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Standard Practice for Testing for Alternative Pathway Complement Activation in Serum by Solid Materials[1]

This standard is issued under the fixed designation F 2065; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

ε[1] NOTE—Editorial corrections were made throughout this standard in June 2001.

1. Scope

1.1 This practice provides a protocol for rapid, in vitro screening for alternative pathway complement activating properties of solid materials used in the fabrication of medical devices that will contact blood.

1.2 This practice is intended to evaluate the acute in vitro alternative pathway complement activating properties of solid materials intended for use in contact with blood. For this practice, "serum" is synonymous with "complement."

1.3 This practice consists of two procedural parts. Procedure A describes exposure of solid materials to a standard lot of C4-deficient guinea pig serum [C4(-)GPS], using 0.1-mL serum per 13 × 100-mm disposable glass test tubes. Sepharose™[2] CL-4B is used as an example of test materials. Procedure B describes assaying the exposed serum for significant functional alternative pathway complement depletion as compared to control samples. The endpoint in procedure B is lysis of rabbit RBC in buffer containing EGTA and excess Mg^{++}.

1.4 This practice does not address function, elaboration, or depletion of individual complement components except as optional additional confirmatory information that can be acquired using human serum as the complement source. This practice does not address the use of plasma as a source of complement.

1.5 This practice is one of several developed for the assessment of the biocompatibility of materials. Practice F 748 may provide guidance for the selection of appropriate methods for testing materials for other aspects of biocompatibility. Practice F 1984 provides guidance for testing solid materials for whole complement activation in human serum, but does not discriminate between the classical or alternative pathway of activation.

2. Referenced Documents

2.1 *ASTM Standards:*

F 748 Practice for Selecting Generic Biological Test Methods for Materials and Devices[3]

F 1984 Practice for Testing for Whole Complement Activation in Serum by Solid Materials[3]

2.2 *Other Document:*

ISO 10993-4: Biological Evaluation of Medical Devices. Part 4: Selection of Tests for Interactions with Blood[4]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *water*—distilled, endotoxin-free.

3.2 *Abbreviations:*Abbreviations:

3.2.1 *Ab*—antibody (hemolysin)

3.2.2 *BBS*—barbital buffered saline

3.2.3 *BBS-G*—barbital buffered saline – gelatin

3.2.4 *BBS-G-EGTA/Mg (Mg Buffer)*—barbital buffered saline – gelatin EGTA Mg^{++}

3.2.5 *BBS-GM (Ca Buffer)*—barbital buffered saline – gelatin metals

3.2.6 *C′*—complement

3.2.7 *C4(-)GPS*—C4-deficient guinea pig serum [serum from guinea pigs genetically incapable of producing C4, the fourth component of complement]

3.2.8 *EDTA*—ethylenediaminetetraacetic acid, disodium salt: dihydrate

3.2.9 *EGTA*—ethylene glyco-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, tetrasodium salt

3.2.10 *HAGG*—heat aggregated gamma globulin

3.2.11 *HS*—human serum

3.2.12 *I*—control tube with serum but no material, kept on ice.

3.2.13 *M*—tube containing serum plus a test material

3.2.14 *NM*—tube containing serum but no material

3.2.15 *PVDF*—polyvinylidene fluoride

3.2.16 *RBC*—red blood cell(s)

4. Summary of Practice

4.1 Solid material specimens are exposed to a standard lot of C4(-)GPS complement under defined conditions, in parallel to appropriate controls (Procedure A). If the alternative

[1] This practice is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.16 on Biocompatibility Test Methods.

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[2] Sepharose™ is a registered trademark of Pharmacia, Inc.

[3] *Annual Book of ASTM Standards*, Vol 13.01.

[4] Available from American National Standards Institute, 11 W. 42nd St., 13th Floor, New York, NY 10036.

complement pathway is activated by the material, complement components will be depleted from the serum. Exposed serum is then tested for remaining functional complement activity, by determining complement mediated lysis of rabbit RBC in buffer containing EGTA and excess Mg^{++}(Procedure B).

5. Significance and Use

5.1 Inappropriate activation of complement by blood-contacting medical devices may have serious acute or chronic effects on the host. Solid medical device materials may activate the complement directly by the alternative pathway. Unlike the classical complement activation pathway (see Practice F 1984), antibodies are not required for alternative pathway activation. This practice is useful as a simple, inexpensive screening method for determining alternative whole complement activation by solid materials in vitro.

