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QUANTITATIVE CELL CULTURE BIOCOMPATIBILITY TESTING OF MEDICAL DEVICES AND CORRELATION TO ANIMAL TESTS

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ABSTRACT

The biocompatibility of a wide variety of biomaterials was quantitatively assessed, in a physiologically normal environment, as to cytotoxicity induced in WI-38 cells by cell culture medium extracts. Materials tested included PVC plastic, rubber, silicone rubber, polyethylene, polypropylene, acetal, polyurethane, Teflon®, nylon, epoxy, and polystyrene. Cell culture test results were correlated to U.S.P. animal tests. Potential test artifacts, lead, barium, cadmium, and endotoxin were tested for cytotoxicity in WI-38 cells.

Cell culture methods yielded more positive tests, particularly rubber, PVC plastic and silicone rubber compounds, than observed in U.S.P. animal tests. Positivity in animal tests did not correlate quantitatively to cytotoxic titers in cell culture. Discrepancies between cell culture tests and animal tests, specifically rubber compounds, were attributable, in some instances, to differentials in elution efficiency between saline, cottonseed oil, and complete MEM cell culture medium. In other instances, particularly PVC plastics, differences between cell culture and animal test results were due to an inherent difference in the two indicator systems to respond to specific toxic moieties.

I. INTRODUCTION

An array of *in vivo* and *in vitro* biocompatibility tests (1-25), for the appraisal of medical devices, has been evolved with the presumptive goal of predicting and preventing adverse

reactions during clinical application in humans. Previous test results have been difficult to correlate and interpret because of the nonquantitative endpoint parameters inherent to many tests, the lack of statistically adequate numbers and generic variety of test specimens, wide biologic variations in indicator systems, and diverse physical/biochemical ambience employed in each test system.

To this end, we have attempted to quantify the biocompatibility of specific formulations of biomaterial specimens in a human (WI-38) cell culture system (26) and have attempted to plot the correlation to U.S.P. animal tests (27). The cell culture test system was designed to encompass physiologically normal biochemical and physical environments in order that insight might be gained into the short-term bioavailability of leachable toxic moieties which could be pertinent to clinical biomedical device usage.

II. MATERIALS AND METHODS

A. Cell Cultures

WI-38 cells (28) were received from American Type Culture Collection and were propagated in 32 oz prescription bottles with growth medium consisting of Minimum Essential Medium (Eagle) with Earle's salts (MEM), 10% fetal bovine serum (FBS) (BBL, Division of BioQuest) (Table 1), 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 mcg/ml), Fungizone (2.5 mcg/ml), and Gentamicin (5 mcg/ml) (the total mixture referred to as complete MEM medium). All cell cultures were maintained in a humidified atmosphere (90% relative humidity) with 5-7% carbon dioxide at a temperature of 37°C. All suspensions were prepared with the aid of 0.25% trypsin solutions and were counted in a haemocytometer. Cells for cytotoxicity scoring were placed in 60 mm polystyrene cell culture dishes (Falcon Plastics) at 250,000 cells per dish and usually reached monolayer

TABLE 1

Chemical Characterization of Fetal Bovine Serum[a]

Specific gravity	1.018
Total protein	4.1 g%
Albumin	2.0 g%
Globulin	2.1 g%
Blood urea nitrogen	14.0 mg%
Total lipids	109.0 mg%
Total bilirubin	0.4 mg%
Hemoglobin	12.2 mg%

[a]data provided by BBL, Division of BioQuest

in 5-7 days. Alternatively, cells for cytotoxicity titrations were plated in Microtest II tissue culture plates (6 mm wells) (Falcon Plastics) at 20,000 cells per well and normally achieved monolayer in 5 days. WI-38 cells were not used beyond the 35th passage.

The cell cultures were free of mycoplasma, as determined by aerobic and anaerobic culture (29), at the beginning and at the termination of the study.

B. WI-38 Cell Culture MEM Elution Test

Test materials were cut into pieces not to exceed 20 mm in any dimension, or specimens made up of particles less than 20 mm in cross-sectional dimension were tested as received. None of the test specimens were sterile at the time of test nor were specimens aseptically handled. Materials were placed into (150 ml) borosilicate glass bottles containing MEM + 10% fetal bovine serum with antibiotics (as described under cell cultures) (hereafter referred to as complete MEM medium) at a ratio of 1 g test material to 5 ml of complete MEM medium. Bottles containing test specimens and complete MEM medium were incubated (stationary) at

37°C for 24 hours. After the 24-hour elution period, the mixtures were centrifuged at 500 x g and supernates were decanted for testing.

