Designation: F 1906 – 98 (Reapproved 2003)

Standard Practice for Evaluation of Immune Responses In Biocompatibility Testing Using ELISA Tests, Lymphocyte Proliferation, and Cell Migration[1]

This standard is issued under the fixed designation F 1906; 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 covers the introduction of a foreign substance into mammalian body that may induce the formation of an immune response. The immune response may lead to inadvertent tissue damage and be an undesirable event. In the standard protocols for biocompatibility testing, various studies in animals are done. These animals or their blood and tissues could be used to determine if immune responses have occurred and what types have occurred. At the current time, the immunologic testing in biocompatibility protocols is very limited. Techniques can be developed in the future which are simple, reliable, and sensitive.

1.2 It is the purpose of this practice to delineate some possible test methods. It must be remembered that these are protocols for use in biocompatibility testing, they are not diagnostic tests for evaluation of human conditions. Diagnostic tests for use on humans must go through evaluation at the regulatory agencies. The tests described here are clearly adaptable for use in humans and can be used for research purposes and provide data in clinical trials, but are not necessarily cleared for diagnostic purposes. This practice present selected methods. Other validated methods may be equally applicable.

1.3 The values stated in SI units are to be regarded as the standard.

1.4 *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]

F 619 Practice for Extraction of Medical Plastics

F 719 Practice for Testing Biomaterials in Rabbits for Primary Skin Irritation

F 720 Practice for Testing Guinea Pigs for Contact Allergens: Guinea Pig Maximization Test

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

F 763 Practice for Short-Term Screening of Implant Materials

F 981 Practice for Assessment of Compatibility of Biomaterials for Surgical Implants with Respect to Effect of Materials on Muscle and Bone

3. Summary of Practice

3.1 Immunologic testing is done using specimens from animals being tested according to the Practice F 748 matrix for irritation and sensitivity, or for implantation. Blood, organs, or tissues from the animals may be used. Blood or biopsies from patients in a clinical trial may also be used. Animals (rabbits or mice) are also immunized with various antigens in this practice. Humans may be immunized with an approved vaccine.

3.2 Immunologic testing is done using materials, known components of the materials, or extracts prepared according to Practice F 619. These materials, components, or extracts may be used for *in vivo* tests or for the *in vitro* tests.

[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 Nov. 1, 2003. Published December 2003. Originally approved in 1998. Last previous edition approved in 1998 as F 1906 – 98.

[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.

4. Significance and Use

4.1 This practice is to be used to help evaluate the biocompatibility of materials used in medical devices in terms of the immune response.

4.2 The appropriateness of the methods should be carefully considered since not all materials or applications need to be tested by this practice.

4.3 The testing suggestions in Practice F 748 and in the matrices of recommended tests issued by regulatory agencies may be considered before proceeding with these tests.

4.4 These tests require the use of blood. Procedures for obtaining whole blood or serum should follow the recommendations of the animal research committee of the institution responsible for the animals. In general serum and plasma behave the same in these tests, but it should be noted which was used.

4.5 *The Testing Protocols*—These will be divided into the two specific areas of humoral immunity and cell mediated immunity, and subdivided from there. The tests for the humoral immune responses will be based on solid phase immunoassays for use with enzyme linked immunoassays (ELISA) techniques.

4.6 *Abbreviations*:

4.6.1 *RPMI 1640*—Specific growth medium (Roswell Park Memorial Institute).

4.6.2 *FCS (FBS)*—Fetal Calf Serum (Fetal Bovine Serum).

4.6.3 *NCS*—Newborn Calf Serum.

4.6.4 *MTT*—(3-[4,5-Dimethylthiazol-2-yL]-2,5-diphenyltetrazolium bromide: Thiazolyl blue.

4.6.5 *LIF*—Leukocyte Migration Inhibition Factor.

4.6.6 *Ig*—Immunoglobulin.

4.6.7 *MIF*—Macrophage Migration Inhibition Factor.