5.2 This practice is composed of two parts. In Part A (Section 10) C4(-)GPS is exposed to a solid material. Since C4 is required for classical pathway activation, activation of complement in C4(-)GPS can only occur by the alternative pathway **(1)**.[5] In principle, nonspecific binding of certain complement components to the materials may also occur. In Part B (Section 11), complement activity remaining in the serum after exposure to the test material is assayed by alternative pathway-mediated lysis of rabbit RBC.

5.3 Assessment of in vitro whole complement activation as described here provides one method for predicting potential complement activation by solid medical device materials intended for clinical application in humans when the material contacts the blood. Other test methods for complement activation are available, including assays for specific complement components and their split products in human serum (X1.3 and X1.4).

5.4 This in vitro test method is suitable for adoption in specifications and standards for screening solid materials for use in the construction of medical devices intended to be implanted in the human body or placed in contact with human blood outside the body.

6. Preparation of Buffers

6.1 Buffers are prepared in accordance with established protocols **(1, 2)**. "Water" refers throughout to distilled, endotoxin-free H_2O. The use of barbital (veronal) buffer is recommended. In the United States, barbital is a class IV regulated substance and requires a DEA **(3)** license for purchase. The use of other buffer systems (such as TRIS) is permissible if they have been demonstrated not to activate complement **(4)**. These solutions are stable for one month at 4°C unless otherwise indicated.

6.2 The 5X stock BBS (barbital-buffered saline) is prepared by adding 20.75 g of NaCl plus 2.545 g of sodium barbital (sodium-5,5-diethyl barbiturate) to about 400 mL water. The pH is adjusted to 7.35 with 1 *N* HCl, then brought to a final volume of 500 mL in a volumetric flask.

6.3 Metals solution is prepared by making a 2.0-*M* solution of $MgCl_2$ (40.66 g $MgCl_2 \cdot 6\ H_2O$ up to a final volume of 100 mL water), and a 0.3-*M* solution of $CaCl_2$(4.41 g $CaCl_2 \cdot\ 2H_2O$ up to a final volume of 100 mL of water), and combining the two solutions 1:1 (v:v).

6.4 The Ca buffer (BBS-GM working solution) is prepared daily, by dissolving 0.25 g of gelatin (type A: Porcine Skin, Approx. 300 Bloom, such as available from Sigma [G-1890]) in 50 mL of water that is gently heated and stirred. The gelatin solution is added to 50 mL 5X Stock BBS plus 0.25 mL metals solution, brought up to about 200 mL then adjusted to pH 7.35 (with 1 *N* HCl or 1 *N* NaOH) before bringing the final volume to 250 mL in a volumetric flask. The Ca buffer contains both Mg^{++} and Ca^{++}, which allows both classical and alternative pathway complement. activation to occur.

6.5 The BBS-G working solution is prepared the same way, but omitting addition of the metals solution.

6.6 10X Stock EDTA (0.1-*M* disodium dihydrate EDTA) is prepared by adding 7.44 g disodium EDTA·2 H_2O to about 160 mL of water, adjusting the pH to 7.65 (with 1 *N* NaOH or 1 *N* HCl), then bringing the volume to 200 mL in a volumetric flask.

6.7 The 0.1 *M* EGTA (tetrasodium salt, EGTA·4.5 H_2O) is prepared by adding 4.683 g tetrasodium EGTA to about 80 mL of water, adjusting the pH to 7.35 (with 1 *N* NaOH or 1 *N* HCl), then bringing the volume to 100 mL in a volumetric flask.

6.8 BBS-G-EDTA (to be used in preparing RBC before being washed out) is prepared by adding 10 mL of stock 10X EDTA to 90 mL of BBS-G in a volumetric flask.

6.9 The Mg Buffer (BBS-G-EGTA/Mg working solution) is prepared daily, by dissolving 0.25 g gelatin in 50 mL water that is gently heated and stirred. The gelatin solution is added to 50 mL 5X stock BBS, plus 0.625 mL 2.0 *M* $MgCl_2$, plus 4 mL of 0.1 *M* EGTA, brought up to about 200 mL, then adjusted to pH 7.35 (with 1 *N* HCl or 1 *N* NaOH) before bringing the final volume to 250 mL in a volumetric flask. The Mg buffer has EGTA to bind Ca^{++}. The presence of Mg^{++} allows the alternative pathway activation to proceed, while the absence of Ca^{++} prevents activation of the classical pathway.