Two modes of testing were employed: cytotoxicity scoring and cytotoxicity titration. To determine the cytotoxicity score, 5 ml of undiluted test eluate was placed into each of two 60 mm cell culture dishes containing monolayers of WI-38 cells from which complete MEM medium had been aspirated. The plates containing test eluates were held at 37°C in a humidified (90% relative humidity) atmosphere containing 5-7% carbon dioxide for 24 hours, after which time all fluids were decanted and cells were fixed in methanol and stained with Papanicolaou hematoxylin stain (Fisher Scientific Co.). Culture dishes were microscopically (100X) scored as to the degree of morphologically discernable cytotoxicity. Cytotoxicity was subjectively scored from 0 to 4, 0 indicating a negative test and 4 indicating virtually complete cell destruction.

Cytotoxicity titers were determined by preparing two-fold dilutions (2-128) of test eluates in complete MEM medium with subsequent application of 0.25 ml of each dilution of test eluate to each of two wells in a Microtest II plate which contained monolayers of WI-38 cells from which all medium had previously been aspirated. Microtest II plates, containing monolayers of WI-38 cells and two-fold dilutions of test eluates were incubated, fixed, stained, and morphologically scored as described under procedure for cytotoxicity score. The cytotoxicity titer was defined as the reciprocal of the highest dilution of test eluate that induced morphologically discernable alteration of WI-38 cells as determined by microscopic (100 X) observation of stained cell preparations.

C. Systemic Injection Tests of Saline and Cottonseed Oil Eluates in Mice

Methods used in the performance of this test have been delineated in U.S.P. XIX (27). Test materials were eluted with

sodium chloride injection, U.S.P. (Cutter Laboratories) (hereafter referred to as saline) and refined cottonseed oil (VWR Scientific Co.)

Random-bred female mice, ranging in weight between 17 and 23 g, were used as indicators of toxicity. Saline eluates were injected intravenously at a dose of 50 ml/kg body weight. In a similar manner, cottonseed oil eluates were injected intraperitoneally at a dose rate of 50 ml/kg body weight. All mice were observed for abnormal clinical signs and death, at 4 hours postinoculation and at 24-hour intervals thereafter for a total of 72 hours.

D. Intracutaneous Tests of Saline, Complete MEM Medium, and Cottonseed Oil Eluates in Rabbits

Test protocol was based on U.S.P. XIX (27) and utilized female New Zealand white rabbits, weighing 2.5-3.5 kg. Test eluates of saline (50 or 70°C), complete MEM medium (37°C), and cottonseed oil (50 or 70°C) were injected (0.2 ml) intracutaneously at ten linearly arranged sites, parallel to the spinal column, on each of two rabbits. The procedure was modified from that described in U.S.P. XIX in that 7 test eluates and 1 control were injected (10 injection sites per eluate) on each rabbit. Injection lines were demarcated by outlines prepared on the shaved skin with picric acid. Injection sites were examined at 24, 48, and 72 hours postinoculation for evidence of erythema, edema, and necrosis and were scored according to the following numerical scale:

0 = no discernable response
1 = trace response
2 = slight response
3 = mild response
4 = moderate response

E. Intramuscular Implantation Test in Rabbits

Female New Zealand white rabbits, weighing 2.5-3.5 kg, were used as test subjects. Test procedure was basically as described

in U.S.P. XIX (27). Test materials and U.S.P. negative controls were prepared in strips 1 x 1 x 10 mm and inserted into the paravertebral muscles, of each of 2 rabbits, through a 15 ga needle, with 4 test strips and 4 control strips per rabbit. After 3-7 days, rabbits were euthanized, muscle surrounding the sample was excised, and the tissues circumscribing the implants were macroscopically examined for evidence of hemorrhage, inflammation, and encapsulation necrosis.

