Culture Characteristics of Four Permanent Lines of Human Cancer Cells*

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The usefulness of human malignant tumor cells grown in tissue culture has been amply demonstrated by the numerous reports in the literature on the HeLa strain of epithelial cell developed by Gey and co-workers (5). This report will describe the culture characteristics of three more human tissue culture cell strains derived from epidermoid cancers, one embryonal rhabdomyosarcoma, and one sarcoma which, as yet, has not been completely adapted to continuous transfer. All these strains are derived from neoplasms which were grown first in the irradiated and cortisone-treated rat by one of the authors (8, 9).

MATERIALS AND METHODS

To begin a culture, the neoplastic tissue was finely minced with scissors, suspended in culture media, and placed in either a test tube, Carrel flask, or a 180-ml. milk dilution bottle, depending on the amount of tissue available. After a few days of incubation at 37° C., an outcropping of sheets of cells could usually be observed surrounding the minced pieces. When the media became acid, the fluid was removed and the cells therein spun out for implantation in another receptacle; fresh media was added to the original culture. When cells had grown sufficiently to form colonies of 1–2 cm. or when they completely covered the bottom of the container, they were removed by the addition of 0.5 per cent trypsin. This, after incubation at 37° C. for 5–10 minutes, was sufficient to float off the cells. After centrifugation the cells were resuspended in either two Carrel flasks or two bottles. In some instances when a very small amount of beginning material was available, cultures from tubes were transferred first to small Carrel flasks, then to large ones, and finally to bottles.

Once the cultures were established they were trypsinized whenever the growth had completely covered the bottom of the bottle. The rate of growth is a characteristic of the particular cell strain and will be described later. To prepare culture tubes for experiments, the "dry" tube method described by Morann and Melnick (7) was used. The solid sedimented cells with a few drops of human serum added were streaked with a capillary pipette along the surface of a tube, previously warmed to 45° C., in much the same way that an agar slant is streaked with bacteria. After 30 minutes at 4° C., 1.5 ml. of culture media was added, and, following 2 days' incubation, a solid sheet of cells formed from the streaked site, and the tubes could be used for experiments.

Daily counts of the cells have been made in an effort to determine the pattern of growth for each neoplasm. After implantation, 1 ml. of trypsin solution was added to each tube, and the cells were counted in a standard hemacytometer after 30 minutes' incubation at 37° C. to provide an estimate of the number of cells implanted. Thereafter, daily counts were made in duplicate on tubes set up at the same time. Table 1 gives the general pattern of growth for each neoplasm as exhibited in this type of culture. Although the number of cells implanted varied with different experiments, the general pattern was characteristic for each tumor. After 1 day of incubation there was usually a decrease in the number of cells, probably owing to the fact that all those implanted were not viable. The most rapid growth occurred within 2 days and in the instance of H.Ep. #2, H.Ep. #3, and HeLa, represented the maximum attained, while the maximum for H.Ep. #1 was attained on the 4th day and by H.Emb.Rh. #1 not until the 7th or 8th day.

TABLE 1

DAILY CELL COUNTS* ON CULTURE TUBES OF HUMAN CANCER CELLS

Incubation period (days)	H.Ep. #1	H.Ep. #2	H.Ep. #3	HeLa	H.Emb.-Rh. #1
0	161	26	31	60	210
1	202	22	15	28	52
2	571	111	54	67	233
3	775	77	46	64	232
4	950	88	27	57	357
7	400	44	24	75	400
8	344		10	44	477

* Expressed in thousands.

When the neoplastic cells have been established in tissue culture, a definite routine is followed to determine if any change occurs during their prolonged cultivation. Every month four bottles are set up with each tumor with two cover glasses in each bottle. The cells settling on the cover glasses are removed after 2 days' growth and stained with May-Grünwald stain for comparison with previously and similarly prepared slides. When the cells in the bottles have attained their maximum growth (usually in 2–4 days depending on the tumor) they are trypsinized, pooled, resuspended in a small amount of media or balanced salt solution, and inoculated into irradiated, cortisone-treated weanling rats. When a tumor has appeared (usually 10–14 days) the animal is sacrificed and the morphology of the neoplastic tissue is compared with that of previous tumors and with the original growth. As

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yet no histological change has been noted; the characteristics of each neoplasm appear to be preserved indefinitely.

