Simple Method To Distinguish between Primary and Secondary C3 Deficiencies
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Due to the increasing numbers of reported clinical cases of complement deficiency in medical centers, clinicians are now more aware of the role of the complement system in the protection against infections caused by microorganisms. Therefore, clinical laboratories are now prepared to perform a number of diagnostic tests of the complement system other than the standard 50% hemolytic component assay. Deficiencies of alternative complement pathway proteins are related to severe and recurrent infections; and the application of easy, reliable, and low-cost methods for their detection and distinction are always welcome, notably in developing countries. When activation of the alternative complement pathway is evaluated in hemolytic agarose plates, some but not all human sera cross-react to form a late linear lysis. Since the formation of this linear lysis is dependent on C3 and factor B, it is possible to use late linear lysis to routinely screen for the presence of deficiencies of alternative human complement pathway proteins such as factor B. Furthermore, since linear lysis is observed between normal human serum and primary C3-deficient serum but not between normal human serum and secondary C3-deficient serum caused by the lack of factor II or factor I, this assay may also be used to discriminate between primary and secondary C3 deficiencies.

The human complement system is formed by more than 30 proteins and plays several important inflammatory and immune functions derived from its activation (10). Complement can be activated by the classical, alternative, and lectin pathways. The activation of the alternative complement pathway occurs at a slow but constant rate and is initiated by the hydrolysis of an internal thioester bond in C3 that leads to the formation of C3(H2O) (12). When factor B (fB) binds to C3(H2O), it is cleaved by factor D (fD), generating the first C3 convertase of the alternative pathway, C3bBb, which cleaves C3 to C3a and C3b. C3b binding to fB, followed by cleavage by fD, generates the second C3 convertase of the alternative pathway, C3bBb. As a consequence, several molecules of C3 are cleaved to C3a and C3b in a short period of time. If this activation is not properly regulated, C3 is converted into C3b and a marked consumption of C3 is observed (secondary deficiency) (1, 2, 19).

Factor I (fI) is one of the most important C3 regulatory proteins since it cleaves C3b to iC3b, which is subsequently cleaved by fI to produce C3c and C3d. fI requires the presence of several cofactor proteins, principally, factor H (fH), to perform its regulatory functions properly.

Sera from individuals with homozygous deficiencies of fI or fH or from individuals presenting with nephritic factors have very low levels of intact C3 and low levels of fB (2, 7, 11, 19). On the other hand, primary C3-deficient patients have little C3 or completely lack of C3 but have normal levels of fB, fH, and fI (18, 19). Impairment of the complement-dependent functions is observed in patients with both primary and secondary C3 deficiencies.

Here we describe the use of a modified hemolytic assay with agarose plates containing guinea pig erythrocytes (5) to discriminate between primary and secondary C3-deficient serum. A late linear lysis is observed among C3-deficient sera, depending on the concentration of fB.

MATERIALS AND METHODS

Serum samples. Blood samples were harvested by venipuncture, immediately transferred to an ice bath for 30 min, and centrifuged at 460 × g for 15 min at 4°C. The serum was aliquoted and stored at −80°C. Serum samples were thawed only once, immediately before use. Normal human serum was prepared from sera from a pool from 42 healthy adult blood donors (Hospital Universitário/USP, São Paulo, Brazil). Complement-deficient sera were obtained after the provision of informed consent. Human serum depleted of fB (iN) was prepared by incubation of normal human serum at 50°C for 15 min.

Buffer. An isotonic barbital buffer (5× Veronal-buffered saline) containing NaCl (717 mM), sodium barbital (4.8 mM), and barbital (12.5 mM) was used as a stock solution. Fresh GVB-EGTA-Mg2+ buffer was prepared immediately before use by mixing 10% gelatin, 1 mM EGTA (pH 7.4), and 5 mM MgCl2 with 1× Veronal-buffered saline buffer.

