A COMPARISON OF THE SENSITIVITY OF CELL CULTURES TO DIPHTHERIA TOXIN BY THE DYE-UPTAKE METHOD

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THE sensitivity to diphtheria toxin of cells grown in tissue culture is directly related to the susceptibility of the animal from which the cells are derived (Gabliks and Solotorovsky, 1962; Pappenheimer, Collier and Miller, 1965). Thus, cultures of cells from mice and rats are much less sensitive to diphtheria toxin than cells from susceptible animals such as guinea-pigs and monkeys (Lennox and Kaplan, 1957; Sousa and Evans, 1957; Gabliks and Falconer, 1966). These differences in sensitivity have been studied by observations on the cytotoxic effect of diphtheria toxin titrated in cell cultures.

In the present paper, the cytotoxic technique was compared with the dye-uptake method, introduced by Finter (1969) for the titration of interferon. The dye-uptake method, which depends on the failure of dead cells to stain with neutral red, gave reproducible results when applied to the titration of diphtheria toxin in cell cultures, and was used to compare the susceptibility of 16 cell cultures from nine animal species. The absorption of toxin to cells of differing toxin sensitivity was also investigated by this technique.

MATERIALS AND METHODS

Cell cultures. Tissue cultures were grown in one or other of three media. The media consisted of Eagle's minimum essential medium (MEM) with 0.44 g per l of sodium bicarbonate, antibiotics (100 units per ml of penicillin and 100 µg per ml of streptomycin) and supplements as follows.

Medium A—10 per cent. inactivated calf serum. Medium B—15 per cent. unheated foetal bovine serum (Flow Laboratories Inc., Irvine, Scotland). Medium C—10 per cent. unheated foetal bovine serum and 10 per cent. tryptose phosphate broth.

Primary-cell cultures were prepared from whole embryos of rabbits, guinea-pigs, Swiss and CBA mice, rats and hamsters. The embryos were minced and treated with 0.25 per cent. trypsin in phosphate-buffered saline (PBS), pH 7.4; the dispersed cells were then grown in medium A. Patas monkey kidney cells, obtained as a cell suspension from Wellcome Laboratories, Beckenham, Kent, and chick-embryo fibroblasts, obtained by trypsinisation of embryos from 10-day eggs were also grown in Medium A.

An established line of normal mouse cells (L-cells, NCTC929) was obtained from Flow Laboratories Inc., and a line of monkey kidney cells (BSC-1 cells) from the Wellcome Laboratories; these cell-lines were grown in Medium B. A line of human tumour cells (HEp-2 cells) was cultured in Medium A, a second continuous line of monkey kidney cells (Vero cells) (Flow Laboratories, Inc.) in Medium C, and a diploid line of normal, human fibroblasts (WI-38) in Medium B. All the cell-lines had undergone a known number of tissue-culture passages in this laboratory, and a further unknown number of passages in other laboratories.

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A continuous line of SV40 virus-induced hamster tumour cells, established from a tumour induced *in vivo*, was obtained from Flow Laboratories, Inc., and grown in Medium B. Adenovirus type 12-induced CBA mouse tumour cells, grown in Medium C, were from a cell-line established in this laboratory from a transplanted CBA mouse tumour induced by adenovirus type 12. These tumour cell-lines were composed entirely of virus-induced tumour cells, since specific virus-induced tumour antigen was demonstrable in all the cells by immunofluorescence tests (Pope and Rowe, 1964).

Most of the primary cell-cultures and continuous cell-lines were grown in 20-oz. (570 ml) medical flat bottles at 37°C. Tube cultures were prepared from bottles with confluent monolayers; the cells were removed with 0.25 per cent. trypsin, diluted to a concentration of $(1-5) \times 10^5$ cells per ml in growth medium and dispensed into $6 \times \frac{5}{8}$ in. (15×1.5 cm) Pyrex test-tubes, which were then incubated in a stationary position at 37°C. The primary monkey kidney cells and the chick-embryo fibroblasts were always grown initially in test-tubes. Confluent monolayer tube cultures for diphtheria toxin titrations were all maintained in Eagle's MEM containing 2 per cent. inactivated calf serum, 0.88 g per l of sodium bicarbonate and antibiotics.