4.6.8 *PEC*—Peritoneal Exudate Cells.

4.6.9 *PMNS (POLYS)*—Polymorphonuclear Leukocytes.

4.6.10 *PHA*—Phytohemagglutinin.

4.6.11 *ConA*—Conconavalin A.

4.6.12 *PBS*—Phosphate Buffered Saline.

4.6.13 *PBS/T*—Phosphate Buffered Saline with Tween 20.

4.6.14 *MW*—Molecular Weight.

4.6.15 *MED 199*—Medium 199, a specfic growth medium.

5. Tests for Production of Humoral Immune Responses

5.1 These tests are generally done with serum or plasma. The use of peritoneal or ascites fluid is also permissible.

5.1.1 *Response to Immunogenic Substances*—Proteins and most carbohydrates will adhere to polystyrene, especially when dissolved in buffer at pH 8 to 9. Other materials also usually will adhere. Solid materials may be used as the solid support substrate with appropriate controls for nonspecific binding.

5.1.2 The use of polystyrene 96-well microtiter plates is recommended. There are several manufacturers (for example, Costar, Falcon, Nunc) and they are available from standard supply houses (for example, Fisher, VWR, Thomas). No specific source is recommended, however the choice should be documented in the report.

5.1.2.1 The configuration of the testing protocol is up to the evaluator but control wells must be included in the matrix. It is recommended that eight wells receive no antigen and serve as reagent controls. A negative control using serum from matched animals not receiving the material should be used in at least four wells. If known positive antisera is available, this should be used in at least four wells. If quantitation is desired, standard solutions of at least three different concentrations should be run in triplicate and a buffer control run in triplicate. Each specimen to be tested should be run at two different dilutions and each in triplicate.

5.1.3 Dissolve the substance in an aqueous based solution. Adjust the pH to between 8.5 and 9.5 or as high as possible before it precipitates. A phosphate buffer is recommended. The final concentration of the substance should be 0.5 mg/mL. (Any extract prepared according to Practice F 619 may be used in this protocol. The pH should be adjusted to pH 8.5 to 9.5 where possible without precipitation of the extract.

5.1.4 Add 100 uL of this to each of the wells except the control wells. Incubate the plates in a humid environment at room temperature (18 to 22°C) or 37°C for 1 h, place in the cold (4 to 8°C) overnight. Wash the plates with phosphate buffered saline with Tween 20 (PBS/T) at least three times. Wash at least twice with coating buffer that is the PBS/T with a protein source such as 1 % gelatin, egg albumin, or serum. This blocks other combining sites on the plates and reduces the background. The plates may be used immediately or stored in the cold until used.

5.1.5 Add 100 uL of the appropriate serum samples or buffer control to the wells. The test serum samples should be run in at least two dilutions. It is recommended that eight wells receive only buffer and serve as an antigen-second antibody control. Incubate at room temperature or 37°C for 1 to 2 h. Wash well with PBS/T.

5.1.6 Add 100 uL of the appropriate antiserum to all of the wells. This second antibody should be labeled with an enzyme (horse radish peroxidase or alkaline phosphatase are recommended). These antisera can be purchased from scientific supply houses and should be used at the dilution recommended by the manufacturer. It is recommended that polyvalent antisera directed against IgG, IgM, IgA of the appropriate species of the animal be used and that IgE specific antisera be used separately. The plates should be incubated at room temperature or 37°C for 1 to 2 h. They should then be washed well with PBS/T.

5.1.7 Add 100 uL of the appropriate substrate to all wells. The substrate specific for the enzyme label and at the correct concentration should be used. This information should be supplied by the supplier of the antisera. Incubate at room temperature (usually in the dark) for the recommended time (usually 20 to 30 min). Read the optical density at the appropriate wave length for the substrate.

5.1.8 *Analysis of the Data*—The results of the optical density achieved with the test serum samples should be compared to the control samples. Significant elevations or depressions would be signified by values outside two standard deviations of the control. If known standards were included, the results can be expressed from comparison with the standard curve.