F. Preparation and Quantitation of Heavy Metal Solutions

Solutions of BaCl (9 g/25 ml), 3 $CdSO_4 \cdot 8H_2O$ (28 g/25 ml) and Pb $(NO_3)_2$ (9g/25 ml) were prepared in complete MEM medium and were allowed to stabilize for 24 hours at 37°C. Solutions were then adjusted to pH 7.2 with 10N NaOH and precipitates were removed by centrifugation. Control media were prepared for each metal solution with a comparable volume of 10N NaOH added with subsequent adjustment of all control solutions to pH 7.2 with 10N HCl. Final concentration of metal ions, in each respective solution, was determined by atomic absorption spectroscopy on an atomic absorption spectrophotometer (Perkin-Elmer Model 303).

Five-fold dilutions of each metal solution, as well as accompanying control solutions, were prepared in complete MEM medium. Each appropriate dilution (0.25 ml) was placed into each of 4 wells of a Microtest II plate containing a monolayer of WI-38 cells from which growth medium had been previously aspirated. Plates containing monolayers of WI-38 cells and dilutions of metal solutions were held for 24 hours at 37°C in a humidified atmosphere (90% relative humidity) containing 5-7% carbon dioxide. Plates were then fixed, stained, and scored for cytotoxicity as previously described.

G. Preparation of Endotoxin Solutions

Endotoxin (E. coli) was purchased (Mallinckrodt) in powder form, and two-fold dilutions were prepared in complete MEM

medium. Only those dilutions without visible precipitate were tested for cytotoxic effect.

Cytotoxicity titer was determined utilizing procedures described for heavy metals.

H. Test Specimens

Materials for test were selected at random from a total group submitted for various forms of biocompatibility testing. Test specimens included finished products and raw materials; however, the majority of the materials tested were unfabricated raw material. Raw materials were tested in a variety of configurations including sheets, tubing, pellets, granules, blocks, cylinders, and screens.

III. RESULTS

A. Cytotoxicity of Barium, Lead, and Cadmium in WI-38 Cell Culture

Cadmium induced morphologic alteration of exposed cells at concentrations as low as 25 ppm, barium was cytotoxic at a higher level of 500 ppm, and cytotoxicity was not observed with lead concentrations up to 1000 ppm (Table 2). Higher concentrations of lead could not be maintained in solution in complete MEM medium without formation of a precipitate.

Barium, lead, and cadmium cytotoxicity was quantified since these heavy metals have historically been present in eluates of some materials used in the fabrication of medical devices and could be a factor in the ingredient-specific interpretation of cytotoxicity.

B. Cytotoxicity of Endotoxin in WI-38 Cell Culture

Cytotoxicity was noted in those test preparations containing 167 µg/ml or more of endotoxin (E. coli) (Table 3). Endotoxin

TABLE 2

Detection Limits of Barium, Lead, and Cadmium in WI-38 Cell Cultures

Concentration, ppm[a]	Cytotoxicity of Compounds Tested		
	Barium	Lead[c]	Cadmium
1,000	4[b]	0	4
500	4	0	4
100	0	0	4
50	0	0	4
25	0	0	2
10	0	0	0

[a]Compounds diluted in MEM + 10% FBS

[b]Cytotoxicity score

[c]Higher concentrations (>1,000 ppm) could not be maintained in solution without precipitation

cytotoxicity was titrated to provide a point of reference in test environments involving contamination of a biomaterial with Gram-negative bacteria.

TABLE 3

Detection Limits of Endotoxin (E. coli) in WI-38 Cell Cultures

Concentration of Endotoxin, µg/ml [a]	Cytotoxicity Score
167	3
83	0
41	0

[a]Endotoxin diluted in MEM + 10% FBS

C. Overall Correlation of WI-38 Cell Culture to In Vivo Tests

Cell culture-positive specimens were identified in all classes of test material except polypropylene and nylon (Table 4). Polyvinyl chloride (PVC) plastics, rubber, and silicone rubbers evoked the highest proportion of positive responses in cell culture tests. A total of 1013 specimens were tested yielding one class of 115 materials positive in cell culture and one or more *in vivo* tests and a second reactivity class consisting of specimens positive in cell culture and negative in *in vivo* tests. Cytotoxicity titers, an approximation of the bioavailability of the toxic moiety, were clearly highest among the rubber compounds. Six hundred thirteen of the specimens were negative by all tests.

