Designation: F2567 – 06 (Reapproved 2010)

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

This standard is issued under the fixed designation F2567; 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.

1. Scope

1.1 This practice provides a protocol for rapid, *in vitro* functional screening for classical 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* classical 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 human serum [HS], using 0.1 mL serum per 13×100 mm disposable glass test tube. Procedure B describes assaying the exposed serum for significant functional classical pathway complement depletion (decrease in amount of C4) as compared to control serum samples not exposed to the material. The endpoint in Procedure B is lysis of sheep red blood cells (RBC) coated with antibody (hemolysin).

1.4 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 F748 may provide guidance for the selection of appropriate methods for testing materials for other aspects of biocompatibility. Practice F1984 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. Practice F2065 provides guidance for testing solid materials for alternative pathway complement activation in serum.

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.7 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F1984 Practice for Testing for Whole Complement Activation in Serum by Solid Materials

F2065 Practice for Testing for Alternative Pathway Complement Activation in Serum by Solid Materials

2.2 *Other Document:*

ISO 10993-4 Biological Evaluation of Medical Devices, Part 4: Selection of Tests for Interactions with Blood[3]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *water*—distilled, endotoxin-free.

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

3.2.5 *C'*—complement

3.2.6 *C4*—the fourth component of complement

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

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

3.2.9 *HAGG*—heat aggregated gamma globulin

[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.

Current edition approved Sept. 1, 2010. Published November 2010. Originally approved in 2006. Last previous edition approved in 2006 as F2567 – 06. DOI: 10.1520/F2567-06R10.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

3.2.10 *HS*—human serum

3.2.11 *I*—"ice" control tube with serum but no material, kept on ice

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

3.2.13 *NM*—tube containing serum but no material

3.2.14 *RBC*—red blood cell(s)

4. Summary of Practice

4.1 This practice is based on a method published by Gaither et al, 1974 (**1**).[4]

4.2 Solid material specimens are exposed to a standard lot of human C' (specially-prepared, commercial human serum [HS]) under defined conditions, in parallel with appropriate controls (Procedure A). If the classical complement pathway is activated by the material, C4 will be depleted from the serum. Exposed serum is then tested for remaining C4 functional activity. An appropriate dilution of the HS, which by itself is too dilute to lyse sensitized sheep RBC, is added to hemolysin-coated sheep RBC in the presence of C4(-)GPS in which all complement components save the missing C4 are present in excess (Procedure B). Hemolysis in Procedure B provides a quantitative measure of the C4 remaining in HS exposed to test material in Procedure A. Depletion of hemolysis indicates specific classical pathway activation in the human serum caused by exposure to the test material.

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 complement directly by the alternative pathway, or indirectly because of antigen-bound antibodies (as with immuno-adsorption columns) by the classical pathway. This practice is useful as a simple, inexpensive, function-based screening method for determining complement activation by solid materials *in vitro* by the classical pathway.

5.2 This practice is composed of two parts. In part A (Section 11), HS is exposed to a solid material. If complement activation occurs by the classical pathway, C4 will be depleted. Activation by the alternative pathway will not deplete C4. In part B (Section 12), C4 activity remaining in the serum after exposure to the test material is assayed by diluting the serum below the concentration needed to lyse antibody-coated sheep RBC on its own, then adding the diluted HS to C4(-)GPS (which is itself at a dilution where all complement components are in excess save the missing C4). Lacking C4, the C4(-)GPS does not lyse the antibody-coated sheep RBC unless C4 is present in the added HS. The proportion of lysis remaining in the material-exposed HS sample versus the 37°C control HS sample (which was not exposed to the test material) indicates the amount of C4 present in the HS, loss of which correlates with classical pathway activation.

5.3 This function-based *in vitro* test method for classical pathway complement activation 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. It is designed to be used in conjunction with Practice F1984 for function-based whole complement activation screening, and Practice F2065 for function-based alternative pathway activation screening.

5.4 Assessment of *in vitro* classical 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, such as immunoassays for specific complement components (including C4) and their split products in human serum (see X1.3 and X1.4).

5.5 If nonspecific binding of certain complement components, including C4, to the materials occurs in part A of this practice, a false positive for classical pathway activation will be observed in step B. Classical pathway complement activation by the test material may be confirmed by demonstrating an absence of C4 bound to the material following removal of the serum, and/or production of complement split-products such as C4d in the serum (as determined by immunoassay). Although immunoassay could be done in place of this screening procedure, determination of C4d production alone may not be functionally significant. This practice does not detect trivial amounts of classical activation unable to affect functional lysis of sensitized RBC.

6. Preparation of Buffers

6.1 *Buffers* are prepared according to established protocols (**2**, **3**). "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 (**4**) license for purchase. The use of other buffer systems (such as TRIS) is permissible if they have been demonstrated not to activate complement (**5**).

6.2 *5X Stock BBS* (barbital-buffered saline) is prepared by adding 20.75 g NaCl plus 2.545 g 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 $MgCl \cdot 6\ H_2O$ into 100 mL water), and a 0.3 M solution of $CaCl_2$ (4.41 g $CaCl_2 \cdot 2\ H_2O$ into 100 mL water), and combining the two solutions 1:1 (v:v). These solutions are stable for one month at 4°C.

