# A Cell Culture Assay to Evaluate the Toxicity of Arlacel A (34444)

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Oil-adjuvant vaccines using Arlacel A (AA) as the emulsifying agent have produced toxic reactions in man (1–6). Some of the reactions were attributed to impurities in certain batches of AA (1, 7) and subsequently *in vivo* tests were developed to evaluate AA toxicity (8). In this report an *in vitro* assay using cell cultures to evaluate AA toxicity is described and compared to a mouse assay.

*Materials and Methods. Arlacel A* (mannide monooleate) is a complex mixture of esterified and free fatty acids, carbohydrates and other substances (9). Nineteen lots listed in Table I were tested. Lots 3589 and 8B were supplied by W. H. Berkeley and lot 6B by M. Potter; they had been stored at the National Institutes of Health (NIH) for approximately 10 years at room temperature until used. The remaining lots were purchased from the manufacturer (Atlas Chemical Industries, Wilmington) or its distributor (Hilltop Laboratories, Inc., Cincinnati) and were stored upon receipt at 4° until used. The year of manufacture and of testing are given in Table I. The date of receipt was 1961 for lot 32B; 1965 for lot 88B; 1966 for lot 95B; 1967 for lots 103, 114B, and 115B; and 1968 for the remaining lots.

*Cell cultures.* Two established lines, L-929 mouse fibroblast (10) and WI-38 human embryonic lung (11), and four primary cell cultures (rhesus monkey kidney, rabbit kidney, chick embryo, and hamster embryo) were used. The primary cell cultures were prepared as described by Youngner (12). All cultures were supplied by the Cell Biology Section, Division of Biologics Standards.

The L-929 cell line was used in the development of the assay and to test each of the 19 lots of AA. The five other types of cell cultures were used to test nine of these lots of AA (3589, 6B, 8B, 32B, 88B, 95B, 103, 114B, and 115B).

*Toxicity assay in cell cultures.* L-929 cells were grown in medium NCTC No. 135 (13) or in Eagle's minimum essential medium (14) containing 2 – 5% fetal calf or horse serum and 50 μg of neomycin per ml. A 1.0-ml suspension containing 125,000 viable cells per ml was inoculated into each tube. The tubes were exposed individually to 5% $CO_2$ in air, closed with rubber-lined plastic screw caps, and incubated at 36° ± 1° in a stationary position. Cultures were used when cell growth reached 60–75% confluency.

Cell cultures were inoculated in quadruplicate with 0.2, 0.1, and 0.05 ml of AA per tube. The AA was layered onto the medium so as to avoid direct contact with the cell monolayer. After incubation at 36° for 3 hr, the AA-medium fluid was decanted, the cells were fed with 1.5 ml of fresh medium, and incubation was continued for 3 days. Cells without AA were treated in an identical manner and served as controls.

The cytotoxic effect (CTE) was scored at 3 days as follows:

4+, complete destruction of the cell culture

3+, destruction of one-half to three-quarters of the cells. Cell destruction began at the periphery of the monolayer and progressed inward. The surviving cells showed a severe CTE characterized by large (2–4 μ) intracytoplasmic refractile bodies (Fig. 1a).

2+, destruction of approximately one-fourth of the monolayer with the most severe damage at the periphery of the cell sheet. The surviving cells contained intracytoplasmic refractile bodies, and the cultures had a granular appearance.

TABLE I. Results of Cell Culture and Mouse Toxicity Assays and the Free Fatty Acid (FFA) Content of Arlacel A.