7. Preparation of Sheep and Rabbit RBC

7.1 Commercially obtained sheep RBC preserved in Alsever's solution and defibrinated rabbit RBC are stored at 4°C. The sheep cells are discarded after eight weeks or when the supernatant from the second wash contains hemoglobin (red color) by visual inspection (as lots of RBCs age, they are more sensitive to complement lysis in parallel with increased spontaneous lysis). The rabbit cells are more fragile than the sheep cells, and should be discarded after four weeks or when the supernatant from the second wash contains hemoglobin by visual inspection.

NOTE 1—All centrifugations are at 4°C. Except when indicated, all reagents, tubes, and cell preparations are kept cold in chipped ice or an ice slurry.

7.2 Five millilitres of sheep or rabbit RBC are centrifuged at 1000 × g, at 4°C, for 10 min.

7.3 The cell pellet is resuspended in 10 mL of cold BBS-G-EDTA and incubated for 10 min at 37°C. The cells are centrifuged, and the pellet resuspended in 10 mL of 4°C BBS-G-EDTA.

[5] The boldface numbers in parentheses refer to the list of references at the end of this specification.

7.4 The cells are centrifuged, the supernatant discarded (first wash), and the pellet resuspended in 10 mL of cold BBS-GM (Ca buffer) or BBS-G-EGTA/Mg (Mg buffer) (cells to be used in absorbing serum are washed in Ca buffer; cells to be used for detecting alternative pathway C′ depletion, Procedure B, are washed and suspended in Mg buffer). Repeat twice (total of three washes.)

7.5 Adjust cell count spectrophotometrically (where an absorbance of 0.75 for sheep RBC and 1.30 for rabbit RBC corresponds to 2.0×10^8 RBC/mL, at a wavelength of 412 nm and a 1.0-cm light path for 1 volume of cells in BBS-GM or BBS-G-EGTA/Mg plus 24 volumes of water) or count with a hemocytometer. Prepare 10 mL of 2.0×10^8 cells/mL in 4°C BBS-GM.

7.6 The washed, diluted RBC can be held on ice and used for at least 12 h.

8. Absorption of Serum (Complement)

8.1 Although human serum would be preferable to guinea pig serum for alternative pathway complement activation by materials to be used in medical devices intended to contact patient blood, genetically deficient human sera are not routinely available. Human sera depleted of components by antibody absorption on columns are unsuitable for this purpose for the following two reasons: (*1*) specific component depletion is incomplete, so significant classical pathway activation remains; and (*2*) column material may activate the alternative pathway, depleting functional activity. Serum from guinea pigs genetically deficient in C4 [C4(-)GPS] has no classical pathway complement activity but is fully competent for alternative pathway complement activation. Although the titers are different (a greater concentration of C4(-)GPS is required to produce the same lysis of rabbit RBC as human serum)**(5)**C4(-)GPS gives equivalent results to human serum in detecting biomaterial complement activation (see Sepharose™ example in Table 1). Thus, C4(-)GPS is suitable as a screen for subsequent confirmatory immunoassays that detect complement components and split-products indicative of alternative pathway activation in whole human serum. The C4(-)GPS suitable as a source of complement may be purchased from biological supply houses, and is generally labeled as reagent-grade complement.

8.2 Serum may be absorbed with sheep RBC and rabbit RBC in order to remove naturally occurring anti-sheep and anti-rabbit RBC hemolytic antibodies. The procedure is as follows:

8.3 Commercially available C4(-)GPS is stored at −70°C.

8.4 The serum is thawed on chipped ice or reconstituted (if lyophilized) with ice-cold water.

8.5 All manipulations are done in chipped ice or in an ice slurry, with ice cold (4°C) reagents and cells. Centrifugations are carried out at 1000 × g at 4°C. It is critical that this entire procedure be done in the cold to avoid activation of complement in this step.

8.6 Cold serum and 4°C, Ca buffer-washed RBC (a 1:1 mixed volume of sheep and rabbit packed RBC) are gently mixed (by slow rocking), 0.1 mL of packed RBC/2.5 mL of serum, incubated for 10 min on ice, then centrifuged at 1000 × g for 10 min at 4°C. The supernatant liquid is carefully transferred to a new container on ice.

8.7 The procedure in 8.6 is repeated twice.

8.8 The absorbed C4(-)GPS is stored in 0.5–1.0-mL aliquots (convenient for one-experiment use), in cold snap-cap microfuge tubes and kept at −70°C until used. Aliquots should be thawed in chipped ice or an ice slurry, used on the day of thawing, and not refrozen.