Diphtheria toxin. A single pool of partially purified diphtheria toxin was used throughout. The toxin was kindly supplied by the Aerobic Bacteriology Department, Wellcome Research Laboratories, Beckenham, and had an Lf titre of 1200 units per ml. Tests showed that the titre of this toxin preparation, estimated in BSC-1 cells, did not alter during the period of the study.

Assay of diphtheria toxin. The cytotoxic titre of diphtheria toxin was measured in monolayer cell-cultures grown in $6 \times \frac{5}{8}$ in. (15×1.5 cm) test-tubes. The growth medium was decanted and replaced with 1.0 ml of maintenance medium containing diphtheria toxin in a range of ten-fold dilutions; six tubes were used for each dilution of toxin. The tubes were incubated in a stationary position at 37°C for 96 hr and were then examined for evidence of cytotoxicity. Cytotoxic effects involving 75 per cent. or more of the cells were scored as 4+, 50-75 per cent. as 3+, 25-50 per cent. as 2+ and less than 25 per cent. as 1+. The cytotoxic titre was calculated by the method of Reed and Muench (1938).

After the cytotoxic effect of the toxin had been recorded, the same cultures were used to measure the uptake of neutral red, and so to calculate the 50 per cent. dye-uptake titre of the toxin (DU50). The maintenance medium was decanted from the tubes and the cells were incubated with 1.0 ml of 0.003 per cent. (w/v) neutral red in PBS, pH 7.4, at 37°C for 2 hr. Excess dye was then decanted, the cells were washed twice with PBS and the tubes were inverted and left to drain for 2 hr at room temperature. After this time the neutral red was eluted from the cells of each tube into 1.0 ml of ethanol-citrate buffer, pH 4.2 (Finter). The neutral red eluates for each dilution were pooled, and the concentration of the neutral red measured with a spectrophotometer at 540 nm. The spectrophotometric readings were plotted against the toxin dilution, and the DU50 titre of the diphtheria toxin for the cells under test was expressed as the reciprocal of the dilution that gave 50 per cent. dye-uptake by optical density, as compared with control cells incubated in parallel without toxin.

Toxin adsorption studies. Diphtheria toxin was added to cells suspended in maintenance media. After incubation for varying periods of time with frequent agitation, the cells were removed by centrifugation at 1000 r.p.m. for 10 min., and the unadsorbed diphtheria toxin content of the supernatant fluid was titrated in BSC-1 cells by the dye-uptake (DU50) technique.

RESULTS

Assay of diphtheria toxin in cell cultures

Diphtheria toxin was first assayed in Vero cells, mouse-embryo cells and rat-embryo cells by the cytotoxic method. Vero cells were highly sensitive to diphtheria toxin and showed complete degeneration (4+) at toxin dilutions of 10^{-8} and some cytotoxic effects (1+) at dilutions of 10^{-9} . The mouse and rat cells were relatively resistant. Thus, complete degeneration (4+) of mouse

cells was observed in the cell cultures exposed to toxin at a dilution of 10^{-2} , and 2+ cytotoxic activity at a dilution of 10^{-3} . Rat cells were completely destroyed (4+) by toxin at a dilution of 10^{-3} , and were slightly affected (1+) at 10^{-4} . The titre of the diphtheria toxin, expressed as the reciprocal of the highest toxin dilution to give detectable cytotoxic effects (Reed and Muench), was $10^{9.5}$ per ml in Vero cells, $10^{4.5}$ per ml in rat cells and $10^{3.5}$ per ml in mouse cells. To judge from the highest toxin dilution to produce a 50 per cent. cytotoxic effect (2+), the titres obtained were $10^{8.5}$ per ml in Vero cells and $10^{3.5}$ per ml in both rat cells and mouse cells.