| | | | Toxicity assays | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Cell culture-CTE | | | Mouse assay | | | | | | | Toxicity assayed by | |
| | | | Dose (ml) | | | % Difference in wt from saline control | | | | | | | | |
| Lot no. | Date of mfr[a] | FFA ($\mu$moles/ml) | 0.2 | 0.1 | 0.05 | Day 1 | Day 2 | Day 3 | Day 7 | Day 14 | No. dead/no. inoculated | No. adhesions/no. sacrificed | Cell culture | Mouse |
| 3589 | ≤1951[b] | >500 | 4.0 | 4.0 | 3.7 | —15.3 | —28.5 | —29.3 | —37.2 | —18.5 | 6/10[c] | 4/4 | + | + |
| 6B | <1960 | >500 | 4.0 | 4.0 | 3.8 | †[d] | † | † | † | † | 10/10[d] | [e] | + | + |
| 8B | <1960 | 282 | 3.4 | 2.6 | 2.5 | —17.7 | —21.5 | —25.3 | —24.2 | —11.1 | 0/10 | 10/10 | + | + |
| 32B | 1961 | 43 | 2.6 | 1.8 | 1.6 | —9.8 | —8.8 | —6.1 | 5.1 | 9.7 | 0/10 | 0/10 | + | — |
| 73B | 1964 | 58 | 1.2 | 1.2 | 1.0 | —4.0 | —3.2 | —4.8 | —3.5 | 5.5 | 0/10 | 0/10 | — | — |
| 74B | 1964 | 54 | 1.0 | 1.0 | 1.0 | —3.1 | —4.1 | —4.6 | —2.9 | 8.1 | 0/10 | 0/10 | — | — |
| 77B | 1964 | 59 | 0.8 | 0.8 | 0.8 | —6.4 | —5.1 | —6.0 | —1.0 | 16.0 | 0/10 | 0/10 | — | — |
| 88B | 1965 | 22 | 1.8 | 1.3 | 1.1 | —6.8 | —5.6 | —2.1 | —3.1 | 7.5 | 0/10 | 0/10 | — | — |
| 95B | 1965 | 23 | 1.8 | 1.1 | 0.6 | —6.8 | —7.5 | —9.3 | —3.5 | 0.8 | 0/10 | 0/10 | — | — |
| 102B | 1966 | 31 | 0.8 | 0.8 | 0.8 | —7.9 | —7.2 | —7.3 | —4.3 | 5.8 | 0/10 | 0/10 | — | — |
| 103 | 1966 | 41 | 1.4 | 0.8 | 0.6 | —8.5 | —6.1 | —6.6 | —2.2 | 6.8 | 0/10 | 0/10 | — | — |
| 114B | 1967 | 11 | 0.9 | 0.8 | 0.7 | —6.6 | —6.1 | —5.6 | —1.6 | 6.5 | 0/10 | 0/10 | — | — |
| 115B | 1967 | 14 | 1.0 | 1.0 | 0.9 | —10.8 | —10.3 | —7.6 | 2.6 | 15.4 | 0/10 | 0/10 | — | — |
| 123B | 1967 | 30 | 0.8 | 0.8 | 0.8 | —6.1 | —5.1 | —6.0 | —4.9 | 3.8 | 0/10 | 0/10 | — | — |
| 124B | 1967 | 36 | 0.8 | 0.8 | 0.8 | —5.7 | —4.7 | —5.7 | —4.8 | 18.0 | 0/10 | 1/10 | — | — |
| 133B | 1967 | 33 | 0.9 | 0.8 | 0.8 | —5.9 | —4.7 | —5.5 | 1.7 | 6.0 | 0/10 | 0/10 | — | — |
| 134B | 1967 | 32 | 0.8 | 0.8 | 0.8 | —2.3 | —5.0 | —6.9 | 14.8 | 14.1 | 0/10 | 0/10 | — | — |
| 139B | 1967 | 44 | 0.8 | 0.9 | 0.8 | —4.7 | —5.3 | —6.1 | —5.7 | 14.6 | 0/10 | 0/10 | — | — |
| 141B | 1967 | 46 | 0.8 | 0.8 | 0.8 | —6.4 | —6.1 | —7.1 | 6.5 | 6.2 | 0/10 | 1/10 | — | — |

[a] Dates kindly supplied by Atlas Chemical Industries, Inc. Tests of each lot were performed concurrently in 1968.

[b] See Ref. 2.

[c] Animals dead within 48 hr after inoculation. Adhesions were present.

[d] Animals dead within 24 hr after inoculation.

[e] Presence of adhesions could not be determined because of autolysis. When other animals were sacrificed adhesions had developed within 6 hr.