TABLE 1 Percent Lysis[A] of Rabbit RBC in Mg Buffer by Human Serum or C4(-)GPS Pre-exposed 1 h at 37 °C, in 100-µL volumes, to Different Amounts of Sepharose™ CL-4B

µL Sepharose, ™ CL-4B[B] [HS/C4(-)GPS]	HS prep. 1[C]	HS prep. 2[D]	C4(-)GPS[E]
50/12.5	3.3 ± 1.2	3.4 ± 1.7	6.5 ± 2.6
25/6.25	14.2 ± 0.6	11.8 ± 0.8	21.3 ± 3.3
12.5/3.13	23.6 ± 5.2	19.6 ± 2.8	20.1 ± 0.9
6.25/1.57	33.2 ± 2.8	29.4 ± 3.8	27.3 ± 1.4
0/0 [37°C control]	49.9 ± 0.2	45.3 ± 4.6	41.3 ± 0.7
0/0 [Ice control]	51.4 ± 1.6	52.9 ± 0.7	58.4 ± 0.5

[A] Mean plus or minus standard deviation of three replicate tubes.

[B] The indicated volume of Sepharose™ CL-4B was added to 100 µL of HS or C4(-)GPS.

[C] Whole human serum [Quidel (NHSC)], diluted 1:8 in Mg buffer.

[D] Reconstituted lyophilized human serum [Sigma (S1764)], diluted 1:4 in Mg buffer.

[E] Whole guinea pig serum, C4-deficient [Sigma (C1038)], 1:3 in Mg buffer.

9. Whole Complement Titration to Determine Optimal Serum Dilution

9.1 If statistical evaluation of results is desired, all conditions should be assayed in triplicate, using three 13 × 100 mm round-bottomed, disposable glass test tubes per condition. Otherwise, single or duplicate tubes are sufficient. Tubes are numbered in advance. Conditions include “total lysis,” “no complement (RBC only)” (no C′), “tests” (dilutions of C4(-)GPS) with and without hemolysin, and “no RBC (serum color)” (complement color control, at highest concentration of serum used). All reagents, tubes, and manipulations are done at 4°C, with tubes held in a rack in an ice slurry.

9.2 Two sets of tubes are prepared in accordance with 9.3–9.4. One set contains diluted sera placed on Mg buffer-washed sheep RBC, and one set contains diluted sera placed on Mg buffer-washed rabbit RBC.

9.3 The Mg buffer-washed rabbit RBC are added to all tubes except “no RBC (serum color)” tubes (0.05 mL/tube of a 2.0×10^8 cells/mL suspension.) Since this is a small volume for the tube area, care should be taken to deliver the accurate volume to the center of the bottom of each tube. “No RBC (serum color)” tubes get 0.05 mL 4°C Mg buffer.

9.4 HS and C4(-)GPS at 4°C are diluted in 4°C Mg buffer to the desired concentrations (with minimal agitation.) It is recommended to test the sera initially at 1:2 to 1:20 dilutions. Diluted serum is added directly to the bottom of each test tube in a 0.05-mL volume. The “RBC only (serum color)” tubes get 0.05 mL of Mg buffer containing no serum. “Total lysis” tubes get 0.05 mL of distilled water in place of buffer.

9.5 Tubes are shaken manually to suspend cells, then the rack is incubated in a 37°C water bath for 1 h, and intermittently shaken (every 15 min) to keep cells in suspension.

9.6 At the end of 1 h, the rack is placed in an ice slurry. All tubes receive the same volume of additional 4°C Mg buffer (if supernatants following centrifugation are to be removed to a

microtiter well plate for reading, an additional 0.4 mL/tube is sufficient; if readings will be done on a flow-through spectrophotometer, then 1.1 mL/tube should be added). The cold tubes are then centrifuged at 1000 × g for 10 min at 4°C, and the supernatants decanted to correspondingly numbered 13 × 100-mm glass tubes (or placed directly in microtiter wells for plate reader scanning).

NOTE 2—Although supernatant liquids can be placed in microtiter plate wells for reading in a plate reader, the alternative C′ pathway test itself can not be carried out in microtiter plates because the plastic may directly activate the alternative pathway.

