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Reproducible Growth in Tissue Culture of Retinoblastoma Tumor Specimens[1]

Brenda L. Gallie,[2] Wendy Holmes, and Robert A. Phillips

Department of Ophthalmology, University of Toronto [B. L. G.], and Ontario Cancer Institute [W. H., R. A. P.], Toronto, Ontario, Canada M4X 1K9

ABSTRACT

Retinoblastoma is a unique embryonic tumor which frequently arises because of an autosomal dominantly inherited mutation. Study of the genetic changes associated with retinoblastomas requires techniques that allow proliferation of fresh tumor specimens in tissue culture. However, until the present study, there were no reported methods for routinely obtaining *in vitro* growth of fresh retinoblastoma tumor cells. When placed on selected fibroblast feeder layers, 65% of fresh retinoblastoma specimens grew; but in the absence of fibroblasts, the same specimens died. Individual fibroblast strains from normal donors, retinoblastoma patients, and mice were effective feeder layers. A fibroblast strain that provided good feeder function for one tumor generally supported growth of other tumors also. Direct contact was required between the tumor cells and the fibroblasts; conditioned media alone did not support tumor growth and immobilization of tumor cells in semisolid media above fibroblasts failed to give colony growth. Cultured tumor cells injected into the eyes of athymic nude mice formed tumors histologically characteristic of retinoblastomas.

INTRODUCTION

Of patients with retinoblastoma, a rare retinal tumor of childhood, 40% have a germ-line mutation which will result in the development of retinoblastomas in approximately one-half of their offspring (16). The autosomal dominant inheritance pattern of this human tumor makes it an ideal model for studies on the genetic changes associated with the malignant phenotype. However, to carry out such studies, it must be possible to grow the cells in tissue culture to apply the techniques of somatic cell genetics. Although the tumor grows readily in nude mice (8), it has been very difficult to grow and to clone *in vitro*. Only 2 established lines are available and both have highly abnormal karyotypes (13, 15), possibly indicating marked chromosome evolution *in vitro*. Other investigators have shown that the use of feeder layers (1, 3, 7, 10) or medium previously conditioned by other cells (9, 10) will enhance the growth of several human tumor cells. In attempts to grow fresh retinoblastomas in culture, Albert *et al.* (2) obtained transient growth, especially in cultures with a layer of adherent cells from which tumor cells seemed to detach. Because the adherent cells in the study of Albert *et al.* looked like fibroblasts and because others have successfully used fibroblasts as feeder layers for human tumors (1, 7), we tested the ability of various cells and factors to stimulate the growth in culture of retinoblastoma tumor cells. The results described below demonstrate that the use of selected feeder layers of fibroblasts will support the *in vitro* growth of 62% of retinoblastoma tumor specimens.

MATERIALS AND METHODS

Tumor Specimens. All of the tumors tested were derived from primary tumor specimens obtained at the time of enucleation of the eyes. Immediately after enucleation, the tumor was removed and made into a cell suspension by passage through a screen (80 mesh). One aliquot was cryopreserved at a controlled rate of 1°/min and stored in liquid nitrogen. To expand the amount of tumor available for study, another aliquot of tumor was injected i.o.[3] into athymic nude mice as described previously (8). In addition, solid chunks of tumor (1 cu mm) were placed s.c. in athymic nude mice. For most experiments, the tumor was obtained from frozen samples or from nude mice; in some instances, cells from the surgical specimen of tumor were placed directly in tissue culture.

Culture Media. The medium used for culture of fibroblasts, retinoblastomas, pigment epithelium, and bone marrow consisted of Iscove's modified Dulbecco's minimum essential medium (11) with 15% fetal calf serum. Most growth curves were performed in this medium. Experiments were also performed with the following factors added: nerve growth factor (2 ng/ml); fibroblast growth factor (100 ng/ml); epidermal growth factor (5 ng/ml); retinoids (retinol acetate, retinal, and retinol; 1 µg/ml); taurine (40 to 80 mM); hyaluronic acid (0.06 to 1.2%; Grade IV from bovine vitreous humor); fibroblast-conditioned medium (50% medium from confluent cultures of fibroblast 356); tumor cell-conditioned medium [50% medium from Y79, an established retinoblastoma line (15)]; mouse spleen cell-conditioned medium (10 to 50% medium from 3-day phytohemagglutinin-stimulated mouse spleen cell cultures); bovine and zinc insulin (0.4 to 2.4 ng/ml); or 2-mercaptoethanol (5×10^{-5} M). Cloning experiments were performed in 0.8% methylcellulose or in 0.25% agar.

