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GROWTH AND TRANSPLANTABILITY OF CLONALLY RELATED TUMORIGENIC MURINE CELL LINES

J. R. WALKER*

From The University of Sheffield, Sub-Department of Medical Genetics, Sheffield

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Summary.—A malignant cell line derived from the s.c. inoculation of Adenovirus 12 into a CBA mouse has been isolated *in vitro*, cloned, and within 10 passages the clones have been investigated for their karyotype, morphology, growth rate, saturation density and response to plant lectin *in vitro*, and their tumorigenicity and growth rate *in vivo*. The cell lines rapidly acquired a highly heterogeneous karyotype, but remained homogeneous with respect to more complex physiological parameters.

Examination of the cellular characteristics has indicated that the rate of growth of the cell lines *in vivo*, but not their tumorigenicity, may be related to their *in vitro* potentials.

The clones responded differently to the cytotoxic effects of concanavalin A, but there was no correlation between the effect of the lectin and the malignant potential of the cells.

TRANSPLANTABLE mammalian tumour cell lines exhibit a variety of *in vitro* characteristics which differentiate them from their non-transformed counterparts. Many studies have attempted to identify which of these characters are indicators of the tumorigenicity of the cells upon inoculation into syngeneic host animals. More specifically, comparisons have been made between tumorigenicity and *in vitro* growth rates (McFarland *et al.*, 1974; Gallimore *et al.*, 1977), *in vivo* growth rates (Hosokawa *et al.*, 1975), saturation density (Stephenson *et al.*, 1973; Berman, 1975), serum dependence (Clarke *et al.*, 1974) anchorage-independent growth (Shin *et al.*, 1975 McFarland *et al.*, 1974; Gallimore *et al.*, 1977) and interaction with the lectin and concanavalin A (Lehman & Bloustein, 1974; Berman, 1975). Many of these parameters are independent; selection pressure to isolate revertants of each character frequently leads to segregation of the different aspects of the transformed phenotype (Vogel *et al.*, 1973; Vogel & Pollack, 1973). However, not one of these studies has utilized cell lines of recent common ancestry, and divergence during culture may complicate interpretation of the results.

This communication describes the growth properties, both *in vivo* and *in vitro*, of a series of clonally related cell lines which were derived, without conscious selection pressure, from a parental adenovirus-12-transformed transplantable murine tumour cell line, CBAT. All experimental work was completed within 10 passages of the cloning event, to preclude, as far as possible, divergence of the clones from the stem cell.

MATERIALS AND METHODS

Cell lines.—The original tumour was obtained by the s.c. inoculation of 0·1 ml high-titre adenovirus 12 into a thymectomized newborn CBA mouse several years ago (Potter and Oxford, 1970) and passaged at 2–3-week intervals *in vivo* prior to the establishment of a tissue culture line by the procedure of Buonassisi *et al.* (1962). At passage 66, when the parental line represented a well adapted experimental tumorigenic cell line, clones were obtained by seeding cells thinly into a

* Now at University of Warwick, Department of Biological Sciences, Coventry, CV4 7AL.

Petri dish and ring-cloning the resultant colonies. All cell lines were grown in Eagle's minimum essential medium supplemented with 20% foetal bovine serum and subcultured 1:3 twice weekly after disaggregation with trypsin/versene.

Karyotype.—Karyotypes of the clones were prepared using conventional techniques: cells were accumulated in metaphase by the addition of 0·1 μg/ml colcemid for 4 h, they were detached from the substrate with trypsin/versene, and treated for 10 min with a hypotonic solution of 25% foetal calf serum in water, pelleted and fixed in 3:1 ethanol: acetic acid. Fixed cells were dropped on to wet microscope slides, air dried and stained with lacto-acetic orcein.

Colony morphology.—The colony morphology of the clones was assessed by inoculating 100mm^2 square Petri dishes containing 4 sterile microscope slides with 10^5 cells. After incubation for 72 h, a temporary mount was made in growth medium and the colonies photographed under phase contrast with a Leitz Photomicroscope II.

Growth properties in vitro.—The population doubling times and saturation densities of the clones were determined in normal growth medium which was changed at 48 h intervals. Eight sterile 22 × 44 mm microscope cover glasses were lightly affixed with silicone grease into 100 mm^2 plastic Petri dishes and each dish inoculated with 3×10^6 cells. At intervals, one coverslip was removed from each dish, the cells were removed with versene and counted with a haemocytometer. This was continued until the saturation density was reached. Each experiment was performed several times and the population doubling time during the exponential-growth phase was calculated by linear regression.

