3-deficient subject fails to synthesize significant amounts of C3. The finding of an elevated frac-

[3] Grayce, J., and G. Vos. Personal communication.

TABLE V

*Passive Hemolysis of Guinea Pig Erythrocytes Induced by Serum and CoF*

| Serum source | Dilution | Hemolysis | | |
|---|---|---|---|---|
| | | With CoF | Without CoF | Corrected |
| | | | % | |
| C3-deficient patient | 1/30 | 89.2 | 2.1 | 87.1 |
| | 1/90 | 33.7 | 0.2 | 33.5 |
| C3b inactivator-deficient patient | 1/30 | 0.5 | 0 | 0.5 |
| C5-deficient patient | 1/30 | 0 | 0 | 0 |
| Normal subject | 1/30 | 87.0 | 27.7 | 59.3 |
| | 1/90 | 38.0 | 6.0 | 32.0 |

tional catabolic rate in this subject is puzzling. It may be that at levels as low as occur in her, the small amount of labeled C3 that she was given is destroyed by normally occurring C3-cleaving enzymes or utilized and taken up by minimal antigen-antibody complexes to a greater extent than in a person with even 2-3% of the normal concentration of C3. A similar mechanism may operate in vitro where increased conversion of labeled C3 was also observed. In distinction to a serum containing nephritic factor, admixture of this patient's serum with normal serum or purified C3 did not potentiate C3 conversion in vitro and, in fact, decreased it.

It has long been known that PNH erythrocytes are exquisitely sensitive to complement lysis (14) and that this lysis requires an intact alternative pathway (20-23). The failure, therefore, of the patient's serum to produce lysis of PNH erythrocytes is no surprise.

The shortened survival of erythrocytes from a patient with PNH in the C3-deficient subject's circulation may have one or both of two explanations. The cells may already have been damaged by complement through the C3 stage in the donor's circulation. Alternatively, complement may play no part in the shortened survival of random PNH red cells, since the bulk of complement-sensitive cells are destroyed shortly after their emergence from the marrow (24).

The lack of a vasopermeability response to the intradermal injection of $C\overline{1s}$ in the C3-deficient person's skin is puzzling. It is known that patients with very low levels of C3 (around 3% of the normal level) give a normal response, whereas C2-deficient subjects (25) give no response and C4-deficient guinea pigs have minimal swelling (26). Nevertheless, the complete absence of a vasopermeability response to the intradermal injection of $C\overline{1s}$ in the C3-deficient patient strongly suggests a requirement for C3 or a protein in the alternative pathway for the elaboration of this response. It is unlikely but not impossible that the normal response to $C\overline{1s}$ is the result of generation of C3a, since it cannot be blocked by antihistaminics (26), which effectively block the vasopermeability action of purified C3a (27). It is conceivable that the patient has end-organ unresponsiveness to whatever is responsible for the reaction to $C\overline{1s}$. This possible unresponsiveness must be prolonged if not continuous, because the patient has been tested on three separate occasions, two of which were 2 yr apart. Studies are in progress to define this problem further.

Since the early work on the anticomplementary effect of cobra venom on serum, attention has been focused on the serum-mediated attack on C3 (28-31). Jensen, however, provided convincing evidence that CoF attacked purified C5 after interaction with a serum protein free of C3 (32). The passive lysis of unsensitized guinea pig erythrocytes by serum incubated with CoF (18) has been assumed to proceed via C3 activation and cleavage (18, 33). The latter is certainly a prominent effect of CoF on serum. The present findings confirm Jensen's observation of a direct attack of CoF-serum complex on C5 and furthermore establish that human C3 is entirely dispensable and irrelevant to the induction of passive hemolysis by serum incubated with CoF. These observations are also consistent with the concept of Lachmann and Nicol (34) that CoF is C3b-like since it can function, as can C3b, in the activation of C5. Alternatively, CoF may activate a pathway to C5 activation independent of the classical or properdin pathways.

The near-normal immune adherence activity of the C3-deficient patient's serum confirms Cooper's finding (35) that only the first two components of complement, C1 and C4, are required for immune adherence. This function, if it has relevance in vivo, may afford some means of resistance to infection in this patient whose serum is devoid of opsonic activity, bactericidal activity for smooth strains of salmonella, antigen-antibody-induced chemotaxis, and hemolytic activity of complement (1).

In the initial report of the C3-deficient patient, the failure of infection with pyogenic organisms to induce a leukocytosis was noted (1), confirming Rother's observation in vitro that C3 is required for the mobilization of leukocytes from the rabbit femur (36). The finding in the present studies that a normal leukocytosis developed in the patient in response to the injection of endotoxin in the form of typhoid vaccine suggests that there are several normal mechanisms whereby bacterial infection results in leukocytosis. Apparently the leukocytosis of infection with some pyogenic organisms is C3-dependent but that induced by endotoxin administration is not.

Although small differences in the thymus-dependent antibody response to sheep red blood cells were shown to occur in C3-depleted mice (37), no deficit in this type of immune response could be shown in the C3-deficient patient. It seems highly unlikely that C3 plays a role in the human humoral antibody response. It is possible that the treatment of animals with CoF and other agents that produced sustained, systemic C3 depletion have other important effects on the immune response than merely lowering the level of C3.

Since the discovery of the present patient, two additional unrelated individuals with C3 deficiency have been found. One is a 4-yr-old patient of Dr. John S. Davis, IV. This patient also lacks a corpus callosum and has had frequent episodes of bacterial infection. Her C3 level is approximately 1% of the normal level and further studies of this patient and her relatives, some of whom are heterozygous deficient, are in progress. Another case (38) has no detectable C3 but family studies

could not be performed. This patient has also had numerous bacterial infections.

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