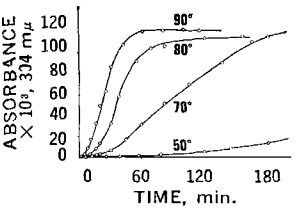

Fig. 7—Formation of 304 mμ absorbing material from degraded 6APA solutions. Initial 6APA concentration, 0.184 M, pH 6.30.

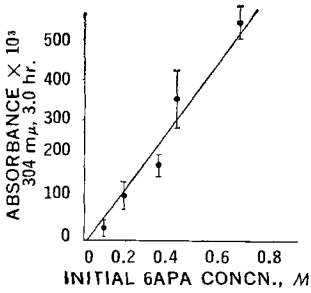

Fig. 8—Maximum level of 304 mμ absorbance as a function of the initial 6APA concentration. 6-APA degraded at pH 6.30, 80°.

DISCUSSION

The rates of β-lactam hydrolysis and dimerization of 6APA are both proportional to the first order of the intact β-lactam content in solutions below pH 7.5. This is not a surprising result since no direct measure of 6APA monomeric concentration is obtained by the assay of intact β-lactam in a system undergoing hydrolysis and polymerization. The dimerization or further polymerization of 6APA involving a β-lactam acylation by another 6APA molecule always leaves one β-lactam intact per two molecules of 6APA. The rate of dimerization would thus be proportional to the square of the 6APA concentration, whereas it would be directly proportional to the first-order loss of β-lactam.

Since the activation energy (E_a) for the hydrolysis of other penicillins is similar to that measured in the degradation of 6APA, it is reasonable to conclude that both β-lactam hydrolysis and intermolecular acylation occur with equal ease. This is one suggested explanation for the proportionately direct increase in the amount of polymer formed as a function of the initial 6APA concentration. Otherwise, one might expect that the rate of dimerization, which is proportional to the square of the initial concentration of 6APA, would be reflected by a proportionate increase in the polymer yield.

In alkaline solution, second-order kinetics of β-lactam loss are apparent from the data, and in these instances the β-lactam content can be visualized to closely approach the actual remaining concentration of 6APA. The increased lability of an acylated β-lactam over that of 6APA affects the apparent loss of two β-lactams per dimer rather than one.

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Cytotoxicity of Rubber Closures in Tissue Culture Systems

By P. J. VASINGTON, H. D. PIERSMA, J. J. CORBETT, and J. L. BITTLE

Rubber closures used in the pharmaceutical industry were tested for cytotoxicity in primary cultures of chick embryo cells and in cultures of cells passaged from human malignant tissue. Assay was by three methods: direct contact, growth-extract, and maintenance-extract. In the first, rubber samples were placed in direct contact with cell monolayers. In the growth-extract system, a sample was incubated in growth medium, and the resultant extract was placed in a cell suspension. In the maintenance-extract method, following incubation of a sample in maintenance medium, the extract was overlayed on a cell monolayer. Tissue culture provided a more sensitive test of toxicity in comparison with the generally accepted implantation of rubber in the paravertebral muscles of rabbits.

TESTING THE toxicity of rubber closures for pharmaceutical applications has been primarily physicochemical. To date, there is no acceptable biological assay system, although the rabbit implantation method of Brewer and Bryant (1) for screening plastics has gained considerable favor.

The need for a rapid, economical, and reproducible *in vitro* test has led to the investigation of

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the use of tissue culture systems. The cytotoxicity of some rubber closures used to stopper cell culture tubes and bottles of synthetic medium became quite obvious to early investigators. Parker *et al.* (2) found a number of rubber closures to be toxic to cell cultures. Cruickshank *et al.* (3) employed tissue explants of chick embryo and guinea pig skin to test the toxicity of rubbers and plastics used in transfusion-giving sets. They concluded that tissue culture explants might prove valuable as a screening method to select suitable materials for clinical trials. The method involved peripheral fibroblastic outgrowths away from the area of toxic samples.

Rosenbluth *et al.* (4) reported the similar use of fibroblastic-like "L" cells for testing the toxicity of plastic materials employed in medical practice. They compared their results with those obtained in rabbit implantation and found the cell culture system to be more sensitive. They placed a sample in direct contact with the cell monolayer. Zones of degeneration developed when samples were toxic.