6.4 *Ca Buffer (BBS-GM 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 0.25 mL Metals Solution, brought 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. Ca buffer contains both Mg^{++} and Ca^{++}, which allows both classical and alternative pathway complement activation to occur.

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

6.5 *BBS-G Working Solution* is prepared the same way, but omitting addition of the metals solution.

6.6 *10× Stock EDTA* (0.1 M disodium dihydrate EDTA) is prepared by adding 7.44 g disodium EDTA. · 2 H_2O to about 160 mL 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 *BBS-G-EDTA* (to be used in preparing RBC before being washed out with Ca buffer) is prepared by adding 10 mL of stock 10X EDTA to 90 mL of BBS-G in a volumetric flask.

7. Preparation of Sheep RBC

7.1 Commercially-obtained sheep RBC preserved in Alsever's solution are stored at 4°C. The sheep cells are discarded after eight weeks or when the supernatant liquid from the second wash contains hemoglobin by visual inspection (as lots of RBCs age, they increase in sensitivity to complement lysis in parallel with increased spontaneous lysis).

NOTE 1—All centrifugations are at 4°C. Except when indicated, all reagents, tubes, and cell preparations are kept on ice or in an ice slurry. In subsequent sections where the word "cold" is used, that denotes tubes in ice or sitting in an ice slurry.

7.2 Five mL of sheep 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 BBS-G-EDTA.

7.4 The cells are centrifuged, the supernatant discarded (first wash), and the pellet resuspended in 10 mL of cold BBS-GM (Ca Buffer). This step is repeated twice more for a total of three washes.

7.5 Adjust cell concentration by counting with a hemocytometer, and prepare 10 mL of 3.0×10^8 cells/mL in cold 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 Serum should be absorbed with sheep RBC in order to remove any naturally-occurring anti-sheep hemolytic antibodies. The procedure is as follows.

8.2 Commercially-available HS and C4(-)GPS are stored at –70°C. Both sera should be absorbed separately.

8.3 Serum is thawed on ice or reconstituted (if lyophilized) with ice-cold (4°C) water.

8.4 All manipulations are done on ice, with ice-cold 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.

8.5 Sheep RBC are washed as in Section 7, centrifuged 1000× g for 10 min at 4°C, and the cold supernatant removed down to the pellet. The cold, packed RBC are then added to the serum in a glass tube on ice, 0.1 mL/2.5 mL serum. The cells are mixed thoroughly into the serum slowly inverting the capped tube several times. The cell/serum mixture is 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 fresh glass tube on ice.

8.6 The procedure in 8.5 is repeated twice, exposing the cold serum to three fresh preparations of cold cells.

8.7 The absorbed HS is stored in 0.5 to 1.0 mL aliquots (convenient for one experiment), in pre-chilled, cold snap-cap microfuge tubes immediately placed at –70°C until used. Aliquots should be thawed cold, on ice (not allowed to warm higher than 4°C), used on the day of thawing, and not re-frozen.

9. Determination of Optimal Hemolysin Concentration

9.1 Determination of optimal hemolysin concentration is necessary in order to conserve expensive reagents and to avoid prozone effects. Commercial rabbit anti-sheep RBC serum (hemolysin) is thawed (or, if lyophilized, reconstituted with distilled endotoxin-free water), heat-inactivated at 56°C for 30 min to inactivate the rabbit complement, aliquoted in convenient volumes, and stored at –70°C until used.

9.2 To cold 13×100 mm disposable glass tubes, placed in a rack in an ice-slurry, 50 µL of washed sheep RBC at 3×10^8 cells/mL is added directly to the bottom of each tube. If statistical evaluation of the results is desired, three replicate tubes for each condition should be used. Otherwise, duplicates or even single dilution tubes are sufficient. One set of three replicate tubes receives only 50 µL of cold Ca buffer/tube ("no RBC" control, for complement color).

9.3 To the RBC-containing tubes, one set of three tubes gets 0.35 mL cold distilled H_2O/tube ("total lysis" control), another gets 50 µL mL Ca buffer ("no hemolysin" control), and the other sets get 50 µL mL each of 1:2 serial dilutions of hemolysin ("tests"). Dilutions between 1:200 to 1:25 600 antibody are recommended, with two sets of 3 tubes each for 1:200. The "no RBC" control receives 50 µL of additional BBS-GM instead of hemolysin. All tubes except "total lysis" controls should each contain at this point a total 0.1 mL.

9.4 Each tube is quickly mixed by gentle shaking to resuspend cells, the rack is placed in a 37°C water bath, incubated 10 min, then returned to the ice-slurry.

9.5 One of the two 3-tube sets of 1:200 hemolysin gets 0.1 mL of cold Ca buffer ("no-complement" control). All other tubes besides the "total lysis" control set get 0.1 mL cold absorbed HS (C') diluted 1:100 or 1:200.

NOTE 2—For a particular lot of human serum, a 1:100 or 1:200 dilution should provide sufficient complement activity. Also, percent lysis in the "no-hemolysin" (complement only) control should not exceed 10 %. If lysis with the 1:100 dilution of complement exceeds 10 %, use 1:200. If the "no-hemolysin" control still exceeds 10 %, a different lot of serum will need to be tested.