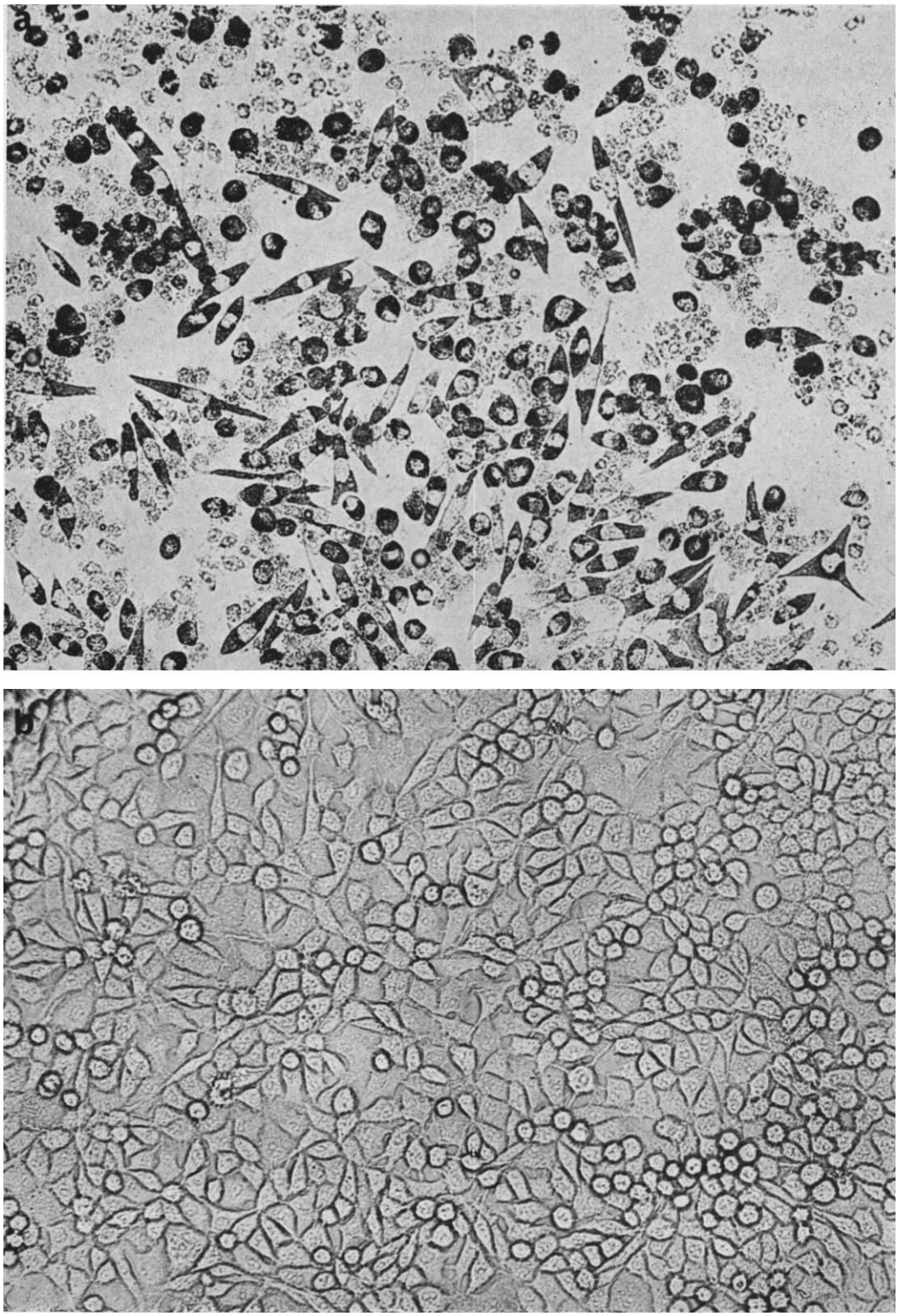

FIG. 1.(a) 3+ cytotoxic effect induced in L-929 cells by a toxic lot of Arlacel A. Note the large characteristic intracytoplasmic refractile bodies and dead cell debris (×165). (b). Uninoculated L-929 cells (×165).