9.7 Absorbance of the supernatants is measured at 412 nm, the percent of lysis is calculated for each test and control tube by subtracting from the 412-nm absorbance of the "no-RBC serum color" control (mean of the three replicate tubes) or proportional value for greater dilutions dividing by the total lysis control value (mean of the three replicate tubes), and multiplying by 100.

$$\% \text{ lysis} = \frac{\text{test absorbance} - \text{"no-RBC" control absorbance}}{\text{total lysis absorbance}} \times 100 \quad (1)$$

9.8 The final percent of lysis for each condition is expressed as mean ± 1 standard deviation of the three "percent" lysis values for each three-replicate set.

9.9 The optimal dilution of a particular lot of serum, for example, the dilution at which serum exposed to a material will be assayed for rabbit cell lysis in Procedure B, can now be determined. The optimal dilution is defined as that in which the percent of lysis for sheep RBC is ≤5 % while the percent of lysis for rabbit RBC is at least 40 % but not greater than 80 % (that is, lysis is on the linear part of the complement titration curve.) A typical optimal dilution for a lot of absorbed, lyophilized HS is 1:4 (used to conform that C4(-)GPS is a good surrogate for human serum) (see Table 1) added as a 0.05-mL volume in the assay. A typical optimal dilution for a lot of absorbed, whole HS is 1:8. A typical optimal dilution for a lot of absorbed, whole C4(-)GPS is 1:3.

10. Procedure A—Exposure of Material to C4(-)GPS

10.1 *Preparation of Material*:

10.1.1 Sepharose™ CL-4B (cross-linked 4 % beaded agarose) is presented here as an example of a solid material used in medical devices that contact patient blood (such as antibody-depleting columns). Sepharose™ CL-4B has a fractionation range (molecular weight) from 60 000 to 20 000 000 daltons for globular proteins and from 30 000 to 5 000 000 daltons for dextrans, with a wet bead diameter of 40 to 165 µm. It is stored as a suspension containing 20 % ethanol between 2 to 8°C, and not frozen. Sepharose™ CL-4B, which is a moderate activator of the alternative C′ pathway, can be used as a positive control material, and is presented here as an example. Other materials shown to activate the alternative pathway may also be used (see Section 12).

NOTE 3—Sepharose™ CL-4B forms a discrete pellet upon centrifugation. For materials where centrifugation in a typical tabletop clinical centrifuge is insufficient to pellet the material following incubation with complement, a filtration step, with appropriate control, is required. The procedure presented in 10.1.2-10.2.5 is specifically designed for Sepharose™CL-4B, although other materials can be assayed in the same way. The main objective is to expose a known quantity of material to a minimum volume of 100 µL serum in a way that allows for the following: (*1*) exposure of the maximum surface area of the material to the serum and (*2*) easy separation of the material from the serum following exposure, for subsequent assay of remaining alternative complement activity against rabbit RBC. Any configuration of material/serum that meets these objects is suitable. For instance, for materials that can easily be weighed and transferred, round-bottomed upright 13 × 100 glass tubes may be more suitable than tilted glass centrifuge tubes needed for the Sepharose™ CL-4B in this example.

10.1.2 Sepharose™ CL-4B is prepared for assay by washing five times with Ca buffer. Put a defined quantity of stock, suspended Sepharose™ CL-4B (enough for the proposed assay, such as 1.4 mL) previously determined to yield a set volume after five washes in a plastic centrifuge tube, on ice. Ten mL of 4°C Ca buffer is added by pipette, squirted down with sufficient force such that the Sepharose™ is well-suspended. The suspension is centrifuged at 4°C, 1000 × g, for 10 min, then the supernatant is drawn out to near the pellet. This is reapeated four times more. At the last wash, all remaining fluid is carefully removed from above the pellet, and a volume of Ca buffer equal to the total volume of the pellet is added (in this case, 800 µL). The suspension of Sepharose™ CL-4B is held in chipped ice until needed.

10.1.3 Mix well the 1:2 dilution of Sepharose™ suspension and place an appropriate volume into the bottom of 15-mL disposable glass centrifuge tubes such that the desired volume of particles is delivered to each tube (for example, 25µL, 12.5µL, 6.25µL, and 3.17-µL beads). (The pointed tips of the glass centrifuge tubes allow for more complete removal of liquid without disturbing or aspirating any pelleted Sepharose™ CL-4B following centrifugation, even when very small volumes are present.) All material (M) tubes, no material (NM) 37°C control tubes, and Ice (I) control tubes receive 1.0-mL Ca buffer, necessary to ensure that none of the Sepharose™ CL-4B in the small volumes dispensed remains adhered to the sides of the tubes. All tubes are centrifuged at 4°C, 1 000 x g, for 10 min, and all fluid cafefully removed from the top of the pellets. The tubes are capped and left at 4°C in chipped ice until serum is added.