9.6 All tubes except the "total lysis" controls receive an additional 0.1 mL of Ca buffer. All tubes except for the "total lysis" control should at this point each contain a total 0.3 mL. Tubes are shaken manually to suspend cells, and the rack is placed in a 37°C water bath for 1 h. Each fifteen minutes into the incubation the rack is shaken to keep cells in suspension.

9.7 At the end of 1 h of 37°C incubation, the rack is placed in the ice-slurry. All tubes except the "total lysis" controls receive 0.1 mL BBS-G-EDTA. All tubes at this point should each contain 0.4 mL. The cold tubes are then centrifuged at 1000× g for 10 min at 4°C. Being careful not to disturb the pellets, 0.2 mL of the supernatant from each tube is transferred to a microtiter well plate and absorbance at 405 nm is measured.

9.8 "% Lysis" is calculated for each test and control tube by subtracting from the 405 nm absorbance the "no RBC" control (mean of the three replicate tubes), dividing by "total lysis" control value (mean of the three replicate tubes), and multiplying by 100.

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

9.9 Final "% Lysis" for each condition is expressed as Mean ±1 Standard Deviation of the three % Lysis values for each three-replicate set.

9.10 A titration curve is obtained by plotting the inverse of the hemolysin concentration on the abscissa versus % specific lysis on the ordinate. Twice the concentration of hemolysin that is just on the plateau of the titration curve is used for sensitizing RBC for subsequent assays ("optimal hemolysin concentration"). Hemolysin is freshly diluted from stock each day.

10. Titration of Human Complement and C4(-)GPS to Determine Optimal Dilutions

10.1 If statistical evaluation of results is desired, all conditions should be assayed in triplicate, using three 13×100 disposable glass test tubes per condition. Otherwise, single or duplicate tubes are sufficient. Tubes are numbered in advance. Conditions include "total lysis," "no complement" (no C'), "no RBC" (complement color control, at highest concentration of serum used—for both HS alone and C4(-)GPS alone), and "tests" (C4(-)GPS alone at a single dilution, or different dilutions of HS in the absence of C4(-)GPS, or different dilutions of HS each added to the single dilution of C4(-)GPS), with and without hemolysin). All reagents, tubes, and manipulations are done ice-cold, with tubes held in a rack in an ice slurry.

10.2 Ca buffer-washed sheep RBC are added to all tubes except "no RBC" tubes (50 µL/tube of a 3.0×10^8 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. In place of the sheep RBC, the "no RBC" tubes get 50 µL cold Ca buffer. The "total lysis" tubes receive 0.35 mL water onto the 50 µL of cells.

10.3 Tubes that are to contain sensitized RBC receive 50 µL cold Hemolysin at optimal concentration (see Section 9), while tubes to contain unsensitized RBC receive 50 µL cold Ca buffer. Each tube is quickly mixed by gentle shaking to resuspend cells. The rack containing the tubes is placed in a 37°C water bath, incubated 10 min, then returned to the ice-slurry.

10.4 Cold HS and cold C4(-)GPS are diluted in cold Ca buffer to the desired concentrations (with minimal agitation). It is recommended to test the HS initially at 1:2000 to 1:40 000, alone and in combination with 1:50 C4(-)GPS. Diluted HS serum (or same volume of Ca buffer) is added directly to the bottom of each appropriate test tube as a 0.1 mL volume. Diluted C4(-)GPS (or same volume of Ca Buffer) is added directly to the bottom of each appropriate test tube as a 0.1 mL volume. "RBC only" tubes get 0.2 mL of Ca buffer containing no serum. All tubes except "total lysis" should at this point each contain a total 0.3 mL volume.

10.5 Tubes are shaken manually to suspend cells, then the rack is incubated in a 37°C water bath for 1 h, and intermittently shaken (each fifteen minutes) to keep cells in suspension.

10.6 At the end of 1 h, the rack is placed on ice. All tubes receive 0.1 mL of BBS-G-EDTA (to chelate calcium ions, and together with the 4°C cold prevent any further complement activation). The cold tubes are then centrifuged at 1000× g for 10 min at 4°C. Without disturbing the pellets, 0.2 mL of the supernatant from each tube is transferred to a microtiter well plate for measuring absorbance at 405 nm.

10.7 % Lysis is calculated for each test and control tube as in 9.8, except the appropriate "no RBC" serum control is used for the appropriate "test" (with proportional color being subtracted for the HS dilutions).

10.8 Final % Lysis for each condition is expressed as Mean ±1 Standard Deviation of the three % Lysis values for each three-replicate set.

10.9 The optimal dilution of a particular lot of HS can now be determined. This is the dilution at which HS exposed to a material will be assayed for its ability to lyse sensitized sheep RBC in subsequent experiments of Procedure B. The optimal dilution is defined as that in which % Lysis for sheep RBC by HS alone is ≤10 % while HS plus C4(-)GPS produces a % Lysis of at least 40 % but not greater than 90 % (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 1:5000 added as a 0.1 mL volume in the assay (see Fig. 1).