With the exception of one specimen, none of the test samples negative in the cell culture test were positive by an *in vivo* test (Table 4). This specimen was a Teflon® polymer, and inadequate quantity was available to confirm a borderline reaction of a cottonseed oil eluate in the intracutaneous rabbit test.

As a measure of the reproducibility of the cell culture test, 50 specimens were selected by random number table to be retested at the conclusion of the study. On the basis of cytotoxicity score, none of the retests deviated more than 2 units from the original score (Table 5).

D. Polyvinyl Chloride (PVC) Plastics

One hundred fifty-two PVC plastic specimens, representing a wide range of formulations, were tested yielding 74 (49%) samples cell culture-positive. Sixty-two (84%) of these specimens were negative in all animal tests (Table 6). Systemic injection tests on cell culture-positive materials were negative with all eluates; intracutaneous tests were negative with saline eluates and positive in 9 of 69 specimens with cottonseed oil

TABLE 4

Summary of Specimens Tested for Cytotoxicity in WI-38 Cell Cultures

	PVC	Rubber	Poly-propylene	Poly-ethylene	Epoxy	Silicone Rubber	Acetal	Nylon	Teflon	Poly-urethane	Poly-styrene	Misc.	Composition unknown	Total
Total number specimens tested	152	296	202	108	17	24	22	9	11	8	20	63	81	1013
No. of specimens positive in cell culture & animal tests	7	66	0	22	0	9	0	0	1	1	0	2	7	115
No. of specimens positive in cell culture & negative in animal tests	62	127	0	14	1	11	3	0	0	1	1	25	28	237
No. of specimens positive in cell culture & untested in animal tests	5	2	0	0	0	0	0	0	0	0	0	0	0	7
No. of specimens negative in cell culture & animal tests	74	101	202	72	16	4	19	9	9	6	19	36	46	613
No. of specimens negative in cell culture & positive in animal tests	0	0	0	0	0	0	0	0	1	0	0	0	0	1
No. of specimens negative in cell culture & untested in animals	4	0	0	0	0	0	0	0	0	0	0	0	0	4
Titer range in cell culture	0-8	0-64	-	0-32	0-2	0-16	0-2	-	0-1	0-2	0-2	0-32	0-32	

TABLE 5

Reproducibility of Cytotoxicity (MEM Eluate) in WI-38 Cell Cultures

	Original Cytotoxicity Score				
	0	1	2	3	4
Number of samples yielding same cytotoxicity score on repeat test[a]	31	0	0	1	13
Number of samples yielding 1 unit deviation in cytotoxicity score on repeat test	0	0	2	0	0
Number of samples yielding 2 unit deviation in cytotoxicity score on repeat test	0	1	0	1	1
Total Number of Samples			50		

[a]Samples selected for repeat test by random number table

eluates (Table 6). Intramuscular implant tests were positive in 1 of 22 tests on cell culture-positive PVC plastics (Table 6). The proportion of cell culture-positive materials yielding positive results on an in vivo testing was not a function of the cytotoxic titer, i.e. the two PVC plastic specimens with the highest cytotoxic titer (1:8) were negative in vivo (Tables 6 and 7). None of the cell culture-negative specimens were positive in vivo.

Patterns and titers of cytotoxicity were formulation-specific; and differences in response, as measured by cell culture and in vivo tests, were attributable to the elution and/or the target phase of the cell culture test. The elution phase of the test was assessed by performing intracutaneous tests with complete MEM eluates and negative results were observed in most instances (Table 8). Clearly, the target phase of the cell

TABLE 6

Quantitative Correlation Between WI-38 Cell Culture Cytotoxicity Titers, Systemic Injection, Intracutaneous, and Intramuscular Implant Tests for PVC Plastics

Cell Culture		Animal Test Results				
		Systemic Injection Tests		Intracutaneous Tests		Intramuscular Implant
Cell Culture Titer	Number Specimens	Saline Eluate	CSO Eluate	Saline Eluate	CSO Eluate	
0	78	0/65 [a]	0/61	0/72	0/74	0/23
1	49	0/39	0/35	0/39	5/44	1/16
2	19	0/18	0/17	0/19	3/19	0/2
4	4	0/3	0/3	0/4	1/4	0/3
8	2	0/2	0/2	0/2	0/2	0/1