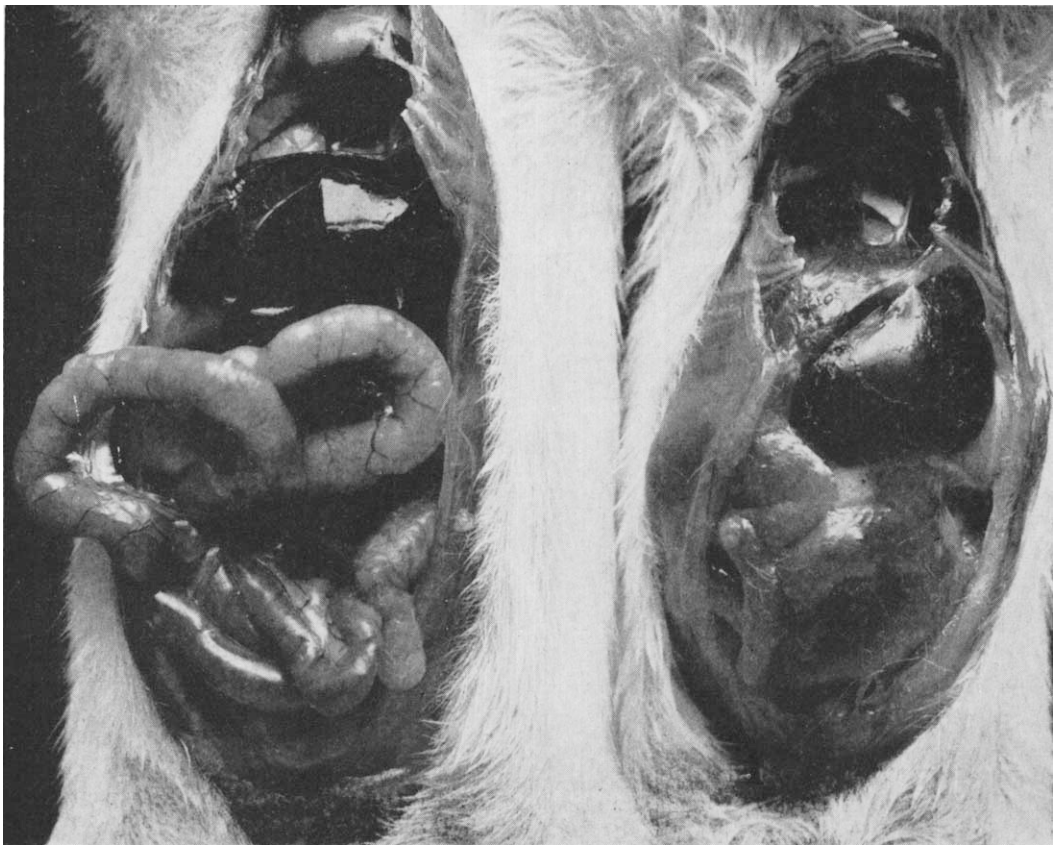

FIG. 2. Left, normal saline control mouse. Right, generalized peritoneal adhesions of viscera in mouse given toxic AA.

1+, no destruction of the culture with some peripheral cells showing very small refractile bodies, and the culture had a diffuse granular appearance.

0, no cellular changes (Fig. 1b).

Intermediate scores were assigned as indicated, *i.e.*, a score of 2.5 was given when the CTE was evaluated between 2+ and 3+. The final score for each dose of a lot was the mean score of the four tubes of each of two or more separate tests. A lot of AA was considered toxic when it caused a 2+ or greater CTE reaction at any dose.