10.2 *Incubation of Material with Undiluted C4(-)GPS*:

10.2.1 200 µL of absorbed, undiluted C4(-)GPS at 4°C is placed onto the pelletted Sepharose™ CL–4B at the bottom of the cold 15-mL conical disposable glass centrifuge M tubes in chipped ice, into the NM tubes, and into the Ice control tubes. In this example, 200 µL is needed instead of the minimum 100 µL of serum in order to provide sufficient volume after dilution for assay in Procedure B. Sufficient surface area is provided by tilting the tubes (see 10.2.3) for good exposure of material to serum. This might not be the case if 13 × 100 glass tubes were being used for another type of material (in which case replicates would be needed to provide sufficient pooled exposed serum).

NOTE 4—A minimum assay requires three tubes, labeled M (material), NM (no material, 37°C control), and I (Ice, maximal complement activity control). For statistical evaluation, a minimum of three replicate tubes/condition should be used. In addition, other controls besides I and NM could include a comparison to another alternative-pathway activating material (with the same unit surface area or other appropriate measurable

parameter), a positive reagent control for alternative pathway complement activation (such as zymosan or inulin), or a negative reagent control for alternative pathway complement activation (such as heat-aggregated human gamma globulin (HAGG) which activates complement by the classical pathway), or a combination thereof. (See Section 12 for a more detailed discussion of how to use Zymosan and HAGG as controls.)

10.2.2 Using a 100-µL pippeter and tips with ends that have been cut back to provide larger apertures (to allow for rapid mixing of the Sepharose™ CL-4B particles), quickly and carefully mix the volumes by pipetting the suspension up and down 4X/tube, starting with I, then going to NM, then going on up the range of concentrations of M tubes from lowest to highest concentration.

10.2.3 All tubes except the I tubes are immediately capped, placed in a rack, tilted at an angle (10–15°), and place in a 37°C circulating water bath such that the tips are submerged but the tops are supported on the rim of the bath. In this fashion, the Sepharose™ CL-4B does not form a compact pellet away from the serum, but instead settles onto a large surface area for maximum contact with the serum. The I tubes are left in chipped ice.

10.2.4 At the end of 1-h incubation, put the M and NM tubes back on ice. Immediately dilute the 100 µL of C4(-)GPS in each tube to its optimal assay concentration (see Section 9) by addition of 4°C BBS-G-EGTA/Mg [Mg buffer]. (For instance, if to be used for assay at a 1:3 dilution, 400 µL of Mg buffer is added to all tubes.) Using separate Pasteur glass pipets, the contents of each tube are slowly drawn up and back down into the tube, insuring that the serum and buffer are mixed well.

10.2.5 The tubes are then centrifuged at 4°C, 1000 × g, for 10 min. 400 µL, drawn by placing a pipet tip at mid height in the liquid, is transferred to another labeled glass tube in chipped ice. Serum should be assayed within 1 h for complement activity (Section 11).

10.3 *Fibers or Solid Pieces*—Assay for whole complement activation by solid fibers or pieces of material is similar to that detailed in 10.2 for Sepharose™ CL-4B, except that a defined amount of fiber or material (milligram amounts, just enough to be fully covered by a minimum of 0.1 mL of serum) is put first into room temperature 13 × 100-mm glass tubes. Then 0.1 mL of 4°C serum is added to the M, NM, and I tubes. Immediately place the M and NM tubes in a 37°C water bath while the I tube is put in chipped ice. At the end of 1 h, take the M and NM tubes out of the 37°C water bath and put them also in chipped ice.

10.4 *Assay Size and Conditions Tested*:

10.4.1 The preceding general format can be used to test differing amounts of material to yield dose-response curves, the same quantity exposed to 37°C for various periods of time (time course), or to compare C′ activation by various materials.

10.4.2 It is recommended that the total number of test samples to be assayed not exceed a number requiring a final assay size of around 100 tubes.

