Adaptation of 6C3HED Tumor Cells to Culture *in Vitro*

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SUMMARY

Cells of the C3H ascitic tumor (6C3HED) of mice have been successfully established in continuous culture *in vitro*, and the ready adaptation of these tumor cells to growth on glass surfaces has been described. Cultivation of the tumor cells *in vitro* appeared to accentuate the already induced pathogenicity of these cells for an albino Swiss strain of mice; and further passage of the tumor cells in the albino Swiss mice appeared to diminish the pathogenicity for the original host mice, the C3H strain.

The inclusion of 6C3HED ascitic tumor cells among various animal tissues being investigated for susceptibility to the enteroviruses rendered it desirable to adapt these cells to growth *in vitro*. This lymphoid tumor had been originally induced in C3H mice by Gardner *et al.* (2) and subsequently, of necessity, was maintained in that mouse strain. Morgan *et al.* (4), however, found that a freezing technic used for preservation altered the mouse strain specificity of the tumor cells; they became pathogenic also for the Connaught strain of Swiss mice. The present paper reports the successful propagation and establishment of the ascitic tumor 6C3HED on glass surfaces.

MATERIALS AND METHODS

The 6C3HED tumor cells were obtained from Dr. J. F. Morgan as a freshly removed ascitic suspension, following a recent passage of the tumor, and upon receipt were inoculated into C3H mice. Ascitic fluid was taken from these mice a week later, and a second passage was made in mice of the same strain. When the tumor cells were removed from the latter animals the fluid was centrifuged, the sediment treated with distilled water to hemolyze erythrocytes present, then washed once in M150 (3), and a cell count was made in a hemacytometer chamber following resuspension. The volume was then adjusted and divided into twelve aliquots, each calculated to contain 4,000,000 tumor cells. A final centrifugation was followed by removal of the supernatant fluid.

Two basic media were prepared: (A) M150 plus 10 per cent calf serum and (B) the lactalbumin hydrolysate medium as formulated by Bodian (1), also with 10 per cent calf serum. Six of the cell aliquots were resuspended in 4 ml. each of medium A and the other six in 4 ml. each of medium B. Each pair (one A plus one B) of tubes received, respectively, supplementary nutrients as follows: (*a*) nil, (*b*) 1 per cent chick embryo extract, (*c*) 1 per cent mouse embryo extract, (*d*) 1 per cent human ascitic fluid, (*e*) 10 per cent mouse serum, and (*f*) 1 per cent mouse ascitic fluid. The contents of each tube was divided between two T-15 flasks, making in all 24 T-15 flasks, each containing 2,000,000 tumor cells in one or the other basic medium; twenty contained various additional nutrients. All were incubated at approximately 35° C.

During the early passages a 0.02 per cent solution of versene (Eastman Kodak Co.) in modified (omission of $MgCl_2$ and $CaCl_2$) phosphate-buffered saline was used to separate the tissue from the glass surface, as well as the individual cells, prior to subculture.

RESULTS

At the end of the first week, after daily renewal of the media, there remained only four flasks in which the cultures appeared healthy and even showed signs of multiplication. These were the two with basic medium A and the two containing basic medium B, all with 10 per cent calf serum. Cultures in the remaining twenty flasks comprised only a few cells still adhering to the glass, and even they appeared to be degenerating; they were therefore discarded.

At this stage the cultures in the four retained flasks consisted chiefly of small mononucleated, lymphocyte-like cells, many of which possessed a barely detectable rim of cytoplasm around the nucleus or eccentrically situated on one side only of the nucleus. The smallest of these cells were about

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the size of an erythrocyte or smaller. There were a few similar though larger cells, about 14–16 μ in diameter, with a larger area of cytoplasm showing. In addition to the small and larger lymphocyte-like cells there were occasional forms resembling fibroblasts, some of which had two or more processes extending variable distances from the cell body. The latter cells were evident a few days after initiation of the cultures and may have arisen, in the course of development of the cultures, from the round cells. On the other hand they may have been present in not so evident form among the original cells.

At the end of the second week the cultures in the two flasks containing medium A appeared to be in better condition than the other two; they were accordingly pooled, and two T-30 flasks were seeded. Daily renewal of the medium was continued over the next 14 days, and it was observed that more of the cells were developing cytoplasmic processes. The cultures in the two T-30 flasks were combined at the end of the 3d week and subcultured in two T-60 flasks. Medium A was used in one flask and medium B in the other. After incubation of these cultures, with daily renewal of the media for 2 weeks, the cultures were combined and used to seed a Roux bottle. This constituted the fourth *in vitro* passage. Although the lactalbumin hydrolysate medium was used in this and some of the subsequent passages, beyond its simplicity of preparation it was not apparent that it provided any nutritional advantage over medium M150 for this tumor cell line.

The cells were now proliferating at a moderate rate and tended more than formerly to form a continuous sheet on the glass surface. The round, lymphocyte-like cells still predominated, and interspersed among them were fibroblast-like cells, some with quite long processes, and occasional epithelial-like cells. When the culture in the Roux bottle (fourth tissue culture passage) was fully developed this represented an increase of approximately 16× in culture area over the initial cultures.