[a] Numerator = number specimens positive
Denominator = number specimens tested

CSO - cottonseed oil

TABLE 7

Correlation Between WI-38 Cell Culture Cytotoxicity Titers, Systemic Injection, Intracutaneous, and Intramuscular Implant Tests for Selected PVC Plastic Specimens

Sample Number	Cell Culture Titer	Systemic Injection Tests		Intracutaneous Tests		Intramuscular Implant
		Saline Eluate	CSO Eluate	Saline Eluate	CSO Eluate	
74T-78	1	0	0	0	+	+
74T-488	1	0	0	0	+	0
74T-82	1	0	0	0	0	0
74T-500	2	0	0	0	+	0
74T-614	2	0	0	0	0	0
74T-349	2	0	0	0	+	0
74T-350	4	0	0	0	+	0
73T-74	4	0	0	0	0	0
74T-460	4	0	0	0	0	0
74T-458	8	0	0	0	0	0
74T-465	8	0	0	0	0	0

CSO - cottonseed oil

TABLE 8

Formulation-Specific Comparison of
PVC Plastic Cytotoxicity Titers in WI-38 Cell Culture
with Intracutaneous Tests on CSO and MEM Eluates

PVC Formulation	Cell Culture Cytotoxicity Titer	Intracutaneous Rabbit Test	
		CSO Eluate	MEM Eluate
AA [a]	1	0	0
BB	8	0	0
CC	1	0	0
DD	2	0	0
DD	1	0	0
DD	1	0	0
EE	1	0	0
FF	1	0	0
GG	1	0	0
HH	2	0	+1
II	2	0	0
JJ	2	0	+1
AA	8	0	0
KK	4	0	0
LL	8	0	0
MM	0	0	0
HH	2	+4	0
AA	1	+3	0
NN	1	+3	0
OO	1	+4	NT [b]

[a] Samples with same letter designations are multiple batches of the same formulation

[b] NT - not tested

CSO - cottonseed oil

culture test, WI-38 cells, detects cytotoxic moieties eluted from PVC plastic formulations that were not demonstrable with the same eluate *in vivo* (Table 8).

E. Rubber

Rubber formulations proved the most toxic compound group, in terms of cytotoxic titer as well as percentage of specimens

positive, in cell culture as well as *in vivo*. One hundred ninety-five (66%) of 296 specimens tested proved cytotoxic in cell culture, and 127 (65% of the cell culture-positive samples were not reactive *in vivo* (Table 9). The systemic injection test yielded negative results with all saline eluates (162) and 130 of 132 cottonseed oil eluates from cell culture-positive specimens (Table 9). Similarly, all 98 saline eluates were negative in the intracutaneous tests corresponding to positive cell culture tests. Intracutaneous tests with cottonseed oil eluates proved the most sensitive of the *in vivo* tests as evidenced by 72 (38%) positive tests of 192 cell culture-positive specimens tested (Table 9). The proportion of specimens positive with the intracutaneous test (cottonseed oil) eluate did not vary as a function of the cytotoxic titer in cell culture (Tables 9 and 10). Intramuscular implantation proved a relatively insensitive test with 5 of 29 tests positive, on materials cytotoxic in cell cultures, without quantitative relationship to cell culture titer (Table 11). None of the specimens negative in cell culture demonstrated reactivity in any *in vivo* test.

In an attempt to elucidate the mechanism of action causing a discrepancy in the toxic reactivity of rubber formulations *in vivo* and *in vitro*, complete MEM eluates from cell culture-positive specimens were injected intracutaneously into rabbits. Twenty complete MEM eluates with cytotoxic titers 16 or greater, prepared from specimens negative in the intracutaneous test by cottonseed oil elution, yielded 14 positive intracutaneous tests (Table 12). Complete MEM medium leached toxic moieties from rubber formulations that were biologically unavailable by saline or cottonseed oil extraction. In addition, the complete MEM extractions were performed at 37°C as compared to 50°C for the saline and cottonseed oil extractions.