Concanavalin A cytotoxicity.—To determine the cytotoxic effect of different levels of the lectin, concanavalin A, 18 50 mm Petri dishes were inoculated with 10^5 cells in growth medium and incubated for 24 h. The medium was aspirated, the cells washed twice with PBS, and serum-free MEM containing 0, 1, 2·5, 5, 10, 25, 50 and 100 μg/ml con A was added to dishes in duplicate. Control cultures with growth medium including serum were also set up. After 24 h the con A was removed, the cell sheet washed twice with PBS, disaggregated with versene and the amount of cellular protein determined by the method of Lowry *et al.* (1951).

To check that the effects observed were con A mediated and tumour-cell specific the assay was performed on mouse embryo primary cell cultures in the presence and absence of a specific con A inhibitor, α-methyl mannoside (So and Goldstein, 1967). In this case the protein was assayed *in situ* by the addition of the alkaline Lowry reagent directly to the washed cells in the dish.

In an experiment in which cell-free Petri dishes were incubated with con A in MEM, up to 30% of the lectin became bound to the dish; consequently all assays were corrected for this non-specific lectin binding.

Growth properties in vivo.—A 10-fold dilution series of cells was constructed from 10^7 to 10^4 cells/ml. Eight syngeneic CBA mice about 7 days after weaning were obtained from Sheffield University SPF Suite and inoculated s.c. with 0·1 ml of each dilution. The site of inoculation was palpated twice weekly and the presence and mean diameter of any tumours was recorded for a period of 12 weeks, by which time tumours had ceased to appear. The TD_{50} of the clones was calculated by the method of Reed & Muench (1938) the growth rate of the tumours arising from the 10^6 inoculum of each clone was subjected to an analysis of variance, and a pooled growth rate calculated where tenable (Rees & Westwood, 1974). The mean latent period was calculated from the time for tumours from the 10^6 inocula to reach minimum palpable size, ~1 mm mean diameter.

RESULTS

Karyological relationship

The karyotype of a typical cell is shown in Fig. 1. All clones analysed showed a hyperdiploid chromosome number, composed mainly of acrocentric and telocentric chromosomes, indistinguishable by orcein staining from normal mouse chromosomes. In addition, a number of metacentric marker chromosomes were present in most cells and, usually in a single copy, a market chromosome of complex morphology (m1) which exhibited 2 further constrictions in addition to the centromere.