RESULTS

Description of Tumors

H.Ep. #1 (Toolan).—This epidermoid carcinoma, the first to be established in serial passage in irradiated and cortisone-treated rodents and described previously by one of the authors (8), originated from a small biopsy specimen[1] obtained from a carcinoma of the cervix. Some difficulty was experienced at first in establishing active growth in tissue culture, since it appeared to be harmed by trypsin; but cells from the nineteenth generation which had grown 13 days in the rat were more resistant to the treatment. Serial transfers in tissue culture have been made since September, 1953. In the first passages the cells grew in colonies, and transfers could be made only at 2- to 3-week intervals. Gradually the growth became more rapid and in single-cell form. Characteristically the cells do not form sheets as HeLa or H.Ep. #2 do. Although the cells are often closely approximated, they appear to be more discrete than in either of the above tumors. The cells are of two types; the most numerous ones are large and angular, and the others are small and round. At present the tumor grows rapidly and doubles its bulk in 2 days. Figure 1 shows the morphology of the tissue when removed from the patient; Figure 2, an unstained tissue culture preparation of the same neoplasm, demonstrates its rather angular cells; and Figure 3 illustrates the growth which arose in an irradiated cortisone-treated rat after re-implantation of trypsinized cells which had been in culture for over 16 months.

H.Ep. #2 (Toolan).—After two generations in rats this tumor (also an epidermoid carcinoma), which had originally come from a man with a primary tumor of the larynx (Fig. 4), was implanted in tissue culture in September, 1952, by Dr. Audrey Fjelde, using the plasma clot, roller drum technic. Its characteristics in this medium have been described (4). Since September, 1953, these cells, which can easily be freed from the clot by trypsinization,[2] have been grown on the glass surface of bottles. They grow rapidly, doubling themselves in 2–3 days and forming sheets in the same way as do the HeLa cells from which they are morphologically almost indistinguishable (Fig. 5). When inoculated into an irradiated cortisone-treated rat, a typical epidermoid cancer forms (Fig. 6), which often contains vacuolated cells. This vacuolation has not as yet been noted in the HeLa cells and may be a distinguishing characteristic of our cell line. Figure 4 shows the original growth, in which the area of vacuolated cells is indicated.

H.Ep. #3 (Toolan).—This neoplasm was derived from a lymph node containing metastatic epidermoid cancer, grade III, primary in the buccal mucosa. It grows readily and rapidly in both rats and eggs and was adapted to tissue culture 1 year ago. The cells can be started in culture at will from the animal implant and, even after prolonged cultivation, readily give rise to a characteristic neoplasm when implanted back into the animal. It grows in tissue culture at a somewhat slower rate than do other lines, doubling itself in about 5 days. It does not form sheets but grows either as single cells or a mosaic of flat clumps. The cells themselves are rather angular and may form a multinucleated mass. Occasionally, very large cells with large nuclei are present in the clumps. Figure 7 shows the original neoplasm; Figure 8, the cells in tissue culture in the mosaic form; and Figure 9, the neoplasm which arose 10 days after inoculation of the cultured cells into the irradiated cortisone-treated rat.

H.Emb.Rh. #1 (Toolan).—This neoplasm, which was originally removed from the chest wall of a 37-year-old male, is an embryonal rhabdomyosarcoma which was established in tissue culture from a 17-day-old growth (20th generation in rats) received in June, 1954. It grew slowly at first, and the first trypsinizations were carried out at approximately 2- to 3-week intervals. It adapted itself well to tissue culture, however, and now is transferred at 3- to 4-day intervals. It grows in thick sheets which cover the bottom of the bottle completely with cells which are so transparent that it would be difficult to detect the growth macroscopically, were it not for the fact that the edges tend to curl. In this state it is impossible to see the individual cells, but they are readily visible in stained preparations where the characteristic cross striations may be demonstrated by suitable technics. The tumor is characterized by large nuclei with many nucleoli and by its faintly staining lace-like cytoplasm (Fig. 11). Figure 10 shows the original growth and Figure 12 that formed on the chorio-allantois of the egg after implantation of tissue culture cells imbedded in a plasma clot.

H.S. #1 (Toolan).—This neoplasm was a soft-part sarcoma, primary origin unknown, removed from the calf of a 43-year-old male. It grows rapidly and attains a huge size both in rats and in eggs (1), has grown very irregularly in tissue culture, and as yet is not in continuous serial passage. The

[1] Obtained through the courtesy of Dr. Chester M. Southam.