*Toxicity assay in mice.* A modified Berlin test (8) was used to determine mouse toxicity. The NIH strain was selected since weight-gain patterns had been established for this strain during the past 15 years (15). Male mice, 9–11 g, from the Rodent and Rabbit Production Section, DBS, NIH, were marked and weighed individually 1–3 days prior to use. The rare mouse which lost weight was discarded. Mice in groups of ten were weighed and injected intraperitoneally with either 0.25 ml AA or saline. In early experiments individual mouse weights were recorded. The mean of the individual percentage of weight change and the group percentage of change appeared to be similar. Each group was weighed again on days 1, 2, 3, 7, and 14 and the percentage of weight changes relative to Day 0 were calculated. On Day 14 the surviving mice were sacrificed and examined for the presence of peritoneal adhesions. A lot of AA was recorded as toxic when there was either death, a 10.5% weight retardation relative to the saline control group, or peritoneal adhesions at Day 14 (Fig. 2).

Berlin (8) determined the percentage of weight change per group of mice by averag-

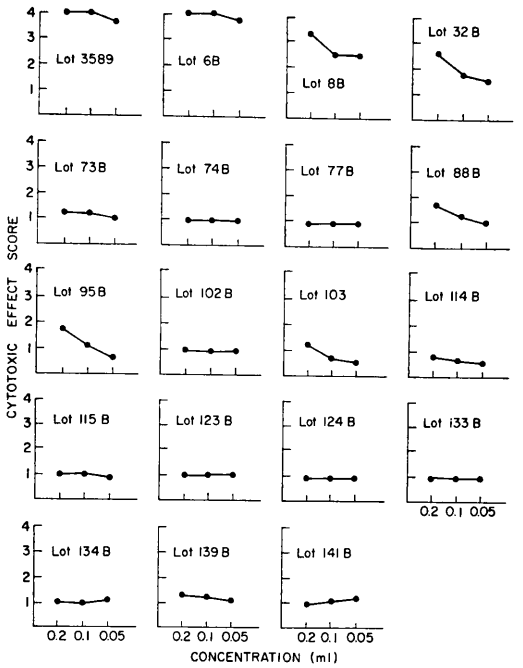


FIG. 3. The results of the cell culture assay of 19 lots of Arlacel A in L-929 cells.

ing the percentage of weight change of the individual mouse at Days 2, 3, and 7 relative to Day 0. If there were either a 10.5% weight retardation relative to the saline control mice or chemical peritonitis, the batch of AA under test was not acceptable.

*Free fatty acid assay.* The free fatty acid (FFA) content of each lot of AA was determined by our modified Duncombe procedure (16).

*Results. Cell culture assay.* The results obtained for 19 lots of AA tested in the L-929 cell assay are given in Table I and Fig. 3. Four lots were toxic. The two most toxic lots, 3589 and 6B, caused complete destruction of the cell cultures. Lot 8B caused marked CTE at each dose and lot 32B caused a marked CTE only at the highest dose. The remaining 15 lots were scored as nontoxic. Their CTE reactions ranged from $>0.5$ to $<2.0$.

Similar scores were obtained with the nine lots tested in each of the six cell cultures. The same characteristic CTE was seen in each of the cell lines. However, primary cell cultures contained excessive amounts of tissue debris and were less suitable for the assay.

*Mouse assay.* The results of mouse assays of the 19 lots of AA are given in Table I. Table II illustrates the procedure used to calculate the percentage of difference in weight change between AA and saline control mice and shows the reaction to toxic and nontoxic AA. Three of four lots toxic by cell culture assay were toxic by the mouse assay (Table I). Lots 3589 and 6B were the most toxic, causing death in 6 of 10 and 10 of 10 mice, respectively. Surviving 3589 mice showed marked weight retardation and marked peritoneal adhesions. Some of the mice that received toxic lots of AA, also, had enlarged spleens, rounded margins of the livers, and hemorrhagic lesions of the lungs. The remaining 16 lots, including lot 32B, were nontoxic although differences in weight gain from lot to lot occurred. A single fibrin band was seen in one animal that received lot 124B and in another that received lot 141B; however, in second experiments no adhesions were found and the lots were considered nontoxic.

*Free fatty acid assay.* The FFA content of each lot of AA is shown in Table I. The largest amounts of FFA were found in the most toxic lots; 3589, 6B, and 8B contained $>500$, $>500$, and 282 $\mu$moles/ml, respectively. The remaining 16 lots contained $<60$ $\mu$moles/ml of FFA.