Fifty cells were analysed from each clone, and the number of metacentric and

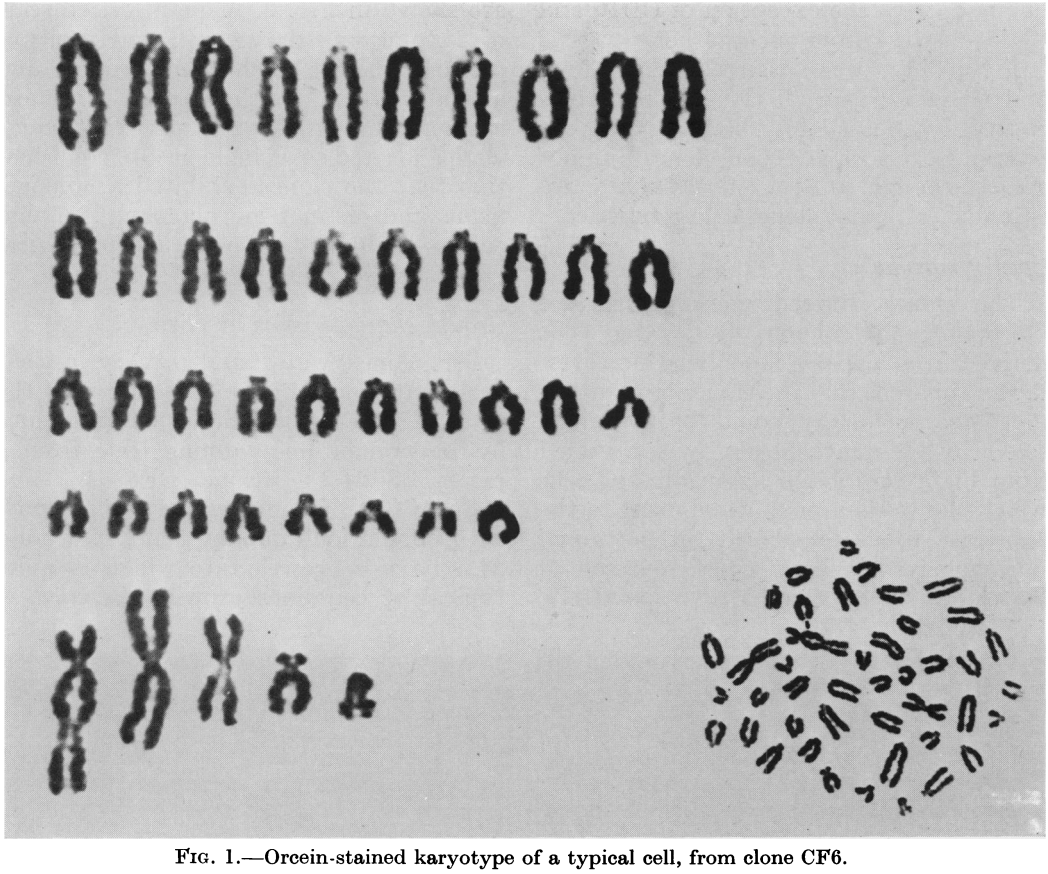

FIG. 1.—Orcein-stained karyotype of a typical cell, from clone CF6.

TABLE I.—*Chromosome distribution and heterogeneity of the parent line and clones*

Clone	Total chromosome mode	Marker m1 mode	Metacentric markers mode	% polyploidy	Total coefficient of variation
CBAT	47	1	3	7·1	3·9
CF5	45	1	3	7·0	4·0
CF6	43	1	2	19·4	6·3
CE11	48	1	3	9·7	2·3
CF4	44	1	3	5·4	3·6
CF10	45	1	3	11·0	6·5
CG6	45	1	6	8·9	3·6
CD11	44	1	2	3·6	5·3
CC11	47	1	3	5·5	3·9

m1 marker chromosomes and the total chromosome number, noted for each cell. The modal numbers are shown in Table I, from which it may be seen that they varied from 43 to 48, whilst the metacentric markers were usually present in 2 or 3 copies per cell. Clone CG6 was a notable exception and displayed up to 11 marker chromosomes and a modal number of 6 per cell. The m1 marker mode was constant at 1 per cell although values of 0–3 were encountered in individual cells.

A $2 \times k$ χ^2 analysis showed all clones to be cytogenetically distinct from the parent

line and, with the exception of CD10 and CD11, distinct from one another.

Polyploidy was assessed separately, and was 3–10% in all clones. The values observed varied slightly between different passage levels of any one clone, but not greatly enough to be a culture characteristic rather than a clonal characteristic.

Colony morphology

The clones differed markedly in the morphology of colonies of growing cells derived from a sparse inoculum. However, it was apparent that the clones were monomorphic and many could be identified solely by their morphology, which varied from highly refractile aggregates of cells which showed minimal attachment to the substrate to flattened cells of polygonal morphology. These extremes, represented by clones CD11 and CF10 respectively, are shown in Fig. 2. A quantitative scale of 1 to 6 was drawn up, and multiple photographs of each clone coded and allocated blind to the groups. In most cases all photographs of a particular clone were allocated to a single group, confirmation that the clones exhibited a homogeneous and distinct morphology. The allocation of the clones to the morphological groups is shown in Table II.

Growth characteristics in vitro

The clones were found to vary in their proliferation *in vitro*; measurement of the rates of growth showed a 2-fold difference in the population doubling time from a mean of 24·2 h (clone CF5) to 45·4 (clone CG6). The fastest growing clones with population doubling times of around 24 h have a growth rate which is quite typical of cell lines growing *in vitro*. A