This report compares the use of primary and continuous cell cultures for evaluating the toxicity of rubber closures using three methods of assay in tissue culture. These are defined as: (*a*) direct contact with the cell monolayer, (*b*) maintenance medium-extract overlay on the cell monolayer, and (*c*) growth medium-extract in a cell suspension. The rubber closures tested were received in the laboratory for routine use. They were composed of natural rubber or synthetic butyl rubber and ranged in size from 13 mm. to 30 mm. in diameter.

EXPERIMENTAL

Direct Contact Method—Primary cell monolayers were prepared as follows. Nine-day-old chicken embryos were decapitated, minced, washed with phosphate buffered saline (PBS, pH 7.4), and the cells dissociated with 0.25% trypsin. Approximately 2×10^6 cells were inoculated into 60-mm. tissue culture plates[1] containing 5 ml. of Eagle's basal medium (BME) supplemented with 10% bovine serum. The plates were incubated at 37° in a humidified CO_2 incubator which maintained a pH of 7.0–7.4. When the cell monolayer was confluent, usually 2–3 days, the medium was evacuated and a rubber sample placed carefully at the center of the monolayer. Growth medium (BME + 10% fetal bovine serum) was added and the plate incubated as above. The rubber samples were tested as received unless the geometry of the closure necessitated cutting them. They were washed in pyrogen-free distilled water prior to testing. Samples were cut to approximately 5 mm.[3] and the cut surface exposed to the cell monolayer. Plates were observed daily for 3 days for evidence of cellular degeneration. When the sample was toxic, macroscopic observation revealed clear zones surrounding the sample, because of degradation of the cell monolayer (Figs. 1 and 2). Microscopic observation was made with a low power objective (10×). A sample was considered to be toxic when cells appeared granular, rounded, and detached from the surface of the culture plate.

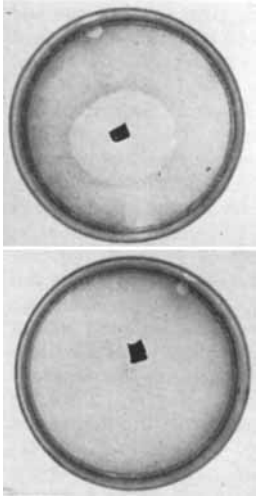

Fig. 1—Direct contact method of assay on cell monolayer. Key: top, toxic rubber sample showing an area of degeneration; bottom, nontoxic rubber sample. Cells stained with neutral red (1:20,000).

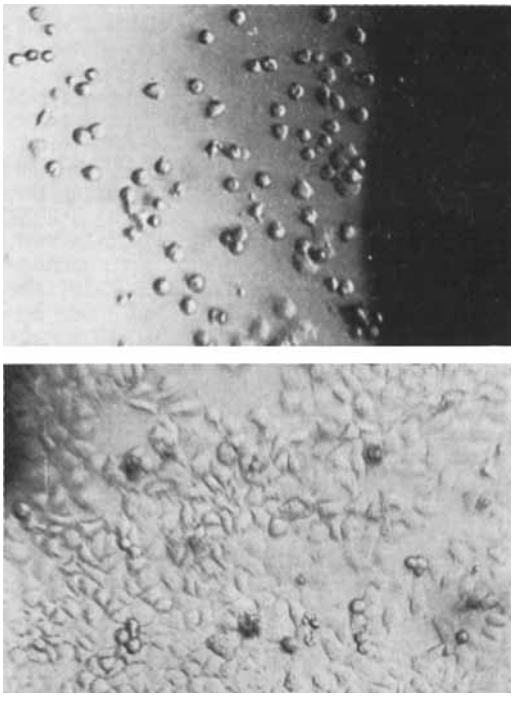

Fig. 2—Microscopic view of areas adjacent to rubber sample shown in Fig. 1. Key: top, toxic sample (shadow) showing degenerative cells; bottom, nontoxic sample (shadow) showing a healthy confluent epithelial cell monolayer. Cells are HEp-2 cultures. 100× magnification.

[1] Falcon Plastics, B-D Laboratories.

Human epidermoid carcinoma cell cultures (HEp-2), originally derived from a carcinoma of the larynx by Toolan (5) in 1955, were grown in 60 cm.^{2} milk dilution bottles (MDB). The cells had undergone an unknown number of passages before receipt. The cells were grown as monolayers by transferring them twice weekly using 0.25% trypsin in phosphate buffered saline (PBS, pH 7.4) to detach the cells from the glass. This was accomplished by removing the maintenance medium from MDB and adding 20 ml. of the trypsin solution to bathe the cell sheet. Following incubation at room temperature for 1 min., the trypsin was removed and the cultures incubated at 37° for 15 min. Sufficient growth medium (BME plus 10% fetal bovine serum) was added to give a cell suspension which was subdivided into MDB or 25 cm.^{2} Falcon plastic flasks at a split ratio of 1 to 6. The concentration of fetal bovine serum was dropped to 2% for maintenance. When the cell sheet became confluent, the tests were performed in the same manner as described for primary cultures except as noted.