F. Polyethylene

Although 36 of 108 polyethylene samples tested were cytotoxic in cell culture, none of the positive specimens repre-

TABLE 9

Quantitative Correlation Between WI-38 Cell Culture Cytotoxicity Titers, Systemic Injection, Intracutaneous, and Intramuscular Implant Tests for Rubber

Cell Culture		Animal Test Results				
		Systemic Injection Tests		Intracutaneous Tests		Intramuscular Implant
Cell Culture Titer	Number Specimens	Saline Eluate	CSO Eluate	Saline Eluate	CSO Eluate	
0	101	0/101 [a]	0/101	0/101	0/101	0/28
1	30	0/28	0/24	0/17	10/29	0/3
2	14	0/13	0/13	0/8	2/14	1/3
4	19	0/19	0/13	0/10	3/19	0/1
8	45	0/39	0/27	0/19	13/44	0/1
16	67	0/52	1/44	0/35	30/66	1/15
32	17	0/9	1/9	0/7	12/17	3/5
64	3	0/2	0/2	0/2	2/3	0/1

[a]Numerator = number of specimens positive
Denominator = number of specimens tested

CSO - cottonseed oil

TABLE 10

Cytotoxicity Profile of Rubber Formulations

Rubber Formulation	Cytotoxicity Cell Culture Titers		Tissue Response Intracutaneous Rabbit Test, CSO Eluate, Mean[b]
	Range	Mean[a]	
U	0-32	7	1.2
A	2-16	6	1
B	1-64	12	4
C	4-32	10	0
E	0-2	1	0
D	16-64	20	0
F	8-32	15	1
G	1	1	3
H	1-64	6	2
I	8-32	18	0
J	1-2	1	0
K	16-32	25	0
L	2-4	3	0
M	4-32	10	1

[a]Mean = geometric mean of a minimum of 3 lots

[b]Mean = arithmetic mean based on a scale of 0 to 4; 4 indicating most severe tissue response

CSO - cottonseed oil

TABLE 11

Correlation Between WI-38 Cell Culture Cytotoxicity Titers, Systemic Injection, Intracutaneous, and Intramuscular Implant Tests for Selected Rubber Specimens

Sample Number	Cell Culture Titer	Systemic Injection Tests		Intracutaneous Tests		Intramuscular Implant
		Saline Eluate	CSO Eluate	Saline Eluate	CSO Eluate	
74T-201	1	0	0	0	0	0
73T-86	2	0	0	0	0	+
74T-297	2	0	0	0	0	0
74T-568	4	0	0	0	0	0
74T-351	8	0	0	+	+	0
73T-39	16	0	0	0	0	+
73T-93	16	0	0	0	0	0
73T-123	16	0	0	0	+	0
74T-159	32	0	0	0	+	+
73T-179	64	0	0	0	0	0

CSO - cottonseed oil

TABLE 12

Formulation-Specific Comparison of Rubber Cytotoxicity Titers in WI-38 Cell Culture with Intracutaneous Tests on CSO and MEM Eluates

Rubber Formulation	Cell Culture Cytotoxicity Titer	Intracutaneous Rabbit Test	
		CSO Eluate	MEM Eluate
C [a]	16	0	0
F	16	0	0
C	16	0	+3
C	16	0	+2
I	16	0	+2
F	16	0	+2
U	32	0	+2
A	16	0	+3
C	16	0	+3
F	16	0	+2
F	16	0	0
F	16	0	0
C	16	0	+2
C	16	0	0
C	16	0	+2
C	16	0	+3
A	16	0	+2
K	32	0	+3
I	32	0	0
I	32	0	+3

[a] Samples with the same letter designations are multiple batches of the same formulation

CSO - cottonseed oil

sented virgin polymer. All of the toxic responses were attributable to pigments, adhesives, or other additives (Table 13). Saline eluates from polyethylene samples were negative in systemic injection tests with the exception of one specimen containing a formaldehyde residue (Table 13). Positive responses were evoked by cottonseed oil eluates in 1 of 108 systemic injection tests and 22 of 108 intracutaneous tests (Table 13). The degree of inflammatory or parenteral response observed in vivo

TABLE 13

Correlation Between WI-38 Cell Culture Cytotoxicity Titers and U.S.P. Tests for Various Polymers

