CELL CULTURE TEST METHODS

S. A. BROWN
editor

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CELL-CULTURE TEST METHODS

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Foreword

The symposium on Cell-Culture Test Methods was presented at Dearborn, Mich., 11 May 1982. The symposium was sponsored by ASTM Committee F-4 on Medical and Surgical Materials and Devices, S. A. Brown, University of California, Davis, Calif., presided as chairman and editor of the publication.

Related ASTM Publications

Medical Devices: Measurements, Quality Assurance and Standards, STP 810 (1983), 04-800000-54

Corrosion and Degradation of Implant Materials, STP 684 (1979), 04-684000-27

A Note of Appreciation to Reviewers

The quality of the papers that appear in this publication reflects not only the obvious efforts of the authors but also the unheralded, though essential, work of the reviewers. On behalf of ASTM we acknowledge with appreciation their dedication to high professional standards and their sacrifice of time and effort.

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Contents

Introduction

The ASTM Committee F-4 on Medical and Surgical Materials and Devices was organized in 1962 to develop standards on materials, test methods, and devices used in medicine and surgery. Since its inception, the committee has continued to address the question of the biological response of tissues to implanted materials and devices. While the actual definition of biocompatibility depends upon the nature of the implantation application, it has always been recognized that the biological response to a material plays a major role in the successful performance of the device made out of that material. Thus, the committee's efforts have focused on developing methods of testing for the biological response or biocompatibility of materials and incorporation of biocompatibility language in material standards.

During the early years of Committee F-4, the Biocompatibility Subcommittee was led and directed by Drs. Jonathan Cohen and Patrick Laing, both of whom were active clinicians and researchers in the field of orthopedics. The major product of their efforts was the development of a mutual trust and understanding between manufacturers, physicians, and academic researchers. This trust, which grew out of the educational interchange debate (heated at times) and familiarity with each others' problems, bore fruit in 1972 with the publication of the first standard for biocompatibility testing, ASTM Practice for Assessment of Compatibility of Metallic Materials on Tissue (F 361). This standard, which was based on the work of Drs. Cohen and Laing, outlines a comparative soft tissue and bone implant model, using various implantation times, control materials, and several animal species to assay for the biological response in situations similar, but not identical, to those encountered by orthopedic implants. Its flavor is orthopedic, as was the flavor of Committee F-4 at the time of its development. Six years later, in 1978, a second biocompatibility test method was approved by Committee F-4. ASTM Practice for Assessment of Compatibility of Nonporous Polymeric Materials for Surgical Implants with Regards to Effect of Materials on Tissue (F 469) was similar in scope and nature to ASTM F 361 with the exception that it was a method for assaying the long-term response of tissue to nonporous polymeric materials. Again, the nature of the method was related to long-term orthopedic applications.

In the mid-seventies, under the initiative of Drs. Jonathan Black and Stanley Brown, the activities of the biocompatibility section expanded beyond consideration of long-term implantation of materials in contact with

muscle and bone, to encompass the questions of other types of materials and devices and the questions of testing of new materials and biological assays for screening materials and devices on a lot-to-lot basis. Our efforts were directed toward the question of how to test materials for biologic response when the intended end use applications were short- or long-term contact with different types of human tissues. In some instances we took documents from the United States Pharmacopia (USP) and modified them for application to medical devices. In 1979 ASTM Practice for Extraction of Medical Plastics (F 619) was published. In 1981 two short-term in vivo screening methods were approved by the Society: ASTM Practice for Testing Biomaterials in Rabbits for Primary Skin Irritation (F 719) and ASTM Practice for Testing Guinea Pigs for Contact Allergens: Guinea Pig Maximization Test (F 720). An additional five documents appeared for the first time in the 1982 edition of the *ASTM Book of Standards*. These included an ASTM Practice for Evaluating Extracts by Intracutaneous Injection in the Rabbit (F 749), Practice for Evaluating Material Extracts by Systemic Injection in the Mouse (F 750), Practice for Assessment of the Hemolytic Properties of Materials (F 756), and Practice for Short-Term Screening of Implant Materials (F 763). The fifth document was ASTM Practice for Selection of Generic Biologic Test Methods for Materials and Devices (F 748).