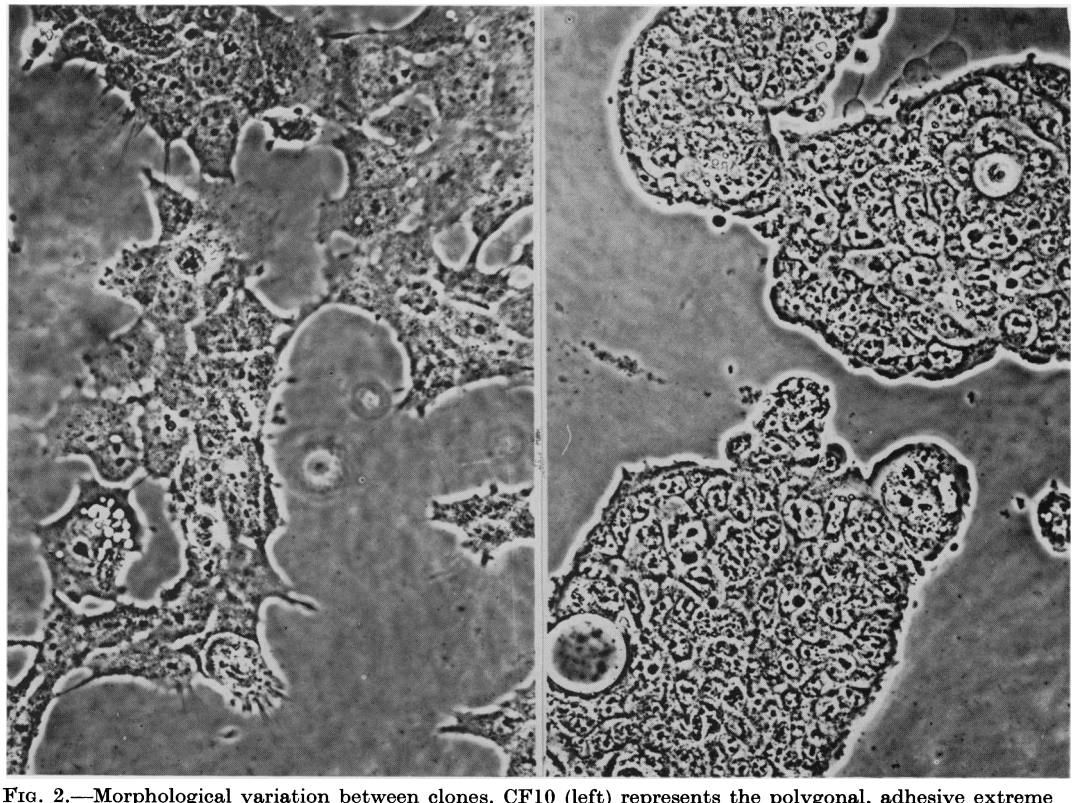

FIG. 2.—Morphological variation between clones. CF10 (left) represents the polygonal, adhesive extreme whilst CD11 (right) grows as tight aggregates of cells.

TABLE II.—*A summary of the properties of the cloned cell lines* in vitro *and* in vivo

	In vitro			*In vivo*		
Clone	Morpho-logical group	Populationg doubling time* (h)	Saturation density cells ($\times 10^4 cm^{-2}$)	TD_{50} cells ($\times 10^4$)	Volume doubling time† (h)	Mean latent period (weeks)
CF5	6	24·2±0·5	ND	2·5	27·6	2·4
CF6	6	40·3±5·8	90	5·0	109·4	5·0
CE11	2	28·0±4·6	31	10	18·5	2·0
CF4	5	ND	ND	32	68·3	5·0
CF10	6	36·2±5·9	111	32	37·5–112·4	4·5
CG6	4	45·4±1·8	ND	50	46·1	3·5
CD11	1	ND	ND	50	34·3–87·4	3·8
CC11	3	30·4±2·7	91	63	47·8	3·4

ND = not done.
* Mean of 3 or 6 determinations ± s.d.
† Pooled rate of 3–7 tumours where statistically valid, otherwise range.

TABLE III.—*Cytotoxic effect of* 24-h *exposure to concanavalin A*

	Clone							
Con A μg/ml	CF6	CE11	CD11	CF10	CC11	CF4	CG6	1° embryo cells
	Cellular protein (% lectin-free controls)							
1·0	92	92	101	112	110	107	118	103
2·5	92	76	108	93	94	109	125	100
5·0	86	60	105	83	87	91	113	95
10	81	48	80	96	87	93	93	100
25	76	41	80	75	85	116	111	107
50	78	30	73	72	91	126	130	105
100	66	19	75	67	76	120	128	108

time of 45 h represents a very slow growing cell line.

A similar variation in saturation density was also observed; the different clones reached their final cell density under the regimen employed in these experiments, at between 31 and 111×10^4 cells/cm^2. In contrast to the unremarkable growth rates observed in this series of clones, saturation densities in excess of 10^6/cm^2 are very high.

The population doubling times and saturation densities achieved by the clones are included in Table II.

Concanavalin A cytotoxicity

The response of the clones to a 24-h exposure to con A at different concentrations is presented in Table III; the levels of cellular protein are expressed as a percentage of those in dishes subjected to serum-free, lectin-free medium, and so reflect lectin-mediated cytotoxicity. Microscopic examination of treated cultures showed that increased lectin caused clumping and detachment of cells from the substrate. It was not determined directly whether the detached cells were viable, but in pulse experiments to high levels (100 μg/ml) of con A the cells did not recover from the lectin treatment.