[2] First done in our laboratory by Dr. Wilbur F. Noyes.

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success or failure of the growth in culture seems to depend on the animal or egg implant selected for a particular experiment, some giving excellent growth and others none at all. A systematic investigation of the factors involved in these variations of growth potential has not been made. When growth occurs, however, the cells are characteristic. They contain many vacuoles, a feature which they share with both the original neoplasm (Fig. 13) and those grown in rat and on the chorioallantois of the egg. Figures 14 and 15 illustrate their appearance in tissue culture.

Growth Characteristics of Neoplastic Cells in Tissue Culture

Media.—The above-described tumors, plus HeLa and human fibroblasts derived from either embryonic or adult testicular tissue, were studied for their ability to grow in five different types of media, the composition of which is shown in Table 2. Tubes were set up in duplicate and their growth followed for 1 month without any change of media. Two such experiments were done. The results, which are shown in Table 3, are arranged according to the best growth, good growth, and lasting growth, to indicate the media in which the cells remained longest in good condition. Since the observations were not quantitative, they must be regarded as a rough measurement of the impressions of two different observers.

In our laboratory all neoplasms and tissues such as the human fibroblasts are routinely kept in media #1, designated BH (Bovine-human). When experiments are to be done, the media is removed and replaced with media #5, designated Enders, since it was first described by that investigator (2). It has the advantage of containing no human serum. The cells of all tumors, with the possible exception of H.S. #1, keep very well for 2 weeks at incubator temperature, at which time our experiments are terminated.

The human serum is obtained from young, fasting adults and is collected by our blood bank[3] in the usual bottles, except that no anticoagulant is added. After sterility tests the serum is placed in tubes at 4° C. until used. We have never noticed any toxic effects from any sera.

Effect of pH.—BH medium (medium #1) was adjusted in steps from pH 5.0 to pH 9.8 by the addition of 0.1 N HCl or NaOH. The adjusted medium was then added to culture tubes in which cells from each neoplasm had been streaked. The tubes were observed for 3 weeks to determine: (*a*) the range of pH in which the cells could survive for a 1-week period, (*b*) the range of pH at which active growth took place, and (*c*) whether any particular pH range was best for growth. The results are given in Table 4.

In the cases of H.Ep. #3 and H.Emb.Rh. #1 it was possible to compare the tissue culture strain of cells with those isolated directly from the same neoplasm grown in the rat. In each instance the cultured cells showed less tolerance to acidity, and best growth seemed to take place in a more alkaline medium. To test the ability of H.Ep. #2 to

[3] The authors wish to thank Miss Mary Channon for her co-operation in collecting the blood.

TABLE 2

Composition of Media* Used in Growth Experiments

	Types of media				
	#1	#2	#3	#4	#5
Bovine amniotic fluid	70	80	85	90	
Bovine embryo extract	10	10	10	5	
Human serum	20	10	5		25
Horse serum				5	
Gey's balanced salt solution					60
Chick embryo extract					15

* Expressed by per cent.

TABLE 3

Summary of Growth Media Experiments

Tumor	Growth		
	Best	Good	Lasting
H.Ep. #1	1*	All	1 and 4
H.Ep. #2	1 and 4	All	All but 5
H.Ep. #3	1	2 and 5	1
HeLa	1 and 5	2 and 3	5
H.Emb.Rh. #1	1	All	1
H.S. #1	1	All	1 and 2
H.Fibroblasts (Adult and embryonic)	5	1	1 and 5

* The numbers indicate the type of media the compositions of which are given in Table 2.

TABLE 4

Growth of Human Tumors in Media at Different pH's

(Range tested: 5.0–9.8)

Tumor	Source of tumor	Survival range	Active growth range	Best growth range
H.Ep. #1	T.C.*	6.0–9.3	7.0–9.0	8.0–8.7
H.Ep. #2	T.C.	6.0–9.7	6.4–9.0	7.6–8.7
H.Ep. #3	Rat	6.0–9.0	7.7–9.0	7.7–8.5
	T.C.	5.5–9.0	6.5–8.5	7.0–8.0
HeLa	T.C.	5.5–9.3	6.4–9.0	6.5–8.0
H.Emb.Rh. #1	Rat	6.5–9.0	7.0–9.0	7.0–8.5
	T.C.	5.5–9.0	6.5–8.5	7.0–8.0
H.S. #1	T.C.	6.5–9.0	6.5–9.0	7.7–8.5
H.Fibroblasts (Adult and embryonic)	T.C.	6.1–8.91	6.4–7.9	7.0–7.5

* T.C. = tissue culture.