Preparation of hemolytic plates. Our assay for hemolysis by the alternative complement pathway is a modification of the protocol described elsewhere (5). GVB-EGTA-Mg2+ buffer (5.1 ml) was mixed with 2% agarose (4 ml) at 56°C. This mixture was then cooled to 45°C and mixed with 0.5 ml of a 10% suspension of guinea pig erythrocytes (Instituto Adolfo Lutz/SP, São Paulo, Brazil) which had previously been washed with GVB-EGTA-Mg2+ buffer. The final mixture (10 ml) was poured onto glass plates (8 by 8 cm). Wells (diameter, 3 mm) separated by 14 mm were cut into the agarose gel. Serum samples (7.5 µl each) were loaded into this gel, and incubation was carried out at 4°C overnight, followed by incubation at 37°C for 2 h to visualize the radial hemolysis around the wells. Finally, the plates were kept at room temperature for 24 h to visualize the linear lysis between adjacent wells. The activity was measured at least in duplicate and was expressed as the percentage of the activity observed for a pool of normal human sera.

Other reagents. Human C3 was purified from fresh human plasma as described by Tack and Prahl (15) and stored as aliquots at −80°C until they were used. Aliquots (7.5 µl) containing 3.3, 1.65, and 0.82 µg of purified C3 (at concentrations below the C3 concentration in normal human serum [1 to 2 mg/ml]...
or 7.5 to 15 µg in 7.5 µl) were loaded into the wells of the gel. Human fB was purchased from The Binding Site (Birmingham, United Kingdom), and 7.5 µl of human fB at a concentration of 200 µg/ml (approximate concentration in normal human serum) was loaded into the wells of the hemolytic plates. fB-deficient serum was obtained after incubation of normal human serum at 50°C for 15 min (8). Where indicated, we also used commercial fB-deficient serum purchased from Calbiochem-Novabiochem Corporation (San Diego, Calif.). Goat polyclonal antiserum against human C3, fB, and fH was purchased from Calbiochem-Novabiochem Corporation.

**RESULTS**

Radial lysis was observed with normal human serum in hemolytic agarose plates incubated at 4°C overnight, followed by incubation for 2 h at 37°C. As expected, no radial lysis was observed with any C3-deficient or fB-deficient serum (data not shown). However, after incubation for an additional 24 h at room temperature, very weak radial lysis was observed for complement-deficient sera with residual C3 concentrations but normal fB levels (P1 sera) (Fig. 1; Table 1). This weak radial lysis was not observed for a complement-deficient serum sample with undetectable C3 and a normal fB concentration (P2 sera) (18) or with sera from patients with low fB levels and residual C3 levels (P3 sera) or undetectable C3 levels (P4 sera) (Fig. 1; Table 1). This 24-h incubation also produced a late linear lysis between normal human serum and P1 and normal human serum and P2 but not between normal human serum and P3 or P4 (Fig. 1; Table 1). Additionally, late linear lysis was never observed between two wells when each well was loaded with the same normal or deficient serum (Fig. 1).

In order to investigate which component of normal human serum (fB or C3) contributed to the formation of late linear lysis, we incubated normal human serum or P1 at 50°C for 15 min to produce iN and iP1, respectively (Fig. 2). At this temperature, fB is labile (8), while C3 remains active. We observed late linear lysis between iN and P1 (Fig. 2), suggesting that late linear lysis is dependent on the diffusion of C3 from iN, while fB is predominantly diffused from P1. For this reason, P4 (with a low fB concentration) (Table 1) was not able to develop a late linear lysis.