11. Procedure B—Assay of Serum for Alternative Pathway Complement Activation in Procedure A

11.1 Procedure B is used to assay serum which has previously been exposed to a material (Procedure A) for possible activation of complement by the alternative pathway. Alternative pathway complement activation in Procedure A (in which complement components are depleted from the serum) is detected in Procedure B as decreased lysis of rabbit RBCs in Mg buffer when the material-exposed serum M is compared to a no-material 37°C control NM.

11.2 All conditions are assayed in triplicate, using three 13 × 100 disposable glass test tubes per condition. Tubes are numbered in advance. Conditions include “total lysis,” “no complement (RBC only)” (no C′), tests, and “no RBC (serum only)” (a color-control.) All reagents, tubes, and manipulations are done ice-cold, with tubes held in a rack in chipped ice or in an ice-slurry.

11.3 Rabbit RBC previously washed in Mg buffer and adjusted to 2×10^8/mL (Section 7) are added to all tubes except, “no RBC (serum color)” tubes (0.05 mL/tube). “No RBC (serum color)” tubes get 0.05mL 4°C Mg buffer.

11.4 To the “no C (RBC only)” tubes, 0.05 mL of Mg buffer is added. The total lysis tubes receive 0.05 mL of water. Then, add 0.05 mL from each of the test or control condition tubes from the material exposure step (Section 10), which are being held on ice and are already diluted to the optimal serum concentration for assay on rabbit cells (Section 9), to each of the three correspondingly marked tubes containing rabbit RBC.

11.5 The tubes are then treated as detailed in Sections 9.5-9.8.

12. Necessary Controls

12.1 Controls needed in each Procedure B assay (Section 11) are: “total lysis,” ”no complement (RBC only)” (background lysis from RBC in buffer, in the absence of serum), “no RBCs (serum color)”, and “no material” (“37°C only” or NM, with no material exposure in part A). In addition, at some point sheep cells should be tested in parallel to rabbit cells to demonstrate lack of nonspecific lysis by the standard serum lot in use, under Procedure B conditions in Mg buffer.

12.2 Controls other than “no material” used in Procedure A (Section 10) may include: (*1*) a known positive material such as Sepharose™ CL-4B (Section 10), (*2*) a known negative material (for which the glass of a test tube without test material can suffice), (*3*) a positive reagent (such as Zymosan or Inulin), and (*4*) a negative reagent (such as heat-aggregated human gamma globulin HAGG)**(6)**.

12.3 Zymosan A (a yeast cell wall component from *Saccharomyces cerevisiae*) may be used as a standard positive control for activation of the alternative pathway. It is stored at 4°C. Ten milligrams of Zymosan is added to 1.0 mL of Ca buffer, then serially diluted to give 1/10 and 1/100 dilutions. Ten microlitres of each of these three solutions will deliver 100, 10, and 1 µg of Zymosan into 100 µL of serum per glass tube for comparison to test materials. The other tubes should receive 10 µL of Mg buffer containing no Zymosan. These amounts of Zymosan should produce complete, partial, and little alternative pathway depletion of complement activity (though percent depletion may vary depending on the lot of serum). Zymosan centrifuges into a tight pellet from which the overlying serum is easily separated following procedure A. Zymosan suspensions should be prepared fresh for each experiment. When final dilution of the serum is done for assay in Procedure B,

compensation with buffer should be made for the 10-µL additional volume.

12.4 Preparation of HAGG: Human γ-globulins (from Cohn Fraction II, III) are stored at 4°C. 10 mg is added to 1.0–mL room temperature BBS in a glass tube. After gentle mixing and setting at room temperature for 10 min, there should be a clear liquid with no precipitate. This preparation is then placed in a 63°C water bath for 20 min. Following incubation, the solution is placed on ice, then aliquoted in 0.1-mL volumes and frozen at −70°C, providing a stock solution of 100 µg/10 µL. For use as a negative control substance (not activating the alternative pathway, only the classical pathway) the heat aggregated human gamma globulin (HAGG) is thawed and 10-µL volumes are added to serum in parallel with tested materials. Although this amount of HAGG will deplete complement by the classical pathway (which can be observed using the whole-complement assay of Practice F 1984, no depletion should occur in C4(-)GPS.

13. Report and Data Analysis

13.1 The 37°C serum-only control (tube NM) should have less rabbit cell lysis than a serum-only control kept in chipped ice (I) because some complement proteins are temperature sensitive (see example in Table 1). If a filtration step is needed to completely remove the material after Procedure A, controlled for by a second ice tube—in which the same procedure was carried out on serum without material, I_2—significant reduction in complement activity from I may also be seen in I_2.