Feeder Layers. Skin or conjunctival biopsies were obtained at the time of surgery from retinoblastoma patients or controls and grown in tissue culture to obtain fibroblast monolayers. Mouse fibroblast lines, 257-3 (a derivative of BALB 3T3) and C3H10T½ (derived from C3H embryonic fibroblasts) were also utilized. Cell suspensions were made by exposing the monolayers to 0.2% trypsin. Adherent cell layers from mouse bone marrow were made according to the method described by Dexter *et al.* (5). The monolayers were allowed to grow for 4 weeks before they were tested as feeder layers. Monolayers of human retinal pigment epithelium were prepared on collagen as described by del Monte and Maumenee (4).

Growth Curves. To measure the growth rate, approximately 5×10^4 retinoblastoma tumor cells obtained either from a cryopreserved sample or from a nude mouse were plated onto a previously established confluent monolayer of fibroblasts in 16-mm Linbro dishes in 1 ml of Iscove's modified Dulbecco's minimum essential medium with 15% fetal calf serum. At various times thereafter, the nonadherent retinoblastoma cells were rinsed off, centrifuged, and resuspended in 0.1 ml of medium. This sample was counted in a hemocytometer to determine

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[2] To whom requests for reprints should be addressed, at Wellesley Hospital, 160 Wellesley Street East, Toronto, Ontario, Canada M4Y 1J3.

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[3] The abbreviation used is: i.o., intraocularly.

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the number of tumor cells. Hemocytometer counting, rather than electronic counting, was used because of the strong tendency of these cells to form large aggregates. Doubling times were calculated by regression analysis of the points on the exponential portion of the growth curve.

RESULTS

Fresh retinoblastoma cells did not give prolonged growth in culture unless the tumor cells were placed on a feeder layer of fibroblasts. There was no difference in growth potential of freshly thawed samples, samples passaged through nude mice, and fresh surgical specimens. Chart 1 shows a typical result for tumor RB-302A. When retinoblastoma cells are placed in tissue culture without a feeder layer, they gradually disappear over 15 days in culture. When the same tumor cells are plated on a confluent monolayer of fibroblasts, in this case fibroblasts from the same patient, the cells grow with a doubling time of 4.8 days. A characteristic feature of retinoblastoma lines in tissue culture is that the cells grow in suspension and do not attach firmly to the substrate (13, 15). When fresh retinoblastoma cells are placed on fibroblast monolayers, they also grow in suspension or loosely attached to the fibroblast monolayer (Fig. 1).

Although the experiment summarized in Chart 1 utilized autologous fibroblasts from the feeder layer, some allogeneic fibroblasts work equally well. The data in Chart 2 show the growth of 2 different tumor specimens on 4 different fibroblasts. For both tumors, the best growth was obtained when the tumor was placed on human fibroblast 356 and the worst growth on 257-3, a murine fibroblast line, and 366, another human fibroblast line.

In a more extensive test of the capability of various fibroblasts to act as feeder layers, 2 tumors, RB-267 and RB-247C, were tested for their ability to grow on 12 different fibroblasts. These data are summarized in Table 1. Three conclusions can be drawn from these results: (*a*) although there is marked variability in the feeding capacity of fibroblasts from various donors, there is a definite but weak correlation (correlation coefficient, 0.45) between the ability of a fibroblast strain to support growth of one tumor and its ability to support growth of another tumor;

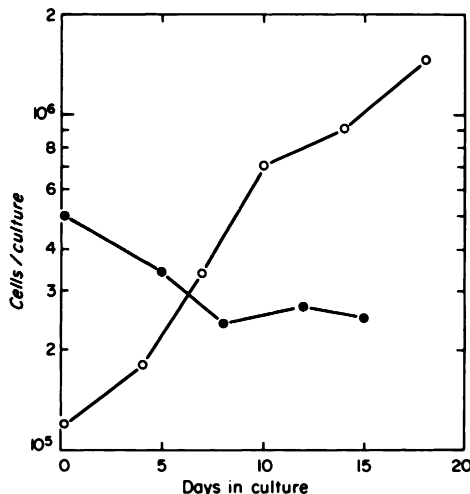

Chart 1. Requirement of feeder layer for growth of retinoblastoma. Tumor RB-302A was placed in culture alone (●) or on fibroblasts derived from the same patient (○).