**TABLE 1. Determination of complement protein concentrations and functional hemolytic activities of complement-deficient patients**

| Serum | C3 (µg/ml) | fB (% N) | fH (µg/ml) | fl (µg/ml) | APH (% N) | CPH (% N) |
|-------|------------|----------|------------|------------|-----------|-----------|
| 1     | <125.0b    | 106.41 (4.24) | 525.50 (39.10) | 59.85 (1.94) | No lysis | No lysis |
| 2     | Zerur      | 120.48 (14.98) | 273.39 (1.40) | 59.38 (5.12) | No lysis | No lysis |
| 3     | 651.25 (22.58) | <12.5b | 175.44 (2.74) | <31.2b | No lysis | No lysis |
| 4     | <125.0b    | <12.5b | <62.5b | 47.65 (2.32) | No lysis | No lysis |

Values are means (standard deviations). All determinations were performed at least in duplicate. N, pool of healthy adult sera; APH, hemolytic activity mediated by alternative pathway; CPH, hemolytic activity mediated by classical pathway. Normal values, expressed as the lower limit, mean, and upper limit, were as follows for C3 (10 to 14 years), 64, 100, and 147 mg/100 ml, respectively (4); for fH (6 to 13 years), 223.26, 695.60, and 1,712.42 mg/ml, respectively (3); for fl (6 to 13 years), 34.03, 57.06, and 91.00 mg/ml, respectively (3).

b A reduced immune precipitation or lysis ring was observed at all.

b The hemolytic activity was below the resolution of the method.
late linear lysis against normal human serum (Fig. 1). While iP1 also formed a late linear lysis with normal human serum, the extent of the lysis reaction was less than that observed between normal human serum and P1 or between iP1 and P1 (Fig. 2). This is because both samples in the reaction between iP1 and normal human serum are partially depleted of fB (normal human serum is depleted because of radial lysis, and iP1 is depleted because of heat treatment).

To confirm that C3 from normal human serum is essential for the development of late linear lysis when C3 reacted with serum from P1 or P2, we performed experiments in which normal human serum was replaced with C3 purified from plasma. As shown in Fig. 3, all concentrations of C3 used (0.82, 1.65, and 3.3 μg) were able to cause late linear lysis upon interaction with P1 but not with normal human serum. Concentrations of C3 lower than 0.82 μg/well were unable to form the late linear lysis against P1 (normal serum contains 1 to 2 mg of C3/ml or 7.5 to 15 μg of C3/7.5 μl). Purified fB (0.2 mg/ml) participates in the formation of late linear lysis with fB-deficient serum (iN; contains ~200 μg of fB/ml) but not with either fI-deficient serum (P3) or primary C3-deficient serum (P1), as shown in Fig. 4. Equivalent results were obtained with 0.1 mg of purified fB/ml but not with 0.05 mg of purified fB/ml. Only a discrete late linear lysis was observed between serum depleted of fB (iN) and normal human serum (Fig. 4). Table 2 summarizes the results found with the different complement C3-deficient sera. Both polyclonal goat anti-human C3 and anti-human fB antibodies were able to independently block the formation of late linear lysis between normal human serum and P1. On the other hand, no inhibition of late linear lysis was observed with anti-human fH (data not shown).

**DISCUSSION**

Most complement deficiencies are associated with higher levels of susceptibility to infections and/or to autoimmune dis-

cases such as systemic lupus erythematosus (13). In the last decade, the investigation of complement deficiencies by clinicians and researchers has been stimulated by the increase in the numbers of patients with diagnosed cases from whom at least one of the complement proteins is missing.

Hemolytic agarose plates have been widely used to study alterations in the classical or the alternative pathway of the complement system (5). In this paper we have described a hemolytic diffusion plate assay which has been specifically modified for the screening of primary or secondary C3 deficiency (caused by fI or fH deficiency or the presence of nephritic factors). This method is based on a modification of the protocol of Lachmann and Hobart (5), in which the alternative complement pathway is activated in the presence of agarose and the membrane attack complex (C5b-9n) is deposited on the surface of the membrane of unsensitized guinea pig erythrocytes (bystander lysis). When complement is properly activated, radial lysis is observed around the well loaded with human serum, and the diameter of the lysis zone is dependent on the functions and concentrations of complement proteins. This methodology is commonly used to screen serum samples after overnight incubation at 4°C, followed by incubation for 2 h at 37°C. Consequently, a primary or secondary C3-deficient serum sample does not develop lysis after incubation for only 2 h at 37°C.