The assay was performed several times on some clones and, as results were similar in each assay, the mean cytotoxicity is presented in such cases.

The response of primary cell cultures of Swiss mouse embryo to the lectin is included in Table III.

Growth characteristics in vivo

The tumorigenic potential of the clones differed, both in their ability to form tumours on inoculation into syngeneic hosts, and in the rate of growth of the tumours once established (Table II). The lines were not highly tumorigenic and exhibited values of TD_{50} between $2{\cdot}5 \times 10^4$ (clone CF5) and $6{\cdot}3 \times 10^5$ (clone CC11) cells per

animal. This represents a 25-fold difference in TD_{50} between the most and least tumorigenic lines.

The rate of growth of the established tumours (those greater than 1 mm diameter) was assessed by palpation and estimation of the mean diameter twice weekly. All tumours grew exponentially to 25–30 mm diameter, when the animals were killed to prevent suffering. No cases of intermittent growth or regression were recorded. Growth rates of all tumours derived from each clone were pooled where statistically tenable, and are presented in Table II; volume doubling times ranging from 18.5h (clone CE11) to 109·4h (clone CF6) were recorded and show that the growth rates of the clones vary more than 5-fold.

Similarly the mean latent period, the average time from the inoculation of 10^6 cells to the development of a palpable tumour, was between 2·4 and 6·8 weeks (Table II).

DISCUSSION

Acquisition of heterogeneity by transformed cell populations

The experiments carried out in this study fall into 2 categories; those which give data on individual cells, and those which give mean values for a large population of cells. The former is represented by the karyotype, morphology and, arguably, the kinetics of tumour growth from low inocula (Porter *et al.*, 1973; Walker, in preparation) whilst the population doubling time, saturation density, con A cytotoxicity and kinetics of tumour growth from high inocula are all determined by a population of cells. It is necessary to establish whether these parameters are indicative of the phenotype of the stem cell before informative analysis of the data is possible.

Investigations into the cytogenetics of most permanent tissue culture cell lines has shown a highly heterogeneous karyotype. In the CBAT system the karyotype appears to be extremely unstable, as the clones isolated from the heteroploid parent line rapidly acquire a comparable level of heterogeneity; the karyotypic diversity of the parental line and the clones after only 10 passages *in vitro* (equivalent to $\sim$40 cell generations is compared) in Table I, expressed as the coefficient of variation of the total chromosome number per cell. Further data (not shown) from later passage numbers and a further 30 cell generations indicate that the numerical heterogeneity remains constant. Thus, it must be concluded that there is a predisposition to a high level of karyotypic heterogeneity, but that this is maintained at a maximal level, perhaps through the death of cells which suffer gross genetic imbalance.

This situation must be contrasted with the observations on the morphology of the cloned cell lines. Whereas the parent line, CBAT, was highly pleiomorphic, and adjacent colonies of cells usually exhibited different morphologies, the clones were monomorphic and even 70 cell generations after cloning they retained a characteristic and uniform mode of growth. The conflict between the morphological and cytogenetic evidence infers that the karyotypic diversity shown by a heteroploid cell line does not necessarily reflect a phenotypic heterogeneity in the cells, but that the functional gene balance of the cells may be conserved.

Conservation of multifactorial cellular characteristics may also be deduced from the growth rates of the tumours derived from the clones. Analysis of variance of the growth rates of tumours from both low inocula, where the tumour has probably grown from a single cell, and those from high inocula, where a population of tumorigenic cells was responsible (Porter *et al.*, 1973) shows good intraclonal agreement but significant difference between clones.

It may thus be argued that, despite their karyotypic heterogeneity, cloned cell lines represent, for the first few cell generations, a homogeneous population with respect to the more complex physiological parameters and that these parameters are an accurate reflection of the

potentials of the stem cell from which the clone was derived.

Cellular potentials in vitro *and* in vivo

Table II shows, for each clone, the population doubling time *in vitro* compared with the mean volume doubling time of the tumours *in vivo*. Examination of these data suggests that, with the exception of clone CF6, which grows rather more slowly than expected *in vivo*, there is a high correlation between the 2 parameters. This is confirmed by a regression analysis which indicates a significant correlation at the 5% level ($r=0{\cdot}98$, $t=4{\cdot}01$, 5 degrees of freedom). Thus, it appears in this system that the rate of growth of tumour cells *in vitro* is quite a good indication of their growth potential as an established tumour *in vivo*.