6. Response to Haptenic Substances

6.1 The immune response to haptens (low molecular weight materials) is similar to the immune response already described. However, this response will be dealt with separately here since there are substantial differences in testing methodologies.

6.1.1 The production of an immune response to low molecular weight degradation, wear, or elution products from materials is an important issue in biocompatibility. These low molecular weight substances may bind to host tissue or protein and become immunogenic. It is not apparent that determination of responses to haptenic materials can be determined using a simple modification of the procedure in Section 5. It is necessary to first coat the plates with a carrier to bind the hapten. For most haptens, albumin serves as an appropriate carrier.

6.2 Step one is to add 100uL of carrier (0.5mg/mL) bovine serum albumin is recommended) to the wells in the plate. Incubate for 1 to 2 h at room temperature or 37°C and then overnight in the cold (4-8°C). Wash the plates well with PBS/T.

6.3 Add 100 uL of the solution containing the hapten. PBS alone should be used as a negative control. Incubate for 1 to 2 h at room temperature. Wash well with the blocking wash (PBS/T containing gelatin).

6.4 Proceed as described in 5.1.5-5.1.7.

6.5 *Analysis of the Data*—The results of the optical density achieved with the test serum samples should be compared to the control samples. Significant elevations or depressions would be signified by values outside two standard deviations of the control. If known standards were included, the results can be expressed from comparison with the standard curve.

7. Stimulation of Immune Responses to Unrelated Antigens

7.1 It is now known that various substances, especially oils, gels, and colloidal suspensions, can stimulate the immune response to unrelated antigens. This is not necessarily a harmful situation, and may be beneficial. The ability of the material or extract to do this needs to be documented.

7.1.1 For this procedure animals should be given an unrelated antigen (such as bovine serum albumin) with and without the material. The antibody levels should be measured at 7 to 10 days, 21 days, and 8 weeks. The antibody levels should be measured using the techniques in 5.1.

7.1.2 *Protocol*:

7.1.2.1 Control animals receiving only the antigen,

7.1.2.2 Test animals receiving the antigen mixed with the material or extract, and

7.1.2.3 Test animals receiving the antigen in one site and the material or extract in another site.

7.1.3 The following controls are recommended but not required:

7.1.3.1 Animals receiving only saline and no material, extract, or antigen,

7.1.3.2 Animals receiving only the material or extract and not antigen, and

7.1.3.3 Animals receiving the antigen and a known adjuvant such as Complete Freunds's Adjuvant or Titer Max.

7.2 An alternative or additional procedure is to use sheep red blood cells (SRBC) as the unrelated antigen. The plaque forming assay can be used with minced spleen cells obtained four days after immunization with SRBC.

7.3 *Analysis of Results*—The antibody responses in groups 7.1.2.2 through 7.1.2.4 should be compared to the results of group 7.1.2.1 to detect enhancement or suppression.

8. Tests for Production of Cell Mediated Immune Responses

8.1 These tests require the use of living cells. These cells may be obtained from peripheral blood, peritoneal exudates, alveolar washes, or minced lymphoid organs. These tests are based on the stimulation of lymphocytes by antigen with the release of various substances (cytokines and interleukins) that affect other cells. The polyclonal T cell stimulants (mitogen) such as PHA or ConA serve as positive controls. It is essential for these tests that strict aseptic precautions be used. The use of antibiotics other than penicillin/streptomycin or gentamicin is not advised because their mode of action may interfere with the cell responses.

8.1.1 *Lymphocyte Transformation Tests*—This detects the production of a cytokine that stimulates other lymphocytes to divide. Thus one lymphocyte responding specifically to an antigen or nonspecifically to a mitrogen will be activated and stimulate other cells to divide, thus amplifying the response.

8.1.1.1 Isolate lymphocytes by differential sedimentation and suspend in growth medium (RPMI 1640 with 10 % fetal or newborn calf serum (FCS or NCS)) at a concentration of 10^6 cells/ mL.