This document (F 748) addressed the issue of what types of tests should be used to demonstrate sufficient safety of materials for particular end use applications. The matrix approach is used similar to that used by Health Industries Manufacturers Association/Pharmaceutical Manufacturers Association (HIMA/PMA) and the British Standards Institution in which end use applications were broken down according to length of time, and types of tissue contact were listed in rows, and particular test methods were listed in columns permitting indication of what types of tests were considered sufficient for particular levels of end use application. A common theme in all of the applications was that the first stage of biocompatibility screening would be some sort of acute in vitro cell-culture toxicity test for identifying materials with toxic extractable moieties.

The subject of cell-culture toxicity has, as with the other methods of biological testing, been one of active debate over the past six to eight years. While there are many industries and commercial laboratories that are currently using cell-culture toxicity methods for screening and testing materials, there is no real consensus as to which methods should be used. There are several ways of conducting a cell-culture test (for example, direct contact, agar overlay, and so forth), and there are a number of different cell types that may be used in any of these methods. During the years of discussing standardization of cell-culture testing, it became apparent that different laboratories were using different methods for good reasons. The nature of the technique of cell-culture testing per se and the nature of the product being tested resulted in the selection of a particular method and a particular cell

line. Many task force meetings seemed to repeat themselves on the subject of "who was doing what and why." At the Nov. 1981 meeting of ASTM Committee F-4, it was decided that the way to resolve the conflicts was to organize a symposium at which laboratories could present their methods and rationales for use of methods, as they pertain to their particular product. The scope of the meeting was laid forth in the letter sent to speakers as an invitation to participate.

> The biocompatibility section of ASTM Committee F-4 on Medical and Surgical Materials and Devices has been struggling for several years with the task of writing standardized methods for cell culture testing of implant materials. It has become clear that there are a number of labs that are using different methods, different cell lines and are doing the testing for different purposes. The approach we would like to take is to develop standards for several methods and to develop recommendations as to when or for what reason a particular test should be used. In order to sort out the differences and to develop standards and recommendations, we are organizing a one-day meeting to which the major labs performing these tests are to be invited to present their methods and rationales for use.
>
> We would like you or one of your technical people with knowledge of the details of cell culture testing to make a presentation and to participate in a workshop on this subject. The presentation should discuss the details of what you are doing (type of test, cell line, etc.) and for what level of testing (initial screen, lot-to-lot, etc.). We would like to know what works for you and why. As part of this we ask that you or your representative also write a paper addressing these issues, which we can use for the workshop, subsequent standards development, and which will also be included in an ASTM publication of the meeting and workshop.

The one-day symposium was organized with presentations being given in the morning and the first part of the afternoon. The second part of the afternoon was devoted to a 2-h open discussion workshop to review and discuss the papers that we had presented, as well as to discuss the role of ASTM Committee F-4 on the development of cell-culture standard testing methods.

The papers presented in this volume are by individuals from laboratories using cell-culture test methods for biomaterial testing and screening. The papers represent, in many instances, commercial input and commercial interest. Their intent is not to say which method is correct, but rather to state what method works for the particular company with its particular products. The papers are organized with the first series discussing specific types of methods that are being used and their particular end use applications, followed by several papers that addressed the most critical question of the correlation between in vitro cell toxicity test results and in vivo performance of materials. Two papers address specific end use application and discuss using radioactive tracers that permit more quantitative assessment of the cellular response to materials or extracts. The final paper discusses the availability and standardization of the cells that are actually used for cell-culture test-

ing. Finally, the proceedings from the workshop are presented in the back of this volume. The text and the workshop has received minor editing for clarity, but essentially represents a free discussion of the issues being addressed. The workshop is organized into four parts, the first three addressing specific questions put to the audience by the Chair: (1) What should Committee F-4 be doing about cell-culture test standardization? (2) Should we develop standard charts or methods for ranking cell reactions? (3) How do we address the problem of standard reference materials for cell-culture testing? (4) And a question and answer period where questions that had been written to specific speakers during the morning session were answered and discussed.