10.10 An assay similar to that in 10.9 for determining optimal HS dilution should also be done for C4(-)GPS. In this case, the HS concentration is kept constant at its optimal dilution (such as 1:5000) while the C4(-)GPS concentration is varied (recommended from 1:50 to 1:150). At the optimal dilution of C4(-)GPS, lysis from the C4(-)GPS alone should be 10 % or below, while addition of the optimal dilution of HS (such as 1:5000) should produce lysis between 40 and 90 %. A typical optimal dilution for a lot of absorbed C4(-)GPS is 1:50 (see Fig. 2).

11. Procedure A—Exposure of Material to Human Serum

11.1 *Preparation of Material:*

11.1.1 The main objective is to expose a known quantity of material to a minimum volume (100 µL) of undiluted 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

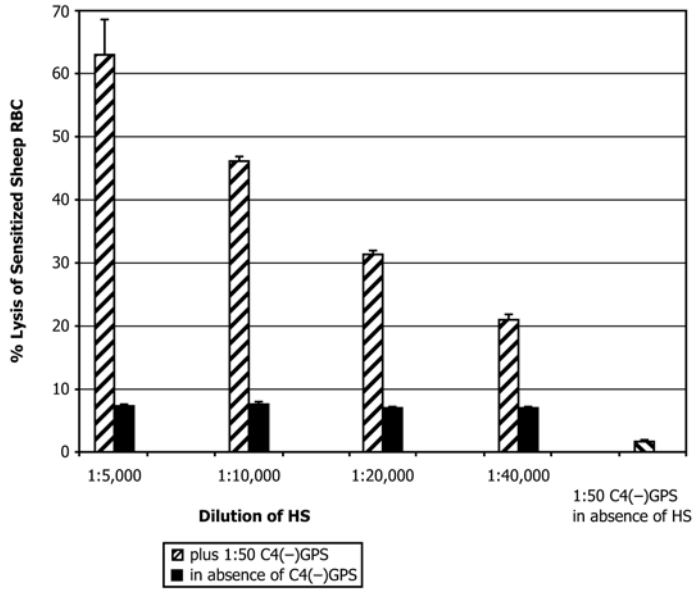

FIG. 1 Example of Determining Optimal Concentration of Human Complement

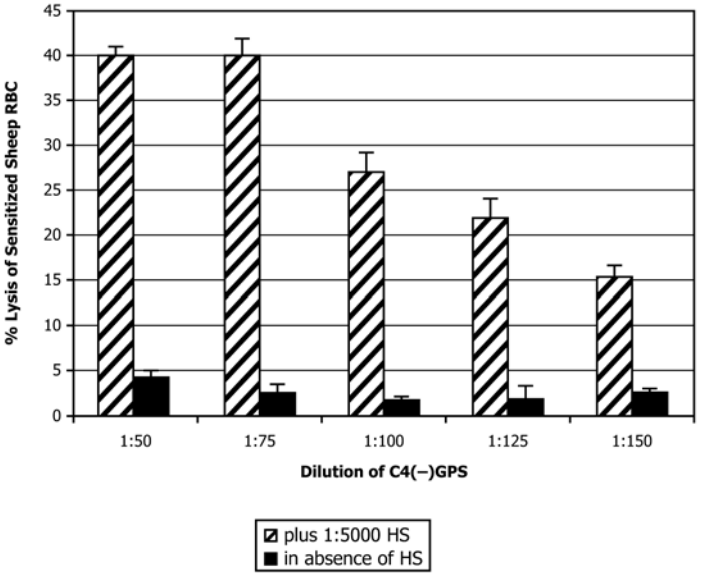

FIG. 2 Example of Determining Optimal Concentration of C4(-)GPS

exposure, for subsequent assay of remaining classical complement activity. Any configuration of material/serum that meets these objectives is suitable.

11.1.2 If particulate material needs to be washed before exposure to serum, cold Ca buffer should be used since it does not add residues that might activate complement. Also, since clumping could change the surface area exposed to the serum by particulates, the physical behavior of particles (particularly nano-particles) should be documented both after any washing step and during subsequent exposure to serum.

11.2 *Incubation of Material with Undiluted HS:*

11.2.1 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 material (with same unit surface area or other appropriate measurable parameter), and/or a negative reagent control for classical pathway complement activation (such as zymosan) and/or a positive reagent control for classical pathway complement activation (such as heat-aggregated human gamma globulin, HAGG, which activates complement by the classical pathway). (See Section 13 for a more detailed discussion of how to use Zymosan and HAGG as controls.) For materials where centrifugation in a typical table-top refrigerated centrifuge is insufficient to pellet the material following incubation with complement, a filtration step, with appropriate control, is also required.

11.2.2 Material is added to undiluted HS in 13×100 mm glass tubes on ice. Depending on the nature of the material, it may need to be suspended in a small volume of Ca buffer (up to 100 µL). In that case, an equal volume of Ca buffer (vehicle) needs to be added to NM and 37°C control tubes. Materials and serum may need to be mixed by very gentle agitation (careful to not have material adhere to the sides of the glass tubes). All tubes except the ice control tubes are placed in a 37°C water bath.

11.2.3 At the end of 1 h incubation, the rack of tubes are taken from the 37°C water bath and put into an ice slurry. Immediately, the 100 µL of HS in each tube is diluted to either its optimal assay concentration or a step towards the final dilution (see Section 10) by addition of cold Ca buffer. (For instance, if to be used in Procedure B at a 1:5000 dilution, 9.9 mL of Ca buffer could be added to all tubes, as an initial 1:100 dilution; later to be followed by an additional 1:50 dilution.) Each tube is capped and gently inverted several times, insuring that the serum and buffer are mixed well.