Cell Culture		Animal Test Results			
		Systemic Injection		Intracutaneous Tests	
Cell Culture Titer	Number Specimens	Saline Eluate	CSO Eluate	Saline Eluate	CSO Eluate
Polyethylene					
0	72	0/72 [a]	0/72	-	0/72
1	17	0/17	0/17	-	12/17
2	9	0/9	0/9	-	5/9
4	4	0/4	0/4	-	0/4
8	1	0/1	0/1	-	0/1
16	1	0/1	0/1	-	1/1
32	4	1/4	1/4	1/2	4/4
Epoxy					
0	16	0/16	0/16	-	0/16
2	1	0/1	0/1	0/1	0/1
Acetal					
0	19	0/19	0/19	-	0/19
1	2	0/2	0/2	0/1	0/2
2	1	0/1	0/1	-	0/1
Teflon®					
0	10	0/10	0/10	-	1/10
1	1	0/1	0/1	-	1/1
Polyurethane					
0	6	0/6	0/6	-	0/6
1	1	0/1	0/1	-	0/1
2	1	0/1	0/1	-	0/1
Polystyrene					
0	19	0/19	0/19	-	0/19
2	1	0/1	0/1	0/1	0/1

[a] Numerator = number specimens positive
Denominator = number specimens tested

CSO - cottonseed oil

did not correlate to cytotoxic titer in cell culture, a phenomenon previously observed with PVC plastic and rubber formulations. No toxic *in vivo* tests were noted among the group of specimens negative in the cell culture test.

G. Epoxies

The epoxy compounds tested were biologically inert with the exception of a single specimen positive in cell culture and negative in all *in vivo* tests (Table 13).

H. Acetals

Twenty-two acetals specimens were tested and found to be devoid of toxicity *in vivo* as assessed by saline and cottonseed oil extraction (Table 13). Three of the 22 specimens evoked low level cytotoxicity in cell culture, and all of the positive specimens were samples of separate lots of the same material formulation.

I. Teflons®

Consistent with past reports of biologic inertness (17), 2 of 11 Teflon® specimens elicited toxic responses in cell culture or animal tests (Table 13). A single specimen was cytotoxic in cell culture and reactive in the intracutaneous test when assessed by cottonseed oil extraction. The second biologically active specimen exhibited a toxicity pattern aberrant to the entire study; a cottonseed oil extract evoked an inflammatory response upon intracutaneous injection; and the cell culture test was negative. This specimen was not representative of the virgin polymer and contained pigmented additives.

J. Polyurethane

Two of eight polyurethane specimens, uncategorized as to polyether or polyester type, were cytotoxic in cell culture; and one of the two was recorded positive by virtue of cottonseed oil extraction and intracutaneous injection (Table 13). All systemic injection tests were negative. None of the specimens negative in cell culture were positive *in vivo*.

K. Polystyrene

Bioincompatibility was noted in a single specimen out of a test group of 20 (Table 13). The single positive response was reported in cell culture, and all in vivo tests were devoid of a toxic response.

L. Miscellaneous and Formulations of Unknown Composition

This specimen category included those formulations for which an inadequate number of lots were tested for meaningful analysis and a more diverse polymer group including those specimens submitted without definitive identification as to formulation. Sixty-two of 144 miscellaneous and unidentified specimens were cytotoxic in cell culture, some attaining cytotoxic titer values of 32 (Table 4). Nine of the 62 cell culture positive specimens were reactive in in vivo tests, all by cottonseed oil extraction and intracutaneous inoculation.

M. Silicone Rubbers

Silicone rubbers, undelineated as to heat cure or room temperature cure, proved a diverse specimen group in terms of biocompatibility. Twenty out of 24 silicone rubber specimens, representative of more than one basic material manufacturer, were cytotoxic in cell culture; and 9 of this cytotoxic group were toxic in vivo (Table 14). Intramuscular implantation was the most definitive of the in vivo tests for silicone rubber, detecting toxicity in 6 specimens negative by cottonseed oil extraction and intracutaneous injection (Table 14). This relationship between toxic response detected by intramuscular implantation and intracutaneous tests is unique to silicone rubber and has not been observed with any other class of polymers.

The severity of the intracutaneous test response, as measured by the cottonseed oil extraction, was not directly proportional to the cytotoxic titers as evidenced by the fact that the 5 specimens evoking the highest cell culture titer were negative by the intracutaneous test with cottonseed oil eluates (Table 14).

