Use of Lymphoblastoid Namalva Cell Cultures in a Toxicity Test. Application to the Monitoring of Detoxification Procedures for Fluorocarbons to be used as Intravascular Oxygen-Carriers

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Abstract: Cultures of lymphoblastoid Namalva cells have been used to check the absence of toxic impurities in fluorocarbons destined for biomedical applications. The test comprises the measurement of growth rate and viability in the presence of the fluorocarbon, and of growth after reseeding. It was used to establish a procedure for the detoxification of a family of fluorocarbons by evaluating the effect on toxicity of a series of individual or combined treatments. A convenient, rapid and industrially feasible procedure was developed which includes washing with 10 % aqueous KOH, shaking with charcoal and filtration over alumina.

Fluorocarbons, when used in blood substitutes, are administered to the patients in huge amounts: several hundred grams at a time. Reliable standard toxicity tests for the control of these and other ingredients involved in the preparation of oxygen carriers for biomedical applications, i.e. in blood substitution, cardioplegia, organ perfusion, diagnosis, etc. (1), are therefore required, as their testing and clinical evaluation develop. While fluorocarbons in the relevant molecular weight range (2) appear to be essentially non-toxic when pure, the crude products are usually found to be more or less toxic, depending on the compound, the source, the batch, etc. This toxicity may be responsible for some of the untoward reactions reported in the earlier stages of this research, as well as for discrepancies in the observations made in distinct laboratories. The need for reliable toxicity tests has therefore been stressed repeatedly (3, 4). It is particularly crucial when mixtures of fluorocarbons, prepared by chemical routes that involve the presence of fluoride ions, are used. Some of these were reported to require refluxing for several days with a KOH/HNR_2 mixture, as well as preparative gas chromatography (5). Even fluorocarbons that seem to be pure according to the standard chromatographic and spectroscopic analytical criteria of the chemists, generally display toxicity, especially toward cell cultures, which appear to be particularly sensitive to toxic impurities. It has therefore been recommended that the first test of biological acceptance of these biomaterials should be that of their innocuousness toward bacteria or cell cultures (3). Such cultures, and especially strain L mouse fibroblasts, have indeed been used for testing the toxicity of fluorocarbons used in blood substitute research (3).

We propose here the use of lymphoblastoid Namalva cell cultures for such tests and, as an illustration, their application to a detoxification procedure that proved satisfactory for a series of 1,2-bis(*F*-alkyl)ethenes selected as O_2-carriers for biomedical applications (6). For this, the efficacy of various detoxification steps, or combinations of steps, has been evaluated by measuring the influence of the treated fluorocarbons on growth rate and cell viability. The same approach is applicable to other fluorocarbons and to some of the other ingredients of oxygen carrying preparations destined for intravascular use.

The Namalva cell strain was chosen because of its availability (it was used to produce human interferon) (7), because of the stability and reproducibility of its growth parameters, and because it grows in suspension without need of solid support, which eliminates one parameter that can interfere with the growth rate when the test compounds display surface-active properties, as is the case with many fluorocarbon derivatives, surfactants, and their emulsions; moreover, cell growth in suspension facilitates the sampling of aliquots. It was shown in a previous study that an underlying layer of F-66E, one of the 1,2-bis(perfluoroalkyl)ethenes developed in our laboratory, can serve as a fluid gas carrier for conveniently controlling the O_2-supply and CO_2-content, and hence the pH of Namalva cell cultures; high cells yield can thus be obtained in the absence of any atmosphere (8). The initial experiments showed, however, that in spite of careful distillation and analytical controls, some batches of the fluorocarbon were toxic in a non-reproducible way, which led us to devise a detoxification procedure that was subsequently extended to other perfluorochemicals and adapted to other components of the blood replacement media. Another study showed that a large variety of chemicals are indeed soluble in minute but definite amounts in such fluorocarbons (9). The use of fluorocarbons as O_2-carrying media for culturing aerobic microorganisms has also been reported (10).

Materials and Methods

The Toxicity Test

The Namalva cell strain was a gift from Dr. H. Marcovich (Laboratoire de Physiologie de la Réplication, Institut Pasteur, Paris). The cells were grown in suspension in RPMI-1640 medium (from Gibco, ref. N 340-1800) containing 360 ppm Fungizone® and 5 ppm Aureomycine® as antibiotics in tissue culture flasks; 10 % v/v of reconstituted fetal calf serum (from Gibco, ref. 629) was used as growth factor.