11.2.4 The tubes are centrifuged at 4°C, 1000× g, for 10 min. A volume is then drawn from mid height in the liquid, and transferred to another labeled glass tube on ice for the final dilution (such as 0.2 mL into 9.8 mL for the final 1:50 dilution). The cold serum should be assayed within one hour for complement activity (Section 12).

11.3 *Fibers or Solid Pieces:*

11.3.1 Assay for whole complement activation by solid fibers or pieces of material is similar to that for particulate material, except that a defined amount of fiber or material (milligram amounts, just enough to be fully covered by a minimum of 0.1 mL serum) is put first into room temperature 13×100 mm glass tubes. Then 0.1 mL of cold serum is added to the bottom of M, NM, and I tubes. Immediately the M and NM tubes are placed in a 37°C water bath while the I tube is put on ice. At the end of 1 h, the M and NM tubes are taken out of the 37°C water bath and also put on ice. In some cases where the material is large, a 0.1 mL volume of serum might be placed directly upon the material, placed in a 100 % humidity incubator (to prevent evaporation) at 37°C in a covered vessel (to prevent condensation from falling into the serum), and retrieved following the incubation period (1 to 2 h).

11.4 *Assay Size and Conditions Tested:*

11.4.1 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.

11.4.2 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.

12. Procedure B—Assay of Human Serum Exposed to Material in Procedure A, for its Ability to Reconstitute C4 Classical Pathway Complement Activity of C4(-)GPS

12.1 *Overview of Assay:*

12.1.1 Procedure B is used to assay human serum which has previously been exposed to a material (Procedure A) for activation by the material of the classical complement pathway. Classical pathway complement activation in Procedure A (in which the complement component C4 is depleted from the serum) is detected in Procedure B as decreased lysis of sensitized sheep RBC.

12.1.2 All conditions are assayed in triplicate unless otherwise indicated, using three 13×100 disposable glass test tubes per condition. Tubes are numbered in advance. Conditions include "total lysis," "no complement" (no C'), "no RBC" (serum only, a color-control) and "tests" (six tubes/condition—three to contain sensitized RBC and three with RBC but no hemolysin). A typical experiment (see Fig. 3) might therefore include: *(1)* 3 tubes for "total lysis;" *(2)* 3 tubes for "no complement" (RBC only, no sera); *(3)* 3 tubes for "no RBC, HS" (HS color control); *(4)* 3 tubes for "no RBC, C4(-)GPS" (C4(-)GPS color control); *(5)* 3 tubes for "no RBC, HS + C4(-)GPS" (HS + C4(-)GPS color control); and *(6)* 6 tubes each (3 of each set of 6 which will contain sensitized RBC and 3 with RBC but no Hemolysin) for the conditions "test,

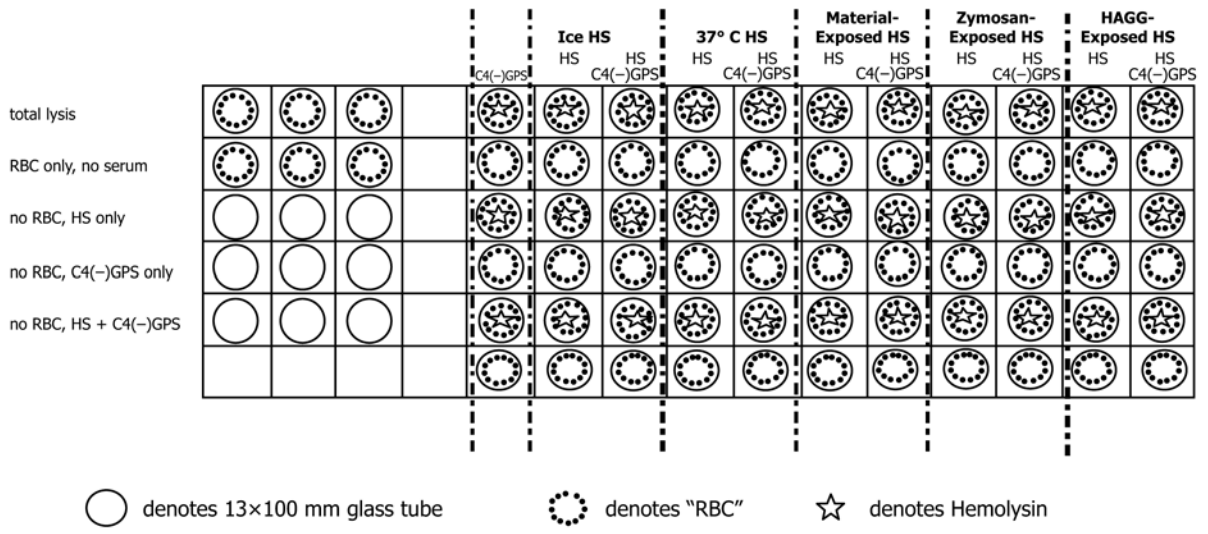

FIG. 3 Typical Matrix of Tubes in a 90-slot Wire Rack for a "Procedure B" Assay

C4(-)GPS only," "test, Ice HS," "test, Ice HS + C4(-)GPS," "test, 37°C HS," "test, 37°C HS + C4(-)GPS," "test, material-exposed HS," "test, material-exposed HS + C4(-)GPS," "test, negative control-exposed HS [such as to zymosan]," "test, negative control reagent-exposed HS + C4(-)GPS," "test, positive control reagent-exposed HS [such as to HAGG, "heat-aggregated human gamma globulin"]," and "test, positive control reagent-exposed HS + C4(-)GPS." All reagents, tubes, and manipulations are done ice-cold, with tubes held in a rack in ice or in an ice-slurry.