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grow in media with an alkaline pH, three continuous transfers have been made into media at pH 9.0. Although the cells grew about 3 times as slowly as the controls kept at the routine pH (7.8), they formed sheets and retained their normal appearance. H.Ep. #3 failed to grow in the alkaline media.

All the neoplastic cells grew actively over a fairly large range of pH, and it was often difficult to discover which was the best. The human fibroblasts grew in the most restricted range. These findings roughly correspond to those reported by other authors (3).

In one experiment employing the growth of H.Ep. #1, H.Ep. #2, HeLa, and HAF (human adult fibroblasts) in a pH range of 5.5 to 9.0, the fluid was removed from the tubes after 1 week and the pH determined again. In each instance a change had taken place in the direction of neutrality. To determine how quickly such an adjustment took place, the pH was determined at intervals after tubes had been inoculated with H.Ep. #2 cells in media of different pH's. The following changes had taken place in 1 hour: pH 5.72 to 6.61, pH 7.7 had not changed significantly, pH 8.9 to 8.5, and pH 9.4 to 9.10. No further significant change in pH was noted thereafter during the period of observation; hence, one may assume that the growing cells had exerted their buffering capacity very rapidly. It is possible that this is the determining factor in the cells' ability to grow over such a wide range of pH.

Effect of heat and ultraviolet on cells.—Experiments were done with all the different strains of cells to determine how long they could be heated at 56° C. in the water bath and still retain their viability. Cells were trypsinized, centrifuged, and re-suspended in sufficient Gey's saline to make one million cells/ml. At the different time intervals 0.1 ml. was removed and streaked on the warmed tube, and 0.9 ml. of BH media was added to the cultures which were placed in a stationary receptacle for incubation at 37° C. The results of these experiments are reported in Table 5, showing the last time interval at which good growth was obtained and the time interval beyond which the cells failed to grow. Most of the cells grew well after being heated to 56° C. for 1 minute; two, HeLa and H.Ep. #1, could survive this temperature for 2½ minutes. The human fibroblasts, whether of adult or embryonic origin, appeared to be the most heat-sensitive of all the cell lines studied.

The effect of exposing similarly prepared cell suspensions to ultraviolet light from a Hanovia 2537 mercury vapor lamp, which delivered 230 ± 15, 10^{-3} ergs Km^2/sec at a 10-cm. distance, was also studied. Three-tenths ml. of the cell suspension containing one million cells/ml was placed in a Maximow slide, and, after exposure at the different time intervals, 0.1 ml. was placed in a tube and 0.9 ml. of media added. The tubes were then inspected twice a week for growth. The last two columns in Table 5 give the results and show that H.Emb.Rh. #1 and the human fibroblasts are very sensitive to ultraviolet radiation and that H.Ep. #1 and H.Ep. #2 are very resistant, whereas HeLa and H.Ep. #3 fall in a middle range.

DISCUSSION

The culture characteristics, morphology, and reaction toward heat and ultraviolet have been described for four human neoplasms (three epidermoid carcinomas and one embryonal rhabdomyosarcoma) which can be grown in large quantities on glass surfaces in tissue culture. Whenever possible, the characteristics of another tumor, H.S. #1, have been described. It is not so completely established in culture, and we have been unable to keep it in serial passage for longer than 6 months. Since the other four neoplasms are readily cultivated, it is assumed that they will find the same uses that have already been described for the HeLa cells. Each has been shown to be different in morphology and in susceptibility to physical agents, and it may be expected that their reactions to viruses or chemicals may also vary.

TABLE 5

EFFECTS OF HEAT AND ULTRAVIOLET LIGHT ON THE GROWTH OF HUMAN CELLS IN TISSUE CULTURE

	Heat		Ultraviolet	
Tumor	Good growth	Survival	Good growth	Survival
H.Ep. #1	1 min.	2½ min.	5 min.	10 min.
H.Ep. #2	1 "	1 "	15 "	15 "
H.Ep. #3	1 "	1 "	30 sec.	50 sec.
H.Emb.Rh. #1	1 "	1 "	5 "	10 "
HeLa	1 "	2½ "	1 min.	3 min.
Fibroblasts:				
Adult	30 sec.	1 min.	1 min.	2 min.
Embryonic	30 "	30 sec.	10 sec.	20 sec.