*Discussion.* For a number of years the Berlin mouse assay (8) has been used to specify the acceptability of a lot of AA for clinical use (17, 18). Criteria of toxicity are weight retardation and peritoneal adhesions.

This report describes an assay in which the L-929 cell line was used to evaluate AA toxicity and compares the results of the cell culture assay to those of our modified mouse assay. Similar results were obtained with 18 of the 19 lots examined: 3 lots were toxic and 15 lots were nontoxic by both procedures. The one exception was lot 32B which was toxic only by the cell culture assay. Lot 32B caused a cytotoxic reaction at the highest test dose but not at lower doses. Moreover, similar results were seen in each of six cell lines employed indicating that the cytotoxic reac-

TABLE II. Example of Average Weight and Average Percentage of Weight Difference Between Mice Injected with Toxic and Nontoxic Arlacel A and with Saline.

| Test material | Day 0 | Day 1 | | | Day 2 | | | Day 3 | | | Day 7 | | | Day 14 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Av wt (g) | Av wt[a] (g) | % Av Δ[b] | % Diff from saline[c] | Av wt (g) | % Av Δ | % Diff from saline | Av wt (g) | % Av Δ | % Diff from saline | Av wt (g) | % Av Δ | % Diff from saline | Av wt (g) | % Av Δ | % Diff from saline |
| Saline | 13.6 | 14.5 | +6.6 | | 15.5 | +14.2 | | 16.4 | +20.7 | | 19.8 | +45.4 | | 23.5 | +72.5 | |
| AA Lot 8B | 13.5 | 12.0 | −11.1 | −17.7 | 12.2 | −7.3 | −21.5 | 12.9 | −4.6 | −25.3 | 16.4 | +21.2 | −24.2 | 21.9 | +61.4 | −11.1 |
| AA Lot 88B | 13.5 | 13.4 | −0.2 | −6.8 | 14.6 | +8.6 | −5.6 | 16.0 | +18.6 | −2.1 | 19.2 | +42.3 | −3.1 | 24.3 | +80.0 | +7.5 |

[a] Average weight of the survivors of ten animals injected with the test material.
[b] Percentage of weight gain (+) or weight loss (−) relative to the average weight of the group of mice on day of injection.
[c] Difference in percentage of average weight change of mice that received Arlacel A and saline.

tion of AA was not cell dependent. The findings show that the cell culture assay is equally if not more sensitive than the mouse assay.

Since a graded dose response was obtained with some lots, the three-dose cell culture assay may provide a useful procedure for comparing relative toxicities of AA lots.

A relationship has been suggested between the FFA content and AA toxicity (7, 19, 20) and is supported by the results of our cell culture and mouse assays. The toxic lots which were the oldest had been stored at room temperature and contained the highest amounts of FFA. The nontoxic lots which were more recently prepared and had been stored at 4° contained the lowest amounts of FFA.

Preliminary studies indicate that palmitoleic and oleic acids can cause severe cytotoxic reactions in cells. The CTE produced by these fatty acids in Drakeol 6-VR were similar or identical to the CTE produced by toxic lots of AA; Drakeol 6-VR did not cause CTE (unpublished data). Hardegree and Kirschstein (20) reported that palmitoleic and oleic acids were the major FFA present in AA. However, their study indicated that there may be additional toxic substances present in AA. Therefore, in addition to FFA, other byproducts of manufacture or hydrolysis and degradation must be considered in evaluating the factors responsible for the toxicity of AA.

*Summary.* An assay using the L-929 mouse fibroblast cell culture line was described to evaluate the toxicity of Arlacel A. The toxic lots of AA produced a characteristic cytotoxic effect consisting of intracytoplasmic refractile bodies. The results obtained by the cell culture and mouse assays were similar for 18 of 19 lots tested; one lot was moderately toxic by the cell culture assay only. A correlation was seen between the amount of FFA and the toxicity of AA. The cell culture assay provides a sensitive and reproducible assay to evaluate AA toxicity.

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