12.2 *Procedure:*

12.2.1 See Fig. 4 for a summary of steps to the procedure. These steps are discussed in detail in the following sections 12.2.2 – 12.2.6.

12.2.2 Sheep RBC previously washed in Ca buffer and adjusted to 3×10^8/mL (Section 7) are added directly to the bottom of all tubes except "no RBC" tubes (50 µL/tube). "No RBC" tubes get 50 µL cold Ca buffer.

12.2.3 The "total lysis" set of three tubes receives 0.35 mL of water. The tubes containing sheep RBC to be sensitized receive 50 µL cold Hemolysin at optimal concentration, while the control RBC tubes (non-sensitized) receive 50 µL cold Ca buffer. All tubes (except "total lysis") should at this point each contain 0.1 mL volume. Each tube is quickly mixed by gentle shaking to resuspend the cells, the rack containing the tubes is placed in a 37°C water bath, incubated 10 min, then returned to the ice-slurry.

12.2.4 The properly-diluted C4(-)GPS and HS samples, or corresponding volumes of Ca buffer, are then added to the appropriate tubes. First, 0.1 mL of cold C4(-)GPS diluted in cold Ca buffer to the optimal concentration (such as 1:50) or 0.1 mL cold Ca buffer is added directly to the bottom of each appropriate test tube. Then, each HS sample (previously exposed or not exposed to test material or control reagent; then diluted to the optimal concentration—such as 1:5000—and held on ice) or equal volume of Ca buffer is added directly to the bottom of each appropriate test tube as a 0.1 mL volume. "RBC only" tubes get 0.2 mL of Ca buffer containing no serum. All tubes besides "total lysis" should at this point each contain 0.3 mL volume.

1. 50 µL of cold Ca buffer or 1.5×10^7 sheep RBC are added to appropriate tubes.
2. "Total lysis" tubes receive 350 µL water.
3. All tubes other than "total lysis" receive 50 µL of Hemolysin or cold Ca buffer.
4. Incubation at 37°C for 10 min.
5. 100 µL of diluted C4(-)GPS or Ca buffer is added to appropriate tubes.
6. 100 µL of diluted HS test samples (previously exposed to material, reagent, 37°C alone, or 4°C alone) or Ca buffer are added to appropriate tubes.
7. Tubes are incubated at 37°C for 1 h, then put back into ice slurry.
8. 100 µL of BBS-G-EDTA is added to all tubes except for "total lysis."
9. Tubes are centrifuged, 0.2 mL of supernatants put into microtiter plate wells, and O.D. measured at 405 nm.

FIG. 4 Sequence of Steps for "Procedure B" Assay

12.2.5 Tubes are shaken manually to suspend cells. Then, the rack is incubated in a 37°C water bath for 1 h, and intermittently shaken (each fifteen minutes) to keep cells in suspension.

12.2.6 The tubes are then treated as detailed in 9.7 – 9.9, except when calculating the % Lysis for each test and control tube, the appropriate "no RBC" serum control is used for the appropriate "test" (with proportional color being subtracted for the HS dilutions).

13. Necessary Controls

13.1 Internal controls needed in each Procedure B assay (Section 11) are: "total lysis," "background lysis" (RBCs in only buffer, in the absence of serum), "serum color" (no RBCs), "37°C only" (no material exposure in part A), and "test on non-sensitized RBC" for each serum condition. In addition, controls other than "No Material" used in Procedure A (Section 10) may include: *(1)* a known positive material, *(2)* a known negative material (for which the glass of a test tube without test material can suffice), *(3)* a negative reagent (such as Zymosan), and *(4)* a positive reagent (such as heat aggregated human gamma globulin, HAGG) **(6)**.

13.2 Zymosan A (a yeast cell wall component from Saccharomyces cerevisiae) may be used as a standard negative control for activation of the classical pathway. It is stored at 2 to 8°C. Ten mg of Zymosan is added to 1.0 mL of Ca buffer, then serially diluted to give 1/10 and 1/100 dilutions. Ten µL 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 Ca buffer containing no Zymosan. These amounts of Zymosan should produce little to no depletion of C4 of the classical pathway (though they will produce large to modest depletion of alternative pathway components) (see Fig. 5). 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 should be made for the 10 µL additional volume.

13.3 Human y-globulins (from Cohn Fraction II, III) are stored at 2 to 8°C. One hundred 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 twenty minutes. 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 positive control substance that strongly depletes C4 (activating only the classical pathway, not the alternative pathway) the heat aggregated human gamma globulin (HAGG) is thawed and 10 µL volumes are added to serum in parallel with tested materials. This amount of HAGG will deplete complement by the classical pathway (see Fig. 6).