8.1.2 Dispense 1 mL of lymphocyte suspension into test tubes for each of four tests. It is recommended that each be run in triplicate.

8.1.2.1 Cell control,

8.1.2.2 Cells plus mitogen (PHA or ConA),

8.1.2.3 Cells plus antigen (material or extract) at first concentration, and

8.1.2.4 Cells plus antigen (material or extract) at second concentration.

8.1.3 The tests should be incubated at 37°C in 5 % CO_2 for seven days. The response to mitogen peaks at four days, the response to antigen peaks at seven days. After seven days of incubation add tritiated thymidine or I-125 thymidine to the cultures. Incubate for 4 to 6 h. Collect the cells (usually by filter wash) and determine the uptake of radioisotope. MTT is being used increasingly as a simple dye marker for proliferation assays. Alamar Blue may also be used.

8.1.4 The use of antigen and mitogen together will detect immunosuppression.

8.1.5 *Data Analysis*—The uptake of radioisotope in the presence of antigen should be compared to that of the cell control. The use of the mitogen confirms the test system was working. However comparison of the response to antigen to the response to the mitogen is permissible.

8.2 *Leukocyte Migration Inhibition (LIF)*—This test is limited to human and rabbit peripheral blood. It does not work with peritoneal exudate cells and may not work with dog blood. Blood must be used within 24 h of drawing. The PMNS are the indicator cell.

8.2.1 *Preparation of Detection Plates*—Prepare 0.75 mg SeaKem agarose in 80 mL distilled water. Autoclave and cool to 45 to 55°C. Add 10 mL newborn calf serum or horse serum and 10 mL of Med 199 10X. Mix and pipette 5 mL into 60 × 15 mm petri dishes. Allow to solidify and leave at room temperature overnight. Place the dishes into a sealed plastic bag and store in the refrigerator until used. The shelf life is 2 to 3 months. Be sure they are stored upright and level.

8.2.2 *Preparation of Antigens*—Prepare suspension of antigens at the appropriate concentration. The use of PHA or ConA is recommended as a control. The cell control is saline or PBS. These may be stored in the refrigerator until use. Shelf life varies but should be several months. Assays should be done to check for purely and activity before using the test protocol.

8.2.3 *Test Procedure*—Obtain 10 mL of peripheral blood that is anticoagulated with sodium or lithium heparin. Mix the blood 2 volumes to 1 volume of 2 % 500 000 MW dextran in PBS or saline. Allow to settle at room temperature for 1 h. Draw off the top layer (buffy coat) and place in a centrifuge tube. Centrifuge at 400 g for 15 to 20 min. Discard the supernatant. Resuspend the cells in 100 uL of PBS.

8.2.4 Place 20 uL of control and antigen solutions in test tubes. Add 20 uL of cell suspension and incubate 15 min at room temperature. Cut wells approximately 2 mm in diameter and 3 to 4 mm apart in the agarose plate. A cork borer or immunodiffusion punch is recommended. Remove the agarose plug if it did not come out with the punch. The use of a 20 gage hypodermic needle is recommended.

8.2.4.1 Pipette 10 uL of the cell-solution mixtures into each of two wells cut into the agarose plate. Incubate overnight at 37°C with 5 % CO_2. Examine the plate with a microscope (inverted scope with 4X lens and 10X eyepiece is recommended) for migration of cells from the well. The amount of migration may determined by area of migration, maximum distance from the well, or estimated as none, very little, some, or good.

8.2.5 *Data Analysis*—Compare the cell migration to that of the control. The cell control should be good and if there is a cell mediated response, the migration from the wells containing cells with material or extract will be none to very little.

8.3 *Macrophage Migration Inhibition (MIF)*—(Cells isolated from the peritoneal cavity (best animals: mouse or hamster), lung (best animals: mouse, hamster, rabbit), or lymphoid organs (any animal) are used. The macrophage is the indicator cell.