13.2 In Procedure B, each tube from Procedure A is assayed in triplicate to allow detection of significant differences ($p \leq 0.05$ by an appropriate statistical test such as ANOVA) between Procedure A tubes. Also, especially when only small differences are present between conditions, each condition in Procedure A may need to be set up as a minimum of three replicates. Thus, in order to obtain statistical significance, materials may need to be tested in triplicate in Procedure A, with each of the three exposure tubes being assayed in triplicate in Procedure B.

13.3 Significant reduction of hemolytic activity in the M tubes as compared to the NM tubes in Procedure B denotes complement activation (with depletion of complement components) by test materials in Procedure A by the alternative pathway.

13.4 Differences in hemolysis are considered significant at $p \leq 0.05$, as calculated by an appropriate statistical test (such as ANOVA.) Results may be presented as a bar graph displaying each condition as a mean and standard deviation.

14. Keywords

14.1 alternative pathway; biocompatibility; blood compatibility; complement testing; materials; medical devices

APPENDIX

(Nonmandatory Information)

X1. RATIONALE

X1.1 The primary purpose of this practice is to describe a simple, inexpensive functional test to screen serum for alternative pathway complement activation by blood-contacting solid materials. Practice F 1984 provides guidance for testing solid materials for whole complement activation, but does not discriminate between the classical or alternative pathways. This practice tests specifically for the complement activation pathway preferred by blood-contacting solid biomaterials, the alternative pathway **(7)**. Confirmation that a material activates complement in vitro by the alternative pathway suggests that in vivo complement activation by this material is a potential concern. Although serum is not the same as the plasma to which a material is exposed in vivo, blood collected with an anticoagulant to give plasma should not be used in this practice because anticoagulants may interfere with complement activation.

X1.2 C4 genetically deficient guinea pig serum can not support complement activation by the classical pathway. Thus, if a material depletes complement activity in C4(-) GPS, it must have occurred via activation of the alternative complement pathway. Rabbit RBC in buffer containing EGTA and excess Mg^{++} are used to assay C4(-)GPS exposed to a material for C′ depletion, because rabbit cells, unlike sheep cells used in classical pathway assays, lack membrane-bound alternative pathway inhibitor molecules **(7)**. Also, the classical pathway requires Ca^{++} and Mg^{++}, whereas the alternative pathway can proceed when only Mg^{++} is present. Therefore, using buffer with EGTA to preferentially bind Ca^{++} in the presence of excess Mg^{++} also ensures that the rabbit cell lysis is only due to alternative pathway activation. (Adding EGTA plus excess $Mg^{++}$to whole human serum in Procedure A can not be used to exclude the classical pathway in this step because this gives substantially different results than when the reagents are used in a buffer-diluted serum.)

X1.3 It is well-recognized that complement activation is an important defense mechanism of the host **(8, 9)**. However, complement activation by material components of blood-contacting devices may be harmful to the host or potentially contribute to failure of devices **(6, 10,11)**. Although complementology has been an active research area for many years, the importance of chronic local complement activation on material/device function and actual impact on patient health is not completely understood.

X1.4 Many investigators have developed tests for whole complement functional activity, depletion of specific complement components, or generation of specific complement split products **(1, 2,4,12,13,14)**. Other validated test methods may

be substituted for the functional alternative pathway complement-depletion assay described in this practice. If immunologic assays for individual complement pathway components are used, consideration should be given as to whether component depletion is by nonspecific binding to a material or by pathway activation.

X1.5 The procedure as presented is intended as a routine screening procedure. It is not represented as being the most sensitive or the most specific procedure for assessing the complement-activation potential of all materials in all applications. Further analysis of materials found to activate the alternative pathway might involve the use of human serum and immunoassays that detect reduction of complement pathway components or elaboration of complement pathway-specific split products (such as Bb and C4d). Complement component-depleted human serum is unsuitable because, (in contrast to genetically deficient C4(-)GPS), depletion by immunoadsorption may result in the following: (*1*) incomplete reduction in the level of classical pathway activity and (*2*) direct activation of the alternative pathway resulting in a reduction in alternative pathway complement titer.

X1.6 Substances that are weak alternative pathway activators might still generate enough relevant split products (C3a, C5a, and so forth) to cause a local inflammatory response in vivo, that may not be reflected by significant changes in whole complement activity. The results obtained with this procedure may be used in conjunction with the results of other tests in assessing the blood compatibility of the test material.

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