The consensus that came from the workshop discussions was that the role of Committee F-4 was to develop documents that could be referred to as "benchmark" standards. While these might not be the specific methods being used on a day-to-day basis by all laboratories, they would be methods that could be used for validating and comparing different methods. The adopting of methods by Committee F-4 was not to be interpreted as an endorsement of anyone's particular method, but rather the development of specific methods that could be standardized and used.

The mood of the biocompatibility section and the subsequent task force meetings was that with this frame of mind we could move forward in developing standardized test methods. Perhaps as a direct result of this symposium the biocompatibility section completed development of its first cell-culture method, which was passed to society ballot at the Nov. 1982 meeting of Committee F-4. It is anticipated that the committee will develop several methods for cell-culture cytotoxicity testing and screening materials over the next few years. With standardized methods, we will be able to move forward in developing a better understanding of the applicability of particular methods, and of the accuracy and reliability of in vitro methods predicting in vivo performance.

As editor and chairman, I would like to thank Thomas Nickel of Travenol Laboratories and Thomas Walker of the Canadian Standards Association for their assistance in organizing the symposium.

Stanley A. Brown
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symposium chairman and editor.

Linda M. Smith[1]

Direct-Contact Cell-Culture Method

REFERENCE: Smith, L. M., **"Direct-Contact Cell-Culture Method,"** *Cell-Culture Test Methods, ASTM STP 810*, S. A. Brown, Ed., American Society for Testing and Materials, 1983, pp. 5–11.

ABSTRACT: A direct-contact cell-culture test method has been developed to provide routine lot-to-lot biological assurance as a part of the quality assurance program during the manufacture and distribution of medical materials and sterile-packaged human-implant devices. It is particularly useful for detecting trace ethylene oxide or catalyst residuals. The method has also been used routinely to provide a first biocompatibility evaluation of proposed new materials or formulation.

Specimens are placed in direct contact with human fibroblastic cells grown to a confluent monolayer. The results are evaluated mircroscopically after 24 h incubation, using appropriate positive and negative controls. An alteration in cellular morphology is considered a cytopathic effect (CPE). Implant materials and devices are approved by the quality assurance department only if they produce no CPE in this test.

This test method has been used continuously in the lot-to-lot quality assurance program at Dow Corning's Medical Products Plant for more than ten years and has provided a fast, reliable, highly sensitive, and inexpensive method for evaluation of biocompatibility.

KEY WORDS: tissue culture, direct contact, biocompatibility, cytopathic effect, medical devices, cell culture, cytotoxicity

Cell culture can be considered an extremely practical and sensitive method of investigating acute biocompatibility for the prediction of toxicity potential [*1*].[2] In our laboratory at Dow Corning in the early 1970s, the use of a direct-contact cell-culture method was initiated for the evaluation of implantable medical devices on a lot-to-lot basis. Over the more than ten years that this method has been used routinely, we have developed a great deal of confidence in its reliability and reproducibility. The use of cultured human diploid cells in a rapid, cost-efficient in vitro test system has provided an effective alternative to animal implant testing. This paper will outline the detailed method of how the test is performed currently in our laboratory, reflect on some of the developmental work that has determined why it is done the way it is done, and, finally, discuss the usefulness of this test in our daily operations.

[1] Quality assurance biology laboratory supervisor, Dow Corning Corp., Midland, MI 48640.

[2] The italic numbers in brackets refer to the list of references appended to this paper.

Method

The direct-contact test-method [*2,3*] currently uses WI-38 human embryonic lung cells (Flow Laboratories, McLean, Va.) and HR-218 human foreskin cells (HEM Research, Rockville, Md.). Two cell lines are used routinely so that a backup is always available in case of problems with one line. Other human cells have been used after qualification to ensure that their sensitivity is equivalent to the previously mentioned lines. All cells are received and propagated in 150-cm^2 flasks that are maintained in a 5% carbon dioxide humidified atmosphere at a temperature of 37 ± 2°C. Cells for biocompatibility testing are seeded from the larger flasks into 35 × 10-mm cell-culture wells and usually reach monolayer within three to four days. Seeding is done by a routine trypsinization procedure. This entails rinsing of the cell sheet with Hank's balanced salt solution (modified) without magnesium and calcium (Flow Laboratories, Rockville, Md.), cell dispersion using 0.5% trypsin at 37°C, centrifugation to harvest the cells, centrifugation to wash the cells, and, finally, seeding by the addition of five to seven drops of cell suspension to the cell-culture wells containing fresh medium. Planting of additional 150-cm^2 flasks for inventory can be also done at this point.