8.3.1 *Direct Test*—Collect cells that include macrophages and lymphocytes from desired site.

8.3.1.1 Wash and resuspend to a dense concentration in medium (RPMI 1640 with 10 % FCS is the recommended medium).

8.3.1.2 Place the cell suspension in capillary tubes (use a syringe and needle to place into sealed tubes or draw up by capillary action into unsealed tubes and gently heat seal one end) Centrifuge at 400 g for 10 to 15 min. Cut the tube at the cell fluid interface.

8.3.1.3 Place the tube into culture wells (six well plates or 35X10 mm petri dishes) using vacuum grease to hold the tube onto the bottom of the dish.

8.3.1.4 Add the medium (same as used above) with and without material extract or mitogen and incubate overnight at 37°C in 5 % CO_2.

8.3.1.5 The recommended test materials are: medium only, medium plus material or extract, medium plus material or extract at a different concentration, and medium plus mitogen.

8.3.1.6 Measure the area of migration from the capillary tube or estimate none, very little, some, good. Compare the response to the material or extract to that of the control or mitogen.

8.3.2 *Indirect Test*—This takes advantage of the accessibility of PEC from the mouse and uses the lymphocytes from another test animal of the same or different species.

8.3.2.1 For this procedure, lymphocytes are obtained (usually peripheral blood) from the test animal. They are isolated and incubated with the material or extract in a manner similar to that in 8.3.1. At the end of 24 h incubation, the media is withdrawn. This medium is then used as one of the tests described in the direct test. Thus the macrophages are obtained from normal mice, prepared as above in capillary tubes and placed into dishes. The test preparations are medium alone, medium from the lymphocyte incubation, and medium with mitogen.

8.3.2.2 Measure the area of migration from the capillary tube or estimate none, very little, some, good.

8.3.3 *Data Analysis*—Compare the cell migration in the culture containing the medium from the lymphocyte stimulation with material or extract to that of the medium only control and the medium with mitogens (PHA or ConA). A decrease in cell migration compared to medium alone will indicate that a cell mediated response was stimulated.

9. Keywords

9.1 biocompatibility; cell migration; ELISA; haptens; immune response; lymphocyte proliferation

APPENDIXES

(Nonmandatory Information)

X1. RATIONALE

X1.1 The primary purpose of this practice is to describe methodologies to determine the propensity of materials to stimulate the immune response specifically or nonspecifically.

X1.2 It is well recognized that the immune response is an important defense mechanism of the host. The interaction of materials with host tissue might alter this response and it is important to understand what materials affect which parts of the immune system. The production of an immune response directed against components of the material (specific immunity) may be favorable to the host. On the other hand, in some circumstances such responses may be harmful.

X1.3 The nature of the immune response has been an active research area for many years. However, not many studies have been done with medical materials. Many investigators have developed procedures for doing immunological studies. This document is intended to delineate some methods for such testing. However, there are many validated test methods that could be substituted for the ones described here as long as their modifications for use with materials, components, or extracts are fully described.

X1.4 The interaction of the immunological system with materials will lead to the production of various responses. It is unknown at this time whether the immune responses which may be stimulated by the materials are favorable or unfavorable to the host. Immune response studies using medical materials, components, or extracts are important so that this information can be obtained.

X2. ADDITIONAL REFERENCES

Burleson, GR, Dean JH, and Munson AE., *Methods in Immunotoxicology*, Wiley-Liss, New York, 1995.

Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, and Strober W. *Current Protocols in Immunology*, N.Y., John Wiley, 1992 (appended frequently).

Price, CP, and Newman, DJ., *Principles and Practice of Immunoassay*, Stockton Press, NY, NY 1991.

Rose NR, and Friedman H., *Manual of Clinical Immunology*, Washington DC, American Society for Microbiology, 1992.

Wild, D., *The Immunoassay Handbook*, Stockton Press, 1994.