Comparison of the *in vitro* characteristics of the clones with their tumorigenicity *in vivo* confirms previous work (McFarland *et al.*, 1974; Gallimore *et al.*, 1977) and shows no such correlation; clones of both high and low tumorigenic potential grow at similar rates in tissue culture. This observation indicates that the factors which determine the chance of tumour formation from an inoculum of cells are different from those responsible for the growth rate of the cell populations either in tissue culture or as an established tumour in the host animal.

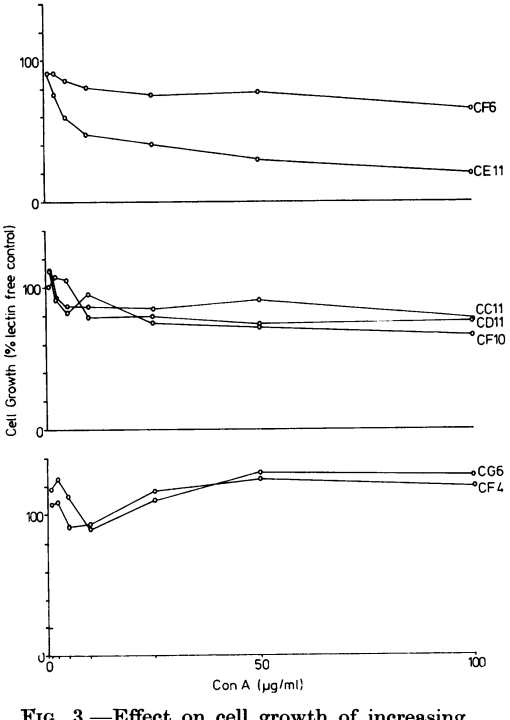

FIG. 3.—Effect on cell growth of increasing concentrations of concanavalin A. Three responses are apparent; progressive cytotoxicity (top), stimulation by low lectin levels (middle), and stimulation at both high and low levels with intermediate toxicity (bottom).

Cellular morphology and malignant potential

The morphology of the clones was scored on a scale of 1 to 6 as described in Materials and Methods and is shown with the tumorigenicity and growth properties of the cells in Table II. No clustering of morphology in relation to tumorigenic potential is seen in these results, indicating that the morphological characteristics of the cells are unimportant in the survival of the cell after inoculation into the host animal.

Interaction of the cells with concanavalin A

The response of the clones to con A in a serum-free culture medium is shown graphically in Fig. 3. Three distinct modes of growth were observed; clones CF6 and CE11 showed a progressive cytotoxic response towards the lectin and, by 100 μg/ml, up to 80% of the cell protein in the dish had been lost. Clones CC11 and CF10 showed similar cytotoxicity of lectin at levels above about 2·5 μg/ml, but lower levels stimulated protein production by the cells in excess of that shown by control cells in lectin-free medium. Clones CF4 and CG6 were stimulated by levels of con A less than 5 and greater than 10 μg/ml, but were depressed by intermediate levels. Thus, stimulation by up to 125% of control values at both 2·5 and 50 μg/ml was observed, whilst a level of growth less than the control occurred in between these peaks.

Whilst Shoham *et al.* (1970) and Thompson *et al.* (1975) have shown that con A is

cytotoxic to transformed murine cells at concentrations of between 20 and 80 μg/ml, none of their experiments detected a complex response to the lectin. However, the former work used only a single concentration, (50 μg/ml) and Thompson's experiments were performed in the presence of serum which contains glycopeptides which may partially inactivate the lectin (Ralph & Nakoinz, 1973).

Cuatrecases & Tell (1973) demonstrated that 20 nM con A bound to the insulin receptors on adipocytes and stimulated glucose transport; 20 nM tetravalent con A is equivalent to 2 μg/ml, which is in good agreement with the con A concentrations which stimulate the growth of some CBAT clones. Higher concentrations of lectin bind increasingly to the cell surface integral proteins, until saturation is achieved by 100 μg/ml (Cuatrecases & Tell, 1973). Addition of further con A leads to competition between bound lectin and free lectin in solution, and releases the membrane organelles from their cross-linking and clustering constraints (Nicolson, 1974). In the CBAT experiments, concentrations between 20 and 50 μg/ml were invariably cytotoxic, whilst higher levels caused, in some clones, a return to the stimulatory condition.

There was no evidence to suggest that the different types of con A response were indicative of the tumorigenicity of the cell lines *in vivo*.

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