From this point onward there was nothing particularly noteworthy regarding the subsequent passage cultures of these tumor cells. Subcultures were usually made at 4 to 5-day intervals, and it was seldom necessary to renew the medium between transfers. There had not been noted at any time after the first two passages a phase of growth retardation, accompanied by deterioration, and followed by a surge in growth activity—events that have been observed during the establishment of other cell lines. This cell line has now been carried through more than 60 successive subcultures.

PATHOGENESIS OF ESTABLISHED TUMOR CELL LINE FOR MICE

Pathogenicity of the 6C3HED tumor cells for both C3H and Connaught Swiss Mice was tested with successful cultures of the 10th, 15th, 20th, and 25th *in vitro* passages. For this purpose 3–4,000,000 cells, washed once in M150 following trypsinization of the cultures, were inoculated intraperitoneally. The behavior of the tumor cells differed in the two strains of mice, although the pathogenicity was very similar at the above specified passages, within the mouse strain (Table 1). The observations regarding pathogenicity will be grouped according to mouse strain.

TABLE 1

PATHOGENESIS OF ESTABLISHED 6C3HED TISSUE CULTURE LINE FOR C3H AND CONNAUGHT MICE

INOCULUM	SERIAL MOUSE PASSAGES*		RESULTS
No. tissue culture passages	No.	Mouse strain	Tumor incidence (per cent)
10–25	1	Connaught	70
	2	"	90
	3	"	90
	4	"	90
10–25	1	C3H	88
	2	"	)
	3	"	)33–42
	4	"	)
10–20	1	Connaught†	100
	2	"	75
	3	"	80
	4	"	89
10–20	1	C3H‡	40
	2	"	30
	3	"	8
	4	"	0
None (6C3HED control)	10	C3H	90

* Ten mice were inoculated in the first passage; 30 mice were inoculated in each of passages 2, 3 and 4.

† Preceded by one passage in C3H mice.

‡ Preceded by one passage in Connaught mice.

Connaught mice.—Four successive intraperitoneal passages were carried out in this mouse strain at each of the tissue culture passages specified above. In the first of the four passages seven of ten mice exhibited well developed ascites 12–15 days after inoculation. In each of the three succeeding passages about 90 per cent of 30 mice developed ascitic tumors, and cell multiplication was readily demonstrated. Essentially similar results were ob-

tained with tumor cells of the 10th, 15th, 20th, and 25th tissue culture passages.

C3H mice.—Serial passage of the tissue culture line of tumor cells in these mice presented a somewhat different picture from that in the Connaught mice. Although the incubation period in the first passage was similar (12–15 days), the degree of abdominal swelling was much less; moreover, if the animals were not sacrificed, the tumors tended to regress and fatalities did not occur. Tumor cell proliferation was demonstrable, though it was probably not more than a quarter of that in the Connaught mice. In the first passage in C3H mice, regardless of the passage source of the tissue culture tumor cells used for inoculation, ascites was induced in 88 per cent of the animals; in the succeeding three passages the incidence of successful inoculations varied between 33 and 42 per cent.

In view of these mouse strain differences noted in the pathogenicity of the tumor cells, it was decided to pass the adapted tissue culture tumor cells of the 10th, 15th, and 20th passages each through (*a*) one passage in C3H mice, followed by four successive passages in Connaught mice, and (*b*) one passage in Connaught mice, followed by four successive passages in C3H mice. The resulting ascitic tumor incidence in the Connaught mice, under (*a*), was 100, 75, 80, and 89 per cent in the four passages, respectively. That in the C3H mice, under (*b*), was 41, 30, 8, and 0 per cent, respectively.

DISCUSSION

It is apparent from the foregoing results that the cells of this ascitic tumor have become well adapted to continuous culture on glass surfaces. During the process of adaptation and the numerous cultures *in vitro*, however, there appeared to take place an accentuation of pathogenicity for a strain (Connaught) of Swiss mice, and a concomitant decrease in that for the original source strain, C3H mice. It may be significant that the tissue culture passages of the 6C3HED ascitic tumor cells reinforced the altered mouse strain specificity described earlier by Morgan *et al.* Possibly the two factors (freezing and artificial culture) combined to effect a genetic charge, although this suggestion can be no more than speculative at present.

Whatever the reason for the altered mouse strain specificity of this tumor, it perhaps should be recorded that no other strain of rodent neoplasm has been carried or transmitted in this laboratory. This precludes the consideration of an accidental contamination in our cultures.

REFERENCES

1. Bodian, D. Simplified Method of Dispersion of Monkey Kidney Cells with Trypsin. Virology, **2**:575–76, 1956.
2. Gardner, W. U.; Kirschbaum, A.; and Strong, L. C. Lymphoid Tumors in Mice Receiving Estrogens. Arch. Path., **29**:1–7, 1940.
3. Morgan, J. F.; Campbell, M. E.; and Morton, H. J. The Nutrition of Animal Tissues Cultivated *in Vitro*. I. A Survey of Natural Materials as Supplements to Synthetic Medium 199. J. Nat. Cancer Inst., **16**:557–67, 1955.
4. Morgan, J. F.; Guerin, L. F.; and Morton, H. J. The Effect of Low Temperature and Storage on the Viability and Mouse Strain Specificity of Ascitic Tumor Cells. Cancer Research, **16**:907–11, 1956.

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