However, a second and later form of lysis, which we termed late linear lysis, between certain complement-deficient sera and normal human serum was observed when the same plates were incubated for a further 24 h at room temperature. Because this second lysis is also dependent on activation of the alternative pathway, the presence of a minimal concentration
of C3 and fB diffused in the space between the two wells is necessary to trigger this reaction.

A phenomenon like late linear lysis between some serum samples was first observed in hemolytic agarose plates by Thompson and Rowe (17). They named it “reactive lysis” as a result of the interaction of at least two factors found in certain serum samples, which they termed “reactor” and “indicator.” Later, it was demonstrated that while both serum samples in a reaction contribute C8 and C9, the reactor serum contributed C5 and C6 and the indicator serum provided C7. The combination of these proteins forms C5b-9, which is deposited on the erythrocyte membranes, causing their lysis (6, 16). The formation of reactive lysis was mainly but not exclusively observed with acute-phase sera (14). It is not clear if the formation of late linear lysis and reactive lysis is necessarily explained by the same phenomenon, since we observed linear lysis between P1 (low C3 levels and normal fB levels) and a series of sera depleted of C5, C6, C7, C8, C9, or fB (data not shown). Another relevant difference is that linear lysis was observed even with P1 and purified C3 and did not require the addition of extra C5 to C9 to form the membrane attack complex. Nevertheless, as far as we know, the formation of linear or reactive lysis has not been systematically applied to evaluations of complement-deficient sera for laboratory diagnosis.

In this work, we reacted a variety of complement-deficient serum samples with a pool of normal human serum, and linear lysis was observed only if the serum was C3 deficient but presented sufficient amounts of fB. Our understanding of this phenomenon is that the contribution of normal human serum was the diffusion of the excess C3 that had not been consumed in the radial lysis. This observation was confirmed when the same linear lysis was observed when C3-deficient serum reacted with purified human C3. In addition, when normal human serum was heated to 50°C in order to inactivate fB (but not C3), linear lysis was again observed with a serum sample with primary C3 deficiency. Linear lysis was never observed between two wells both loaded with normal human serum, even though excess C3 was present, probably because most of the fB (and other complement proteins in limiting concentrations) is consumed in the radial hemolysis reaction (during activation at 37°C for 2 h). Similarly, no linear lysis was observed when both wells were loaded with the same complement-deficient serum sample. Serum from patients with low levels of fB (<25% of the normal concentration) due to fH or fI deficiencies, even though they had low but detectable amounts of C3, did not react with normal human serum to form late linear lysis. The importance of C3 and fB was confirmed by the fact that polyclonal anti-human C3 or anti-human fB serum completely blocked the formation of late linear lysis.

In conclusion, we have described the application of a simple functional assay useful for the screening of patients with impairment in the alternative complement pathway. This method has the power to discriminate between primary and secondary C3 deficiencies. The application of this technique requires only that the deficient test serum reacts against normal human serum for an additional 24 h, representing no additional expense to the routine laboratory investigation. Ideally, one would wish to determine the fB, fI, or fH concentration. However, for economic reasons, most clinical laboratories in developing countries are not equipped or prepared at present to perform these assays. The assay presented here is extremely inexpensive and easy to perform, and the results are easy to interpret. Use of the methodology described here could provide rapid information on the nature of the complement deficiency at hand. Additionally, the protocol is reliable and inexpensive and has the advantage of requiring only a few microliters of an immunodeficient patient’s serum.