14. Report Section and Data Analysis

14.1 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)

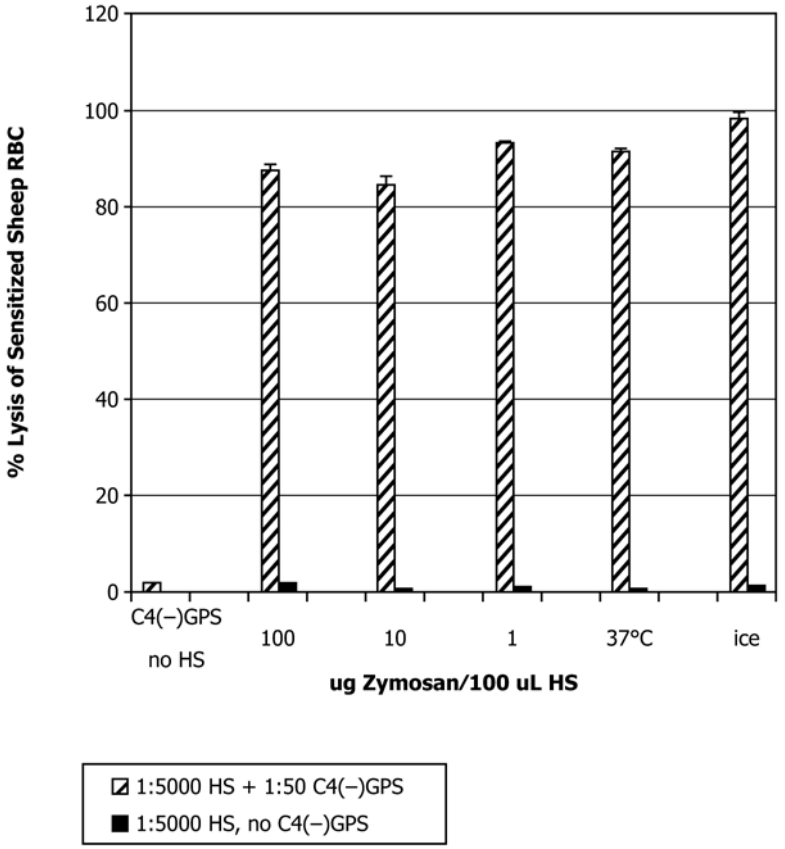

FIG. 5 Example of Negative Classical Pathway Control Reagent

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 material-exposure tubes being assayed in triplicate in Procedure B.

14.2 Significant depletion of control hemolytic activity observed in Procedure B by a human serum sample previously exposed in Procedure A to a material as compared to the 37°C human serum control denotes possible complement activation by test materials in Procedure A via the classical C pathway. A lack of significant difference of the exposed serum sample versus the 37°C serum sample says that C4 was not depleted, the classical complement pathway was not activated.

14.3 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 (see Fig. 1, Fig. 2, Fig. 5, and Fig. 6).

15. Keywords

15.1 biocompatibility; blood compatibility; classical pathway; complement testing; materials; medical devices

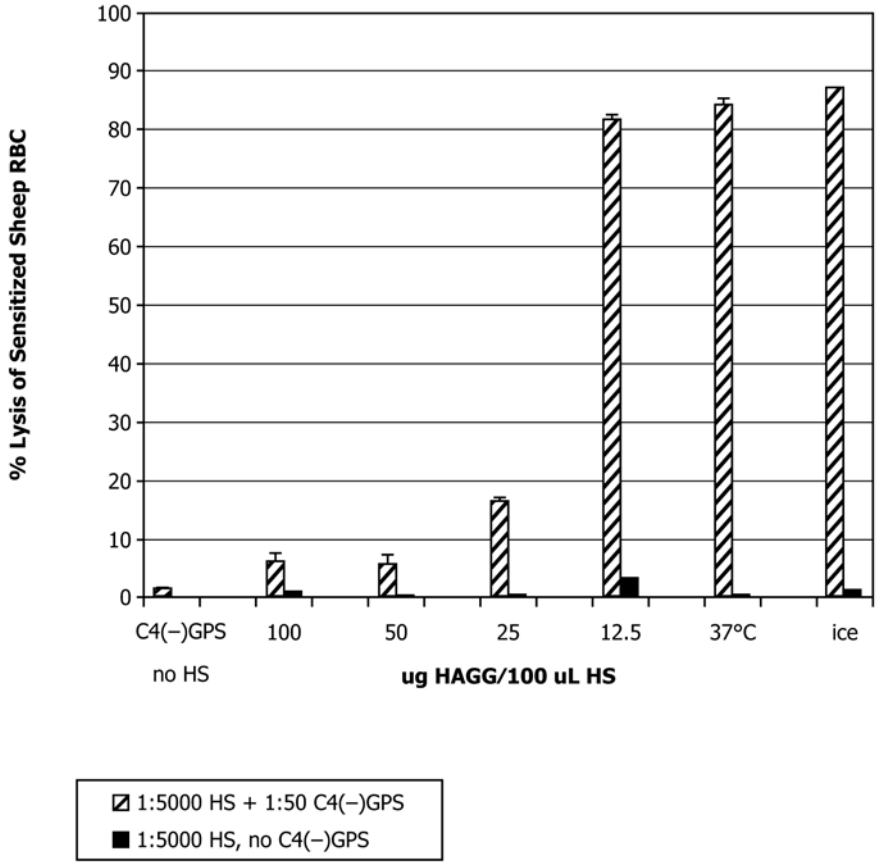

FIG. 6 Example of Positive Classical Pathway Control Reagent

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 classical pathway complement activation by blood-contacting solid materials. Whereas assays identifying levels of individual complement components or split products are also valuable in identifying complement-activating potential of materials, this screening procedure does not register a positive result unless actual complement function (lysis of sensitized cells) can be significantly affected. 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 standard because anticoagulants may interfere with complement activation.