FIG. 1.—Sensitivity of mouse, rat and Vero cells to diphtheria toxin by the dye-uptake method. ▲—▲ = Swiss mouse cells; ○—○ = rat cells; ●—● = Vero cells. The dotted lines measure the sensitivity of the cells, expressed as the highest dilution of toxin required to reduce uptake of neutral red by 50 per cent., as compared with normal cells untreated with toxin.

The titre of diphtheria toxin for Vero, rat and mouse cells was also calculated by the DU50 method. These results are given in fig. 1. The dilution of toxin that reduced dye-uptake by 50 per cent. (DU50) for Vero cells was $10^{-8.78}$, and $10^{-3.68}$ and $10^{-2.78}$ for rat cells and mouse cells respectively. This technique allowed titres of diphtheria toxin to be measured continuously over the whole range of toxin dilutions rather than in logarithmic steps as in the cytotoxic test. The method also clearly showed a difference in the susceptibility of rat and mouse cells. The results obtained by the DU50 technique were highly reproducible; in four independent titrations of diphtheria toxin in BSC-1 cells, the titre varied between $10^{8.95}$ and $10^{9.27}$ DU50 per ml, with an average of $10^{9.06}$ per ml.

Comparison of the sensitivity of primary cells from different species to diphtheria toxin

Table I shows the in-vitro sensitivity to diphtheria toxin of cells from eight animal species. Sensitivity is expressed in terms of the highest dilution of toxin giving 50 per cent. cytotoxic effect, or a 50 per cent. reduction in uptake

of neutral red by the dye-uptake method, as compared with normal cells untreated with toxin. By the cytotoxic end-point technique, monkey kidney cells were the most sensitive, and cells from Swiss and CBA mice the least

TABLE I

Comparison of the cytotoxic and dye-uptake (DU50) methods for determining the susceptibility of eight cell-cultures to diphtheria toxin

Primary cell-cultures from	Number of in-vitro passages	Log ₁₀ dilution of diphtheria toxin giving 50 per cent. end-point* in the	
		cytotoxic method	DU50 method
Monkey	1	-9.5	-8.57
Guinea-pig	2	-7.5	-7.67
Rabbit	2	-7.5	-7.17
Hamster	2	-7.5	-6.42
Chicken	1	-6.5	-6.33
Rat	2	-4.5	-3.68
Mouse (Swiss)	2	-3.5	-2.78
Mouse (CBA)	2	-3.5	-2.74

* Expressed as the highest dilution of toxin to cause a 50 per cent. cytotoxic effect, or a 50 per cent. reduction in dye-uptake, as compared with normal untreated cells.

TABLE II

Comparison of the susceptibility of primary cells and established cell lines to diphtheria toxin

Animal species	Source of cell-culture	Number of in-vitro passages	Log ₁₀ dilution of diphtheria toxin giving 50 per cent. end-point* in the	
			cytotoxic method	DU50 method
Mouse	Embryo Swiss mice	2	-3.5	-2.78
	Embryo CBA mice	2	-3.5	-2.74
	L cell-line	46†	-2.5	-1.96
Rabbit	Embryo rabbit	2	-7.5	-7.17
	RK-13 cell-line	>200†	-8.5	-7.73
Monkey	Monkey kidney	1	-9.5	-8.57
	BSC-1 cell-line	28†	-9.5	-9.06
	Vero cell-line	43†	-9.5	-8.78

* Expressed as the highest dilution of toxin to cause a 50 per cent. cytotoxic effect, or a 50 per cent. reduction in dye-uptake, as compared with normal, untreated cells.

† Number of passages in this laboratory.

sensitive. Monkey cells were also the most sensitive by the DU50 technique. However, although guinea-pig, rabbit and hamster cells appeared to be equally sensitive to the toxin by the cytotoxic test, the guinea-pig and rabbit cells proved to be more sensitive than the hamster cells by the DU50 method. The

cytotoxic test showed that cells from Swiss and CBA mice were equally sensitive to diphtheria toxin, and this was confirmed by the DU50 test.