The question is often raised whether these neoplasms have changed during their prolonged cultivation outside the human body. The following facts indicate that the original characteristics are retained: (*a*) When the long-cultivated cells are inoculated into an irradiated and cortisone-treated animal, growth takes place which is identical histologically with the tissue originally removed from the patient; (*b*) the human cells grown for long periods in the rat, hamster, and egg have been studied immunologically and have been shown to

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maintain a steady human protein pattern (6); (*c*) studies of the chromosomes[4] of the cultured cells show the persistence of characteristic human patterns; (*d*) finally, most of the cultivated cell types have been inoculated subcutaneously into human volunteers and have grown with the same histological characteristics as did the original tissue.[5]

The neoplastic cells have been shown to grow in a wide variety of media. Although we routinely use 20 per cent human serum, it may be possible to adapt the tumors to smaller amounts or to horse serum. Contrary to the experience of most workers, we have not found any batches of toxic serum. The rather wide range of pH over which the cells will grow is surprising. The limited experiments reported here seem to indicate that such pH changes as take place occur very rapidly and that the pH is stabilized shortly after the cells begin to grow. The reason for studying sensitivity to heat and ultraviolet was to determine similarities and differences between the various cell lines. There were great differences in regard to the ultraviolet sensitivity and lesser ones as far as heat was concerned, but these appeared to be due to the individual characteristics of the cell strain itself rather than to the type of neoplasm. For example, one epidermoid cell strain (H.Ep. #2) was extremely resistant to ultraviolet, whereas another (H.Ep. #3) was relatively sensitive. These differences may also be useful in establishing any change which has taken place during cultivation.

SUMMARY

The cultural and morphological characteristics of three strains of human epidermoid cancer cells (H.Ep. #1, H.Ep. #2, and H.Ep. #3) and one human embryonal rhabdomyosarcoma (H.Emb.Rh. #1) have been described. These tumors have been passed serially for periods of 8 months to 2½ years and have maintained their original histological characteristics. One other tumor (H.S. #1), not yet permanently established in passage, has also been described. The pH range and sensitivity of the tumor to heat and ultraviolet have been recorded.

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[4] Done by the courtesy of Dr. A. R. T. Denues.

[5] The reinoculation experiments will be published by Dr. Chester M. Southam.

Fig. 1.—Epidermoid carcinoma from inguinal lymph node biopsy specimen (H.Ep. #1). H & E stain. ×400.

Fig. 2.—H.Ep. #1 cells from a cellular colony grown in tissue culture in BH media. Unstained. ×400.

Fig. 3.—H.Ep. #1. Epidermoid cancer formed from the inoculation of cells grown continuously in tissue culture for 16 months. H & E stain. ×400.

Fig. 4.—H.Ep. #2. Epidermoid carcinoma of the larynx. Arrow indicates the vacuolated cells. ×120.

Fig. 5.—H.Ep. #2 cells from a cover glass stained with May-Grünwald stain. ×160.

Fig. 6.—H.Ep. #2. Epidermoid carcinoma produced in an irradiated cortisone-treated rat 12 days after the subcutaneous inoculation of H.Ep. #2 cells which had been maintained for over 2 years in tissue culture. Arrow indicates vacuolated cells, also seen in the original tumor. ×140.

Fig. 7.—H.Ep. #3. Microscopic appearance of the original epidermoid carcinoma from a metastatic neck node, primary in the buccal mucosa. ×160.

Fig. 8.—H.Ep. #3 after cultivation on cover glasses in tissue culture. Stained with May-Grünwald. ×120.

Fig. 9.—The section of the neoplasm formed when cells grown in tissue culture for 6 months were inoculated into irradiated and cortisone-treated rats. ×140.