When the cells in the test wells are grown to a confluent monolayer, as confirmed microscopically, the medium is aspirated and replaced with a minimal amount (1.5 to 2.0 mL) of fresh medium. Minimum essential medium with Earle's salts, 10% fetal bovine serum, and 5 mg/500 mL L-glutamine (Gibco Laboratories, Grand Island, N.Y.) is used routinely. Earle's basal medium (BME) can be also used. Specimens to be tested are gently but firmly placed directly on the monolayer of cells, and the wells are incubated 24 h at 37°C and 5% carbon dioxide. Each group of specimens must include a positive and a negative control. A good, reliable positive control is a freshly cut surface of clean, sterilized, latex rubber. The negative control is a Dow Corning® brand MDX4-4515 silicone elastomer cured 6 min at 155°C and post cured 2 h at 250°C. This negative control is a product that has a long history of biocompatibility based on a large amount of historical data.

After 24 ± 1 h incubation, each cell well is examined at ×10 magnification for any change in cellular morphology. Any alteration in morphology, ranging from focal clumping and granulation of the cells to complete destruction of the cell sheet, is considered a cytopathic effect (CPE). Figures 1 and 2 show WI-38 cells with no cellular change (Fig. 1) and with a cytopathic effect (Fig. 2), after 24 h of contact with a specimen. Material being tested is reported as producing either "no CPE" (no change in the cell morphology) or "CPE" (any alteration in cell morphology). An alternative grading system from one to five may be also used, in which one is the least amount of cellular change, and five is the greatest amount. A specimen that has produced a CPE can be retested using two specimens of the material. If neither of the second series produces a reaction, the test is reported as "no CPE" (retest was required).

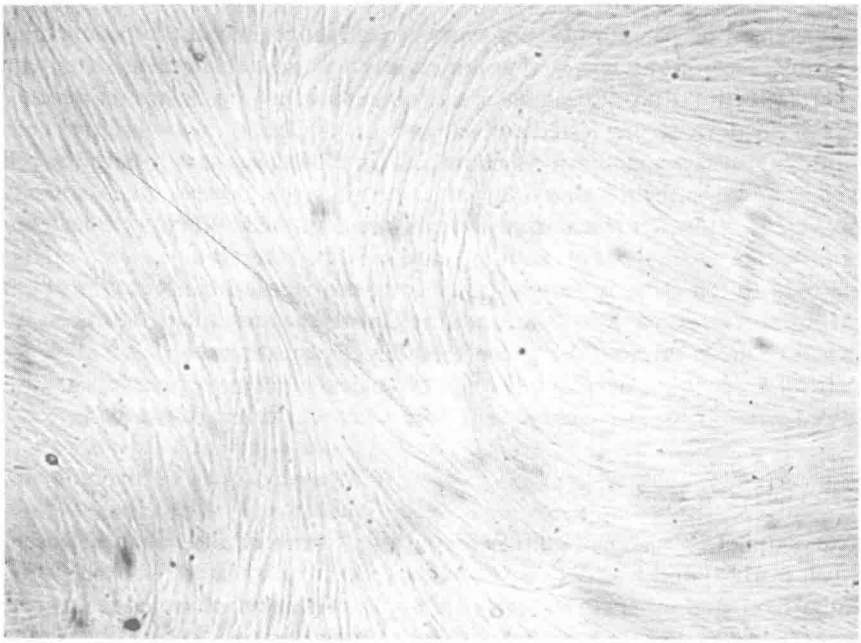

FIG. 1—*WI-38 human embryonic lung cells* (×*10 magnification*) *showing no cytopathic effect.*