The initial cell count, determined before filling the flasks, was 2 to 4×10^5 ml^{-1}. The flasks were incubated at 37 °C under an atmosphere of air containing 7 % (v/v) carbon dioxide. The cells were counted in Malassez cells after dilution to 1/10 in physiological serum. The growth rate (GR) was calculated from the cell counts by the expression $GR = (N_2-N_1)/N_1$. The viability of the culture was estimated by the Trypan Blue dye exclusion test (12) and was expressed as the ratio of living (non-colored) cells to the total number of cells.

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In a typical toxicity test, a series of ten flasks was filled with 2 ml of the culture medium containing N_1 cells and 2 ml of the compound to be tested (or, for the reference series, 2 ml water detoxified over Amberlite IRA 400 (anionic) and IR 120 (cationic) ion-exchange resins. After 3 days of incubation, the cells were counted (N_2) and the viability of the culture determined in each flask of both series. In the test series, the fluorinated compound was then removed, the cells washed with saline, reinoculated at a concentration of 2 to 4 × 10^5 ml^{-1} in 4 ml culture medium and resuspended by gentle shaking. After 3 days, the growth rate and viability were measured again. A statistical study of 60 experiments showed that, in the above mentioned conditions, the average growth rate was 9, with a mean standard deviation of 3. Thus, the results of a toxicity test were taken into account only when the growth rates in the reference series were within these limits.

Typical Detoxification Procedure

The perfluorochemical (1 liter) was first stirred for 2 h at room temperature in the presence of 250 ml 10 % (w/v) aqueous KOH. The underlying fluorocarbon phase was separated by decantation, then stirred for 2 h at room temperature with 10 g activated charcoal (from Sigma, ref. C 5385), decanted and filtered through a phase-separation Whatman paper. Finally, it was filtered over a small (25 mm diameter) column of 100 g Al_2O_3 (from Merck, ref. 1097). The detoxified fluorocarbons were stored in tightly stoppered bottles.

Results and Discussion

The human Namalva cell line was derived from a Burkitt lymphoma biopsy and has been studied since 1972 (11). The establishment of the strain is poorly documented, but its culture conditions are well known (7). In our hands (Fig. 1), the growth rate remains nearly constant with a doubling time of approximately 20 h at 37°C over one week, and the viability stays near 100 % for 5 days. From these results, the standard procedure for a cellular toxicity test was defined as the comparison of the growth rate and viability of two series, each of 5 to 10 samples of 2 ml of culture medium containing initially ca 3 x 10^5 cells, after 3 days in the presence or in the absence of 2 ml of the fluorocarbon to be tested.

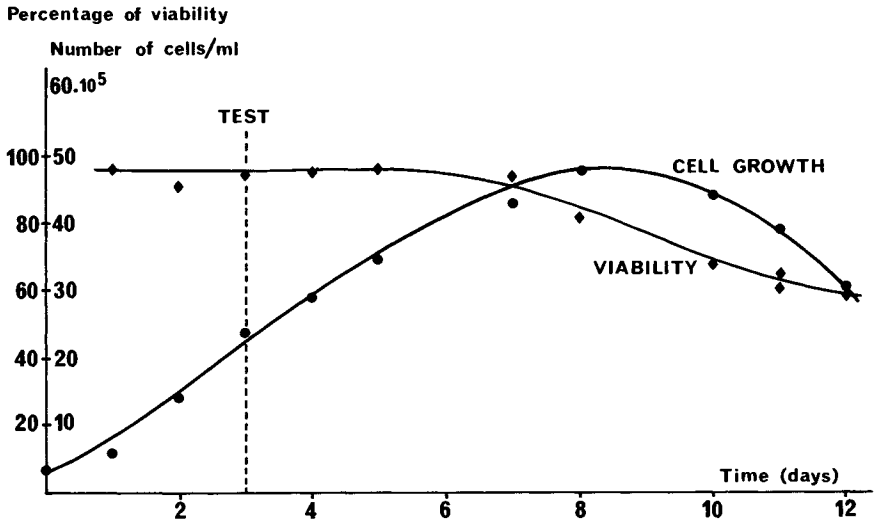

Fig. 1 Time course of the growth and viability of a typical Namalva cell culture at 37°C.