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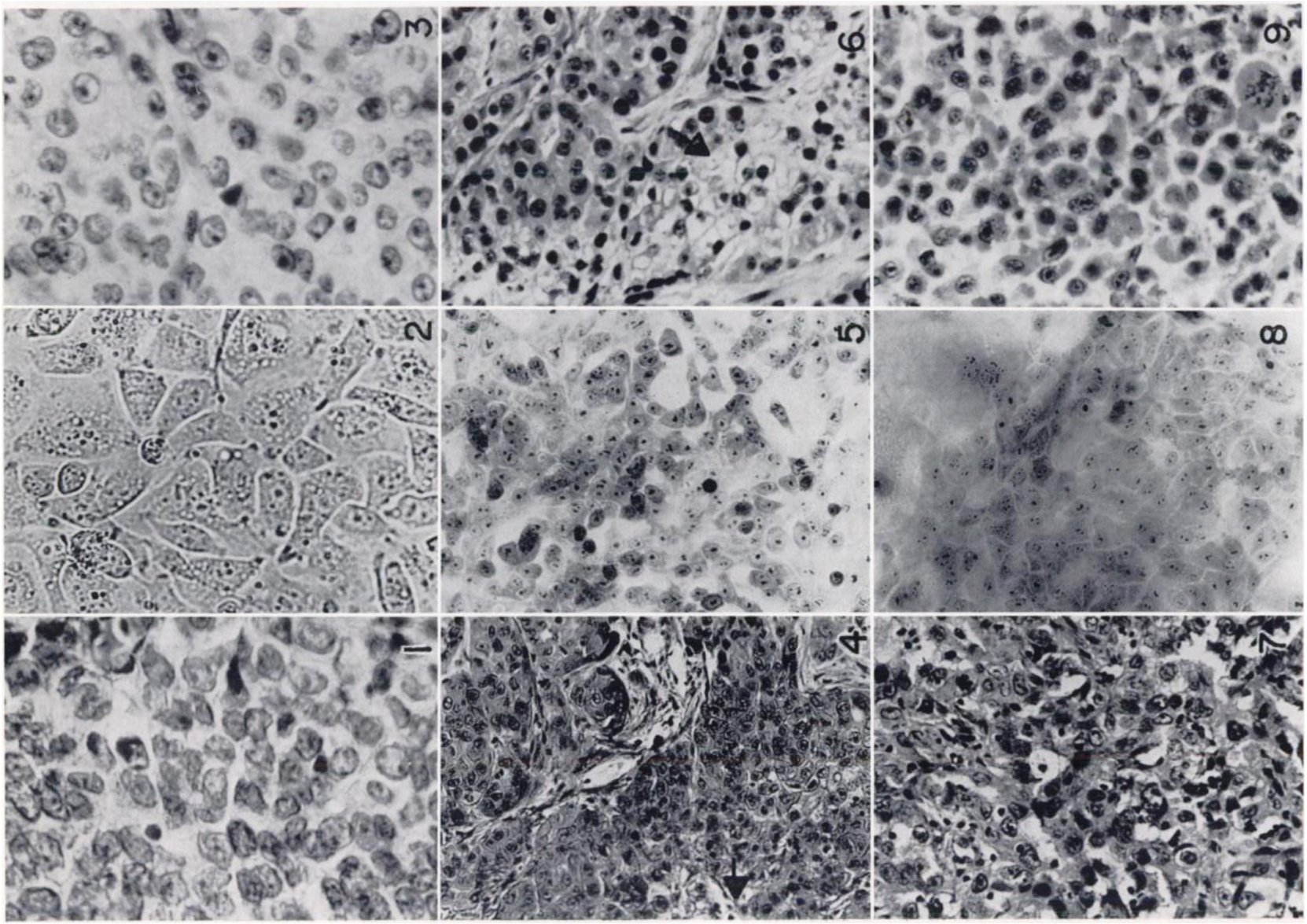

FIG. 10.—Histological section of an embryonal rhabdomyosarcoma removed from the chest wall. ×360.

FIG. 11.—Tissue culture preparation on glass of H.Emb.Rh. #1. Stained with May-Grünwald. ×180.

FIG. 12.—Histological section of H.Emb.Rh. #1 formed when cells from tissue culture were implanted on the chick chorio-allantoic membrane. ×360.

FIG. 13.—Soft-tissue sacroma (H.S. #1), showing characteristic vacuolar cells. ×330.

FIG. 14.—H.S. #1 grown in tissue culture after 40 generations in rats. May-Grünwald stain. ×360.

FIG. 15.—H.S. #1 grown in tissue culture. Higher magnification (×1000) to show characteristic vacuoles.

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