Maintenance-Extract Method—Primary and continuous cell cultures were prepared as described above. When the cell monolayer was confluent, the growth medium was removed and replaced with a maintenance medium to which a sample extract had been added. The extract was prepared by suspending a rubber sample, as received, into 2 ml. of maintenance medium and incubating the suspension at room temperature for 24 hr. The medium was aseptically drawn off and added to sufficient maintenance medium diluent to give a final extract concentration of 25%. The cell cultures were incubated at 37° and examined daily for 3 days under low power magnification (10×). Cellular granulation, rounding, and detachment from the surface of the container were indicative of a toxic response.

Growth Medium-Extract Method—The sample extract was prepared as described above except that growth medium was used in place of maintenance medium. Cell suspensions of chick embryo and HEp-2 cultures were prepared by detaching the cells from MDB or plastic flasks with 0.25% trypsin as previously described. The cells were suspended in the growth medium-extract mixture to give a planting concentration of 1.6×10^5 cells per ml. for chick embryos and 75×10^3 cells per ml. for HEp-2 cultures. The resulting cell suspensions were inoculated into plastic plates, incubated at 37°, and observed daily for 3 days. Absence of cell growth and attachment indicated a toxic response.

Rabbit Implantation—Intramuscular implantation of rubber samples was performed and evaluated by the method described in the USP XVII or in the NF XII for plastics.

Samples used for rabbit implantation were prepared by cutting strips from the top of the rubber closure with a razor blade to a size approximating $1 \times 1 \times 10$ mm. This method resulted in smooth cut surfaces on all sides of the sample.

RESULTS AND DISCUSSION

A comparison between HEp-2 and primary chicken cell cultures was made using the direct contact method to determine the effect of subcultivation on the sensitivity of the cell cultures to toxic samples. Differences were noted by measuring the approximate diameter of the zones of degeneration surrounding the samples as shown in Figs. 1 and 2. Variation in cell sensitivity occurred in both types of cell culture. However, it was interesting to note (Figs. 3 and 4) that the degree of sensitivity of the sample and positive control varied in the same direction with HEp-2 cells as the substrate, whereas there was random variation in primary cell cultures. This may be attributed to the uniformity of epithelial-like cell populations from continuous culture, even though sensitivity changes with different passage levels. In contrast, primary cell cultures are generally disorganized and contain cells of many types. The degree of cytotoxicity in primary cultures may be dependent on the differing distribution and concentration of cell types with each culture preparation.

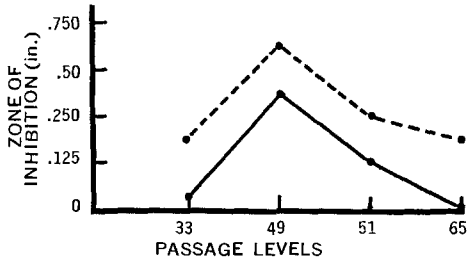

Fig. 3—Effect of passage levels of continuous (HEp-2) cell cultures on rubber closures. Key: - - - -, positive control; ——, sample 2342-2.

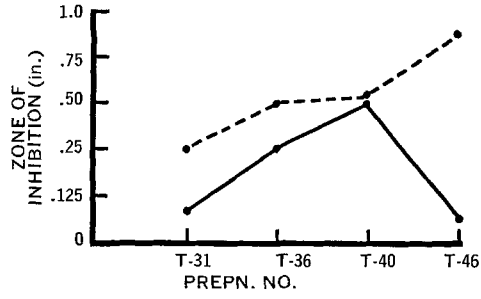

Fig. 4—Effect of different preparations of primary chick embryo (ChE) cell cultures on rubber closures. Key: - - - -, positive control; ——, sample 2342-2.

This study suggested that HEp-2 cells provided more reproducible results than did primary chicken cell cultures, although primary cells appeared more sensitive than HEp-2 cells as judged by the generally larger zones of degeneration.