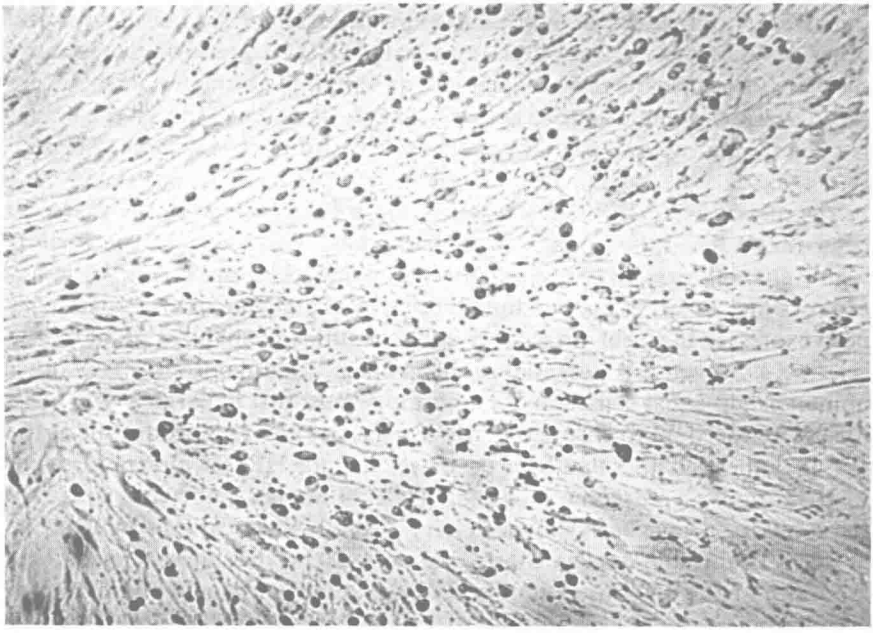

FIG. 2—*WI-38 human embryonic lung cells* (×*10 magnification*) *showing a cytopathic effect.*

Specimen preparation is very important. Specimens must be as clean as possible, free of any bacterial contamination or particulate debris that can interfere with the test. Specimen size is not critical as long as it is representative of the device being tested and enough space is left in the well so that cells adjoining the specimen can be examined. Small devices that fit into the cell well are used in their entirety. Specimens from larger devices are aseptically prepared so that representative surfaces are obtained. When a device contains two or more types of material, care must be taken that each material is exposed to the cells. In testing liquids, 10-mm sterile fiberglass filter pads (Millipore, Bedford, Mass.) are saturated with the specimen, the excess allowed to drain off, and the pad placed directly on the cell.

In all procedures, normal safe microbiological laboratory practices are followed strictly. Aseptic technique is used whenever cells are handled.

Developmental Work

Our direct-contact cell-culture method was designed after that of Rosenbluth et al [*4*] and was developed at Dow Corning by E. A. Abbott, G. W. Robertson, and others in the early 1970s [*5*]. A number of varied test procedures and parameters were evaluated in our laboratory before the direct-contact technique was deemed to be the method of choice. Chief among the other procedures evaluated were many variations of the agar/agarose overlay method. It was determined that this method was a poor choice because of the inability of the agar or agarose to allow diffusion of organosilicones and other nonaqueous or fat-soluble materials [*6*]. This greatly decreased the sensitivity because the test is dependent on the ability of materials to diffuse through water-soluble agar to the cell surface. By comparison, in the direct-contact method all the specimen components are directly available to the cell layer.

The principle cell line that has been used routinely through the years has been the WI-38 human embryonic lung cells. This cell line is the most highly characterized normal human-cell population known and is the standard by which all other human diploid cell strains are compared. Concern has been expressed in recent years concerning the future availability of the WI-38 cell line; however, this potential problem has not yet manifested. Any other human-cell line that is used in our laboratory must first undergo a rigid qualification procedure to ensure that it is as sensitive and as reproducible as the WI-38. Other cell lines that have been used include HR-218 human foreskin, MRC-5 human embryonic lung, and IMR-90 human embryonic lung. All these cell lines have been shown to have virtually identical sensitivity as the WI-38 cells through duplicate testing of over 400 production specimens. Our laboratory has always used diploid cell populations of human origin for biocompatibility testing. As opposed to cells of other animals or of malignant origin, these human cells contain a normal diploid chromosome count, do