Table I. Testing of Various Detoxification Procedures by Means of Namalva Cell Cultures. The Results are Presented as Follows: growth rate with respect to controls / viability with respect to controls " growth with respect to controls after reseeding.

Treatment	Compounds				
	F-44E	F-46E	F-66E	F-i34E	F-i36E
None	0/ 0" 0	0/ 0" 0	0/ 0" 0	0/ 0" 0	0/ 0" 0
Dialysis 24 h	43/79" 82	0/ 0" 0	10/46" 0	41/69" 16	0/ 0" 0
aq. $NaHCO_3$ 0.025 N + aq. NH_4Cl 0.025 N washings	0/ 0" 0	0/ 0" 0	0/ 0" 0	0/ 0" 0	0/ 0" 0
aq. KOH 5 w/v % washing	0/ 0" 0	32/ 0" 0	26/ 0" 0	23/ 3" 0	54/ 0" 0
aq. KOH 10 w/v % washing	14/55" 54	23/70"46	23/70"31	50/94" 18	29/22" 0
aq. KOH 10 w/v % washing, then dialysis	94/93"106	56/81"78	43/78"56	62/88" 83	41/85" 73
aq. KOH 10 w/v % washing, then shaking with activated charcoal	68/91" 93	47/82"86	43/80"52	53/70" 50	71/90" 20
aq. KOH 10 w/v % washing, then shaking with activated charcoal, then filtration on alumina (n = number of samples)	95/94"107 (n = 10)	103/90"90 (n = 2)	92/92"95 (n = 6)	103/90"100 (n = 2)	85/96"108 (n = 4)

In addition, an aliquot of the tested cell cultures was freed from fluorocarbon and regrown in the reference medium; the cells were counted after 3 more days, in order to detect a possible accumulation of toxicity in the cells, or a toxicity that blocks cell reproduction. This event was observed in a few cases (see for example F-i36E in Table I), although the growth rates and viability parameters appeared close to normal after the first part of the test.

Table I presents examples of chemical treatments applied to one sample (chosen among the definitely toxic ones) of each of the fluorocarbons listed. It shows the efficacy of each treatment, and how a standard detoxification procedure was established. The latter was then applied to all available samples from different batches, some produced in our laboratory, others obtained from a commercial source (Atochem). The figures given in the last line of Table I represent the *average* measurements found for all these samples, their number being shown in parentheses.

The chemical purity of the fluorocarbons tested was found by GLC to be over 99.7 %. Despite comparable

chromatograms, different batches of the same compound sometimes displayed considerably different degrees of toxicity. It was also noted that there is no direct relation between chemical purity and toxicity; some batches of 98 % pure F-44E were less toxic to the cell cultures than others that were chromatographically 99.99 % pure. Owing to their preparation procedures the 1,2-bis(*F*-alkyl)-ethenes can contain trace amounts of $R_FCH_2CHIR'_F$ and I_2 that may be toxic. These impurities are expected to be eliminated by alkaline treatment; such treatment was indeed found necessary, but was usually not sufficient. Prolonged stirring with 5 w/v % aqueous $NaHCO_3$ (then NH_4Cl) or 5 w/v % aqueous KOH was, however, not efficacious; washing with 10 % w/v % of aqueous KOH was more effective. Better results were obtained when the washing with 10 % KOH was followed by dialysis against distilled water, which allows the removal of water soluble material, including traces of KOH. A similar result was obtained when the alkaline washing was followed by shaking with activated charcoal. The latter treatment was preferred because it is easier to perform and to scale up. A practical requirement is indeed that the detoxification procedure must be economical and industrially feasible on a multi-ton scale. Full satisfaction was finally reached when this treatment was followed by filtration over a small column of Al_2O_3, which retains not only water soluble polar impurities and traces of iodine, but also the charcoal particles. Alumina alone was not sufficient; washing with 10 % KOH, then filtration over alumina, sometimes was. The final procedure included the following steps: washing with 10 % aqueous KOH, then shaking with charcoal, then filtration over alumina. It has the advantage of removing a wide spectrum of impurities with a convenient and rapid procedure. The complete procedure was successfully applied to 24 samples (last line of Table I. Each value given in this last line is an average of 20 to 60 measurements).

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