Table I summarizes the results of an experiment designed to compare the three tissue culture assay systems in the more sensitive primary chicken cells. Sample 7 was completely nontoxic in all three systems, and sample 5 was toxic, and only slightly, by the direct contact method. The growth-extract system was the least sensitive, with little inhibition of cell proliferation by samples that were toxic in the direct contact and maintenance-extract tests. It may be significant that in the last two methods samples are introduced into established cell monolayers. Since the degree of sensitivity

between the direct contact and maintenance-extract methods did not vary markedly, the former was chosen because of its simplicity for a comparison of toxicity determinations in tissue culture and by rabbit implantation.

TABLE I—COMPARISON OF THREE TISSUE CULTURE METHODS IN EVALUATING TOXICITY OF RUBBER SAMPLES

Sample	Contact (Zone in.)	Extract—Growth (Confluency %)	Extract—Maintenance (Confluency %)
1	.25	95	10
2	.25	80	10
3	.125	80	25
4	.25	70	40
5	.0625	100	100
6	.25	95	75
7	Nontoxic	100	100
8	.50	95	10
9	.50	90	5
10	.25	90	40
Normal control	...	100	100
Neg. control	Nontoxic	100	100
Pos. control	.25	10	25

Table II shows results of implants in rabbits and direct contact in both HEp-2 and chick embryo cell cultures. The findings in the two types of cell cultures correlate well, although the primary cells were significantly more sensitive. The first five samples varied in toxicity between the two preparations, while samples *6*, *7*, and *8* were unquestionably toxic in both. The tissue culture system compared favorably with intramuscular implantation and appeared to be more sensitive, since *2A* and *4*, negative by implantation, were positive in both culture systems. It is also interesting to note that the nonchlorinated segment of sample *2* (*2A*) and the chlorinated (*2B*) were both toxic for tissue culture, but only the chlorinated sample was positive in rabbits.

TABLE II—COMPARISON OF RABBIT IMPLANT AND TISSUE CULTURE TESTING OF RUBBER CLOSURES

Sample	Rabbit Implant	Direct Contact HEp-2	Direct Contact ChE
1	+	3/3[a](1-ST)[b]	3/3
2A	−	3/3(1-ST)	4/4(1-ST)
2B (chlorinated)	+	3/3(1-ST)	3/3
3	Questionable	1/3(ST)	3/3
4	−	3/4(ST)	4/4(1-ST)
5	+	4/4(3-ST)	4/4(1-ST)
6	+	4/4	4/4
7	+	4/4	3/3
8	+	3/3	3/3
9	Questionable	0/1	0/1
Neg. control	− (Sometimes reactive)	0/7	0/4
Pos. control	+	5/5	4/4

[a] Denotes number of times the sample was toxic over the number of tests performed. [b] Slightly toxic.

Another study was designed to determine the effect of autoclave sterilization on rubber closures. Eight samples shown to be toxic by the direct contact method were compared in the growth-extract system. The results (Table III) showed two samples, *N* and *O*, to be negative when autoclaved and allowed to stand 1 hr. prior to assay. There was good correlation between the direct contact method and the growth-extract method for samples which were not autoclaved, except for sample *K*.

TABLE III—EFFECT OF AUTOCLAVE STERILIZATION ON CYTOTOXICITY IN HEp-2 CELL CULTURES

Sample	Direct Contact	Autoclave Sterilized Growth-Extract	Growth-Extract
H	Very toxic	No cell attachment	No cell attachment
I	Nontoxic	75% confluent	65% confluent
J	Very toxic	No cell attachment	No cell attachment
K	Sl. toxic	30–35% confluency	40% confluent
L	Nontoxic	75% confluent	65% confluent
M	Sl. toxic	60% confluent	70% confluent
N	Toxic	75% confluent	30–35% confluency
O	Sl. toxic	75% confluent	55% confluent
Neg. control	Nontoxic	75% confluent	75% confluent

The results of this investigation suggest that tissue culture may be useful as a biological assay to screen rubber closures prior to use in the manufacture of parenteral drug products. The two methods of assay involving established cell monolayers were in general agreement with each other but not with the growth-extract method. The direct contact method appears to be the most useful because of the ease of performance and evaluation. Although it would be difficult to equate cytotoxicity with reactions in man, such a screening mechanism could reasonably be assumed to reduce the risk of potential danger in man.

Pilot studies on a number of plastics using the same methods described for rubber closures compare favorably with the results of Rosenbluth *et al.* cited earlier in this report (4).

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