X1.2 C4 genetically-deficient guinea pig serum cannot support complement activation by the classical pathway unless the missing C4 component is added. Thus, C4(-)GPS at a concentration where all the complement components except for the absent C4 are in excess can serve as a sensitive assay for detecting the presence of C4 in an added test serum when that test serum is at a concentration which by itself is incapable of causing lysis of sensitized RBC (**1**). Thus, if a material depletes the ability of human serum to restore C4 activity to C4(-)GPS, then complement activation may have occurred via activation of the classical complement pathway (or C4 was removed from the serum by being nonspecifically bound to the material). Materials found to not alter the C4 level by this screening procedure can be determined as not being activators of the classical pathway. A positive result using this screening procedure can be confirmed by assay for generation of complement split products (such as C4d) or by other means (**6**).

X1.3 It is well recognized that complement activation is an important host defense mechanism (**7, 8**). However, inappropriate complement activation by material components of blood-contacting devices may be harmful (**6, 9, 10**). Classical complement pathway activation normally requires the presence of antibodies (**11**), so blood-contacting medical devices which do not contain antigen-bound antibodies should be negative in this assay (**10**). Blood-contacting medical devices such as extracorporeal immuno-adsorption devices may involve classical C' activation by antigen-antibody complexes.

X1.4 Many investigators have developed tests for whole complement functional activity or immunoassay detection of specific complement components or split products (**2, 3, 5, 11, 12, 13**). Other validated test methods may be substituted for the functional classical pathway complement C4-depletion assay described here. The procedure as presented here, then, is intended as a routine screening procedure. It is not represented as being the most sensitive or the most specific procedure for assessing the classical pathway complement-activation potential of all materials in all applications.

X1.5 Substances that are weak classical pathway activators might still generate enough relevant split products (C3a, C5a, etc.) 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 should be used in conjunction with the results of other tests in assessing overall blood compatibility of the test material.

REFERENCES

(1) Gaither T. A., Alling, D. W., and Frank, M. M., "A New, One-Step Method for the Functional Assay of the Fourth Component (C4) of Human and Guinea Pig Complement," *The Journal of Immunology*, 113(2), 1974, pp. 574–583.

(2) Giclas, P. C., "Complement Tests," *Manual of Clinical Laboratory Immunology*, fifth edition, eds., N. R. Rose, E. C. de Macario, J. D. Folds, H. C. Lane, and R. M. Nakamura, ASM Press, 1997, pp. 181–186.

(3) Gee, A. P., "Molecular Titration of Components of the Classical Complement Pathway." *Methods in Enzymology*, Vol 93, Immunochemical Techniques, eds., J. J. Langone, H. V. Vunakis, Academic Press, 1983, pp. 339–375.

(4) United States Drug Enforcement Agency, Washington, DC.

(5) Lin, W. Q., White, Jr., K. L., "Complement Assays to Assess Immunotoxicity," *Methods in Immunotoxicology*, Vol I, eds., G. R. Burleson, J. H. Dean, and A. E. Munson, Wiley-Liss, 1995, pp. 357–375.

(6) Chenoweth, D. E., "Complement Activation Produced by Biomaterials," *Trans. Am. Soc. Artif. Intern. Organs*, 32, 1986, pp. 226–232.

(7) Sakamoto, M., Fujisawa, Y., and Nishioka, K., "Physiologic Role of the Complement System in Host Defense, Disease, and Malnutrition," *Nutrition* , 14, 1998, pp. 391–398.

(8) Law, S. K. A., Reid, K. B. M., *Complement*, second edition, Oxford University Press, 1995.

(9) Kazatchkine, M. D., Carreno, M. P., "Activation of the Complement System at the Interface Between Blood and Artificial Surfaces," *Biomaterials* , 9, 1998, pp. 30–36.

(10) Hakim, R. M., "Complement Activation by Biomaterials," *Cardiovasc. Pathol.*, 2, 1993, pp. 187S–197S.

(11) McLean, R. H., Welch, T. R., "Complement," *Handbook of Human Immunology*, eds., M. S. Leffell, A. D. Donnerberg, and N. R. Rose, CRC Press, 1997, pp. 267–318.

(12) Labarre, D., Montdargent, B., Carreno, M. -P., and Maillet, F., "Strategy for In Vitro Evaluation of the Interactions Between Biomaterials and Complement System," *J. Applied Biomat.*, 4, 1993, pp. 231-240.

(13) *Complement Methods and Protocols*, ed., B. P. Morgan, Humana Press, 2000.