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REFERENCES

1. Abramson, N., C. C. Alper, P. J. Lachmann, F. S. Rosen, and J. H. Jand. 1971. Deficiency of C3 inactivator in man. J. Immunol. 107:19–27.
2. Amadei, N., G. V. Baracho, V. Nudelman, W. Bastos, M. P. C. Florido, and L. Isaac. 2001. Inherited complete factor I deficiency associated with systemic lupus erythematosus, higher susceptibility to infection and low levels of factor H. Scand. J. Immunol. 53:615–621.
3. Ferreira de Paula, P., P. R. Jr., V. P. L. Ferriani, V. Nudelman, and L. Isaac. 2003. Concentrations of regulatory complement proteins in Brazilian healthy children and adults. Mol. Immunol. 38:88.
4. Ferriani, V. P. L., J. E. Barbosa, and I. F. Carvalho. 1999. Complement haemolytic activity (classical and alternative pathways), C3, C4, and factor B titers in healthy children. Acta Paediatr. 88:1–4.
5. Lachmann, P. J. and M. J. Hobart. 1978. Complement technology, p. 5A.12–5A.13. In D. M. Weir (ed.), Handbook of experimental immunology, vol. 1, 3rd ed. Blackwell Scientific Publications Ltd., Oxford, United Kingdom.
6. Lachmann, P. J., and R. A. Thompson. 1970. Reactive lysis: the complement-mediated lysis of unsensitized cells. II. The characterization of activated reactor as C56 and the participation of C8 and C9. J. Exp. Med. 131:643–657.
7. Leitão, M. F., M. M. S. Vilela, R. Rutz, A. S. Grumach, N. A. Condino, and M. Kirschfink. 1997. Complement factor I deficiency in a family with recurrent infections. Immunopharmacology 38:207–213.
8. Lynen, R., V. Brade, A. Wolf, and W. Vogt. 1973. Purification and some properties of a heat labile serum factor (VP): identity with glycine-rich beta-glycoprotein and properdin, factor B. Hoppe Seyler’s Z. Physiol. Chem. 354:37–47.
9. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235–254.
10. Morley, B. J., and M. J. Walport. 2000. The complement system, p. 7–22. In B. J. Morley and M. J. Walport (ed.), The complement facts book, 1st ed. Academic Press, Inc., New York, N.Y.
11. Naked, G. M., M. P. C. Florido, P. Ferreira de Paula, A. M. Vinet, J. S. Inostroza, and L. Isaac. 2000. Deficiency of human complement factor I associated with lowered factor H. Clin. Immunol. 96:162–167.
12. Pangburn, M. K., and H. J. Müller-Eberhard. 1980. Relation of a putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. J. Exp. Med. 152:1102–1114.
13. Pickering, M. C., M. Botto, P. R. Taylor, P. J. Lachmann, and M. J. Walport. 2001. Systemic lupus erythematosus, complement deficiency, and apoptosis. Adv. Immunol. 76:227–324.
14. Roither, U. 1988. Innocent bystander lysis by C5-C9, p. 230–236. In U. Roither and G. O. Till (ed.), The complement system. Springer-Verlag, Berlin, Germany.
15. Tack, R. F., and R. F. Prahl. 1976. Third component of human complement: purification from plasma and physicochemical characterization. Biochemistry 15:4513–4521.
16. Thompson, R. A., and P. J. Lachman. 1970. Reactive lysis: the complement-mediated lysis of unsensitized cells. I. The characterization of the indicator factor and its identity as C7. J. Exp. Med. 131:629–641.
17. Thompson, R. A., and D. S. Rowe. 1968. Reactive haemolysis—a distinctive form of red cell lysis. Immunology 14:745–762.
18. Ulbrich, A. G., M. P. C. Florido, Y. Nudelman, E. S. Reis, G. V. Baracho, and L. Isaac. 2001. Hereditary human complement C3 deficiency owing to reduced levels of C3 mRNA. Scand. J. Immunol. 53:622–626.
19. Vyse, T. J., P. J. Späth, K. A. Davies, B. J., B. J. Morley, P. Philippe, P. Athanassiou, C. M. Giles, and M. J. Walport. 1994. Hereditary complement factor I deficiency. Q. J. Med. 87:385–401.