Comparison of the sensitivity of primary cells and cell-lines to diphtheria toxin

Tests were carried out to see if prolonged growth *in vitro* led to a change in the sensitivity of cells to diphtheria toxin. Established cell-lines and primary cells from mice, rabbits and monkeys were compared, and the results are

TABLE III

Comparison of the susceptibility of normal and tumour cells to diphtheria toxin

Animal species	Source of cell-culture	Number of in-vitro passages	Log ₁₀ dilution of diphtheria toxin giving 50 per cent. end-point* in the	
			cytotoxic method	DU50 method
Mouse	Normal CBA mouse Adenovirus type 12-induced tumour cells (CBA)	2	—3·5	—2·85
		32	—3·5	—2·32
Hamster	Normal hamster SV40 virus-induced tumour cells (hamster)	2	—6·5	—6·42
		40†	—6·5	—6·4
Man	WI-38 HEP-2	12†	—9·5	—9·16
		>200†	—9·5	—8·72

* Expressed as the highest dilution of toxin to cause a 50 per cent. cytotoxic effect, or a 50 per cent. reduction in dye-uptake, as compared with normal, untreated cells.

† Number of passages in this laboratory.

recorded in table II. Titrations of diphtheria toxin in primary cells from Swiss and CBA mice gave DU50 titres of $10^{2.78}$ per ml and $10^{2.74}$ per ml respectively, whereas L-cells, after 46 passages in this laboratory, were less sensitive to toxin, giving a titre of $10^{1.96}$ DU50 per ml. However, in the case of primary rabbit-embryo cells and RK-13 cells the cell-line was the more sensitive to toxin. Similarly, the line of BSC-1 cells was more sensitive to toxin than primary monkey kidney cells, but the line of Vero cells had approximately the same sensitivity as the primary monkey cells.

Comparison of the susceptibility of normal cells and tumour cells to diphtheria toxin

The susceptibility of normal cells to diphtheria toxin was compared with that of tumour cells derived from the same animal species. The results are shown in table III. By the cytotoxic test, cells from a transplanted CBA

mouse tumour, induced by adenovirus type 12, and cells from a transplanted hamster tumour, induced by simian virus SV40, showed the same sensitivity as normal fibroblast cells from the homologous species. Similarly, normal human fibroblasts (W1-38) showed the same sensitivity to toxin as HEp-2 cells. By the DU50 technique, however, both mouse and human tumour cells were demonstrably less sensitive than the homologous normal cells; simian virus SV40-induced hamster tumour cells showed the same sensitivity as normal hamster fibroblasts.

FIG. 2.—Adsorption of diphtheria toxin to mouse, rat and monkey cells. The curves show the amount of free toxin, measured by the dye-uptake method, still present in the supernatant fluids after a 3-hr period of adsorption with cells. ▲—▲ = Monkey BSC-1 cells; △—△ = rat cells; ○—○ = mouse cells; ●—● = control, unadsorbed toxin.

The possible role of adsorption in determining the susceptibility of cells to diphtheria toxin

Tests were carried out to find out whether the susceptibility of cells to diphtheria toxin was related to the ability of the toxin to adsorb to the cell surfaces. In preliminary experiments, it was observed that the adsorption of toxin to sensitive cells was very rapid. Thus, when 100 DU50 units of diphtheria toxin (measured by titration in BSC-1 cells) were added to BSC-1 cells and the mixture centrifuged immediately (1000 r.p.m. for 10 min.), all the toxin was found to have been removed with the cells.

To compare the adsorption of toxin by sensitive and insensitive cells, approximately 10^5 DU50 units of diphtheria toxin were added to 4.0×10^5

BSC-1 cells, and to the same numbers of L-cells and rat fibroblasts. After incubation at 37°C for 3 hr, the cells were centrifuged as before and the supernatant fluids titrated in BSC-1 cells. The results are shown in fig. 2. The DU50 titre of diphtheria toxin used in this test was $10^{6.8}$ per ml. Adsorption on L-cells removed less than 10 per cent. of it. In contrast, BSC-1 cells and rat cells removed 99.99 per cent. and 99.5 per cent. of the toxin respectively. Thus, the sensitivity of cells to diphtheria toxin appeared to be related to their ability to adsorb toxin.