TABLE 14

Quantitative Correlation Between WI-38 Cell Culture Cytotoxicity Titers, Intracutaneous, and Intramuscular Implant Tests for Silicone Rubber

Cell Culture		Animal Test Results	
Cell Culture Titer	Number Specimens	Intracutaneous Test CSO Eluate	Intramuscular Implant
0	4	0/4 [a]	0/2
2	1	0/1	0/1
4	5	0/5	4/5
8	5	2/5	3/4
16	3	1/3	2/3

[a] Numerator = number of specimens positive
Denominator = number of specimens tested

CSO - cottonseed oil

Analysis of test results for the silicone rubber group was impeded by the inability to identify specific formulations. It is reasonable to assume that silicone rubber formulations are not monolithic in terms of biocompatibility, but exhibit formulation-specific toxicity profiles as previously demonstrated for rubber and PVC plastic.

IV. DISCUSSION

Cell culture methods have been utilized and reported for over a decade, in a wide variety of modes, to assess the bioincompatibility of medical devices and materials. Data presented in this report is intended to extend previous reports and to accommodate those variables most critical to the extrapolation of in vitro data to the clinical environment. Conceptual elements of the study include: (A) use of a normal human cell, unmodulated by virus (30) or mycoplasma contamination (31), as a target system since the products tested are intended for human use; (B) use of large number

of specimens to measure biologic compatibility of a variety of formulations and multiple lots of specific formulations; (C) quantitative assessment of cytotoxicity to allow correlation studies; (D) use of physiologically normal elution menstrua and temperatures to permit an evaluation of the bioavailability of toxic moieties; and (E) provide for the correlation of all in vitro data with accepted in vivo systems in U.S.P. animal tests.

The WI-38 cell culture test employed in this study (26) defined a larger proportion of toxic specimens than any of the animal tests. Cytotoxic profiles were unique to classes of materials; and in many instances, were quantitatively and qualitatively characteristic of specific material formulations. If a small number of formulations had been tested for each material class, an array of contrasting conclusions on test methods could have been tendered depending on the particular formulation tested.

Rubber specimens yielded the highest percentage of cytotoxic samples (65%) in vitro and fewest discrepancies (43%) between in vitro and in vivo test results. Disparities in cell culture and animal test results were attributable, in some instances, to an elution differential; wherein complete MEM medium eluted toxic moieties at 37°C not manifested by elution with cottonseed oil or saline at 50-60°C. Toxic molecules in rubber were the most readily leachable as evidenced by the cytotoxic titer range. Cytotoxicity profiles, in vitro and in vivo, were formulation-specific and reproducible from lot to lot.

PVC plastics manifested a high percentage (45%) of cell culture-positive samples with a concomitantly low proportion (11%) of these specimens positive in animal tests. The differential in test results is circumstantially ascribable to the target phase (cell cultures or animals) of the test system since efforts to delineate differences in the elution phases were negative. Positivity in animal tests was not proportional to cytotoxic titers in cell culture; however, none of the materials negative in cell culture were positive in animals.

Polypropylenes, polyethylenes, most epoxies, nylons, most Teflons®, and most polystyrenes were biologically inert, in vitro and in vivo, under the ascribed test parameters. Positive specimens in the polyethylene group were artifactual in the sense that none were virgin polymer and toxicities were ascribable to additives. A Teflon® sample proved the aberrant specimen of the entire study by virtue of a positive inflammatory response (intracutaneous) to a cottonseed oil extract and a negative cell culture response.

Overall, the WI-38 cell culture test proved a sensitive and quantitatively reproducible test. In comparison, animal tests were relatively insensitive, particularly systemic injection tests (6 of 944 positive); whereas the intracutaneous test with cottonseed oil extraction proved the most definitive in vivo test. Intramuscular implantation proved uniquely sensitive (among in vivo tests) for the bioassessment of silicone rubbers, detecting more positive specimens than the other animal tests. The biological significance of the cell culture-positive tests, unconfirmed in animals, is dependent in part on the intended use of the tested product. Realistically, these cell culture-positive and animal-negative tests cannot be shunted as biologically false since they represent toxic moieties eluted by physiologically normal menstrua under physiologically normal environments, physical and biochemical, that destroy normal human cells during the 24-hour exposure.

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