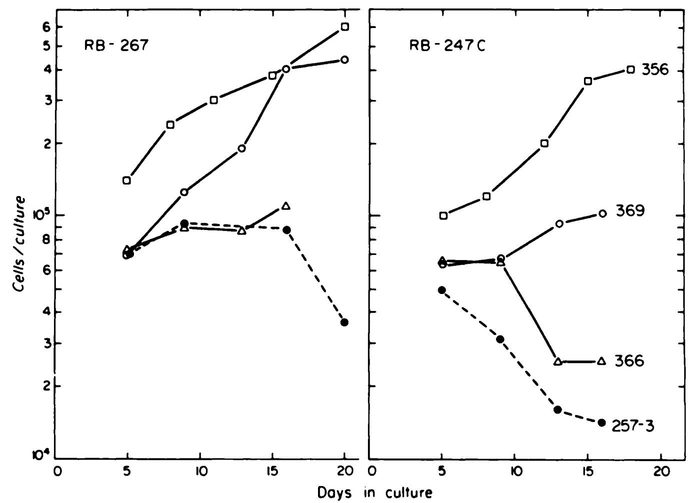

Chart 2. Growth of tumors on different fibroblasts. Tumor cells from 2 different patients (RB-267 and RB-247C) were placed on fibroblast feeder layers of human origin (□, ○, △) or of mouse origin (●). The origin of the fibroblasts is indicated on the chart; the designations are the same as those used in Table 1.

Table 1

Growth rate of retinoblastomas on different feeder layers

Approximately 5 × 10^4 tumor cells were placed in 16-mm wells with confluent monolayers of fibroblasts from the sources shown in the table. For 3 weeks, the number of tumors cells was counted twice weekly in quadruplicate cultures; the doubling time was calculated by regression analysis of the growth curves.

Experiment	Fibroblast feeder		Doubling time (days)	
	No.	Source	RB-267	RB-247C
1	257	Bilateral RB[a]	38	67
	303	Unilateral RB	9.4	17
	369	Unilateral RB	4.5	14
	366	Bilateral RB	21	−6.8[b]
	235	Bilateral RB	16	−36
	257-3	Mouse fibroblast	−17	−3.8
2	357	Bilateral RB	5.4	5.6
	362N	Normal	8.6	8.5
	236	Bilateral RB	13	6.8
	302	Bilateral RB	5.0	11
	367	Bilateral RB	24	7.4
	356	Unilateral RB	7.3	4.4
3	235	Bilateral RB	12	NT
	303	Unilateral RB	10	NT
4	356	Unilateral RB	6.4	10
	303	Unilateral RB	7.0	32
	235	Bilateral RB	9.5	∞[c]

[a] RB, retinoblastoma; NT, not tested.
[b] Negative numbers indicate cells that died on the feeder layer. In these cases, the values shown are the half-life of the tumor cells with feeder layer.
[c] There was no change in the number of tumor cells over 20 days.

(*b*) there is no apparent difference between the ability of fibroblasts from unilateral retinoblastoma patients to support growth compared to those from bilateral retinoblastoma patients. The fibroblasts from unilateral retinoblastoma patients have an 85% chance of being genetically normal, since 85% of unilateral tumors are thought to arise from somatic mutations affecting only a single cell in the retina (12): (*c*) the same fibroblasts give similar doubling times in different experiments. For example, fibroblast 356 was tested in Experiments 2 and 4, and fibroblasts 303 and 235 were tested in Experiments 1, 3, and 4. Comparable results were obtained in each of these experiments.

In addition to the marked heterogeneity among fibroblasts in

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their ability to support tumor growth, tumors show a marked heterogeneity in their ability to grow on a previously selected, good fibroblast monolayer. Chart 3 shows growth curves for 3 different tumors growing on 2 different fibroblasts, selected for their ability to support tumor growth. RB-355 grew well on both fibroblasts; RB-328 grew well on the mouse fibroblasts, but poorly on the human fibroblasts; RB-385 did not grow on either fibroblast. In addition to demonstrating tumor variability, these data demonstrate that some mouse fibroblasts can be feeder layers. In fact, C3H10T½ has reproducibly supported growth of several different retinoblastoma tumor specimens. Murphree *et al.* (14) have also stated that C3H10T½ cells support the growth of retinoblastoma tumor cells.

One of the unique features of the data presented above is the extremely slow growth rate of retinoblastoma tumor cells in tissue culture. We initially considered that the slow growth rate might result from the depletion of medium over the long periods of time that the cells were in culture. However, a test on the effect of regular medium changes, twice per week, showed no effect on the growth rate.

All of the cells which grow on fibroblasts can be maintained in culture for many passages. An example of the maintenance of growth rate following subculture is shown in Chart 4. In this case, both tumors maintained their growth during a second passage. In fact, tumor RB-247C grew better in the second passage than in the first. Several tumors behave similarly to RB-247C and grow faster at low than at high cell concentrations. Over a large number of experiments, we have had no difficulty in maintaining the cultured retinoblastoma tumor cells despite doubling times of 10 days or greater. Tumors which have been maintained in culture for many passages have shown no indication of senescence.