DISCUSSION

The in-vitro cytotoxic effect of diphtheria toxin has been used by many workers to measure the sensitivity of different cells to the toxin (reviewed by Pappenheimer *et al.*, 1965; Solotorovsky and Gabliks, 1965). The dye-uptake method gives an objective assessment of the 50 per cent. end-point and as such was preferable to the cytotoxic method in which the end-point reading is more subjective.

In our hands, the DU50 method gave consistent results, and showed differences in the sensitivity of certain animal cells that were not discernible by the cytotoxic test. It is reasonable to suggest that the DU50 method would provide a more sensitive means of titrating serum antibody to diphtheria toxin than the cytotoxic method.

Using the DU50 technique, cell cultures from eight different animal species were compared. Human and monkey cells were found to be the most sensitive to diphtheria toxin. Guinea-pig and rabbit cells were less sensitive than hamster and chicken cells, and rat and mouse cells were least sensitive. These results confirmed and extended previous observations (Gabliks and Solotorovsky, 1962; Gabliks and Falconer, 1966). When established cell-lines and primary cells from the same animal species were compared mouse L-cells were found to be more resistant to diphtheria toxin than primary cells from Swiss or CBA mouse embryos. Gabliks and Solotorovsky reported similar findings with L-cells and also found that rabbit kidney cell-lines were more resistant to diphtheria toxin than homologous primary cell-cultures. However, in the present study, primary rabbit cells proved to be more resistant to toxin than established RK-13 cells. In addition, primary monkey kidney cells were more resistant than the line of BSC-1 cells. Thus, the lower sensitivity of some cell-lines to diphtheria toxin as compared with homologous primary cells may not be directly related to in-vitro passage. Long-term tissue culture can lead to either increase or decrease of sensitivity of the cells to diphtheria toxin.

In two instances tumour cells were found to be more resistant than homologous normal cells. In the third case, the tumour cells and the homologous normal cells were equally sensitive to toxin. However, these variant results may have been due to differences in the in-vitro passage history of the various cell-lines tested. Alternatively, the differences may have been due to specific changes at the cell surface or to the loss of cell receptors coincident with malignant transformation.

Previous studies have shown that the lethal action of diphtheria toxin on

cells is caused by interference with amino acid incorporation into polypeptides in the presence of nicotinamide adenine dinucleotide (Collier and Pappenheimer, 1964). This is due to the inactivation of transferase II, a labile, peptide bond-forming enzyme (Gasior and Moldave, 1965; Collier, 1967). Diphtheria toxin has been shown to adsorb very rapidly to sensitive HeLa cells (Duncan and Groman, 1969), and, in the present study, to BSC-1 cells. However, the quantity of toxin adsorbed varies with different cell-systems. Thus, L-cells adsorb less diphtheria toxin than BSC-1 cells, and this is directly related to the sensitivity of the cells to the lethal effects of the toxin. This finding confirms the earlier observations of Pappenheimer and Brown (1968) who used autoradiographic techniques with ^{125}I -labelled toxin. Whether the variable sensitivity of cells to the action of diphtheria toxin can be explained entirely by differences in the adsorption of toxin to cell receptors cannot be deduced from the present results.

SUMMARY

Diphtheria toxin was titrated in primary cells, cell-lines and known tumour cell-cultures from nine animal species. The effects of toxin were assayed by the conventional cytotoxic test and by a dye-uptake method. Both tests placed the various cell-cultures in the same order of sensitivity to toxin, reflecting the sensitivity of the donor animals. The dye-uptake technique was found to be as reproducible as the cytotoxic test, and more precise, for calculating the sensitivity of cell cultures to diphtheria toxin.

Comparison of primary cells and cell-lines from the same animal species showed no constant pattern of sensitivity to toxin. For example, primary mouse cells were more sensitive than a mouse cell-line, and vice versa with rabbit cells. There was also variability in relative sensitivity of normal and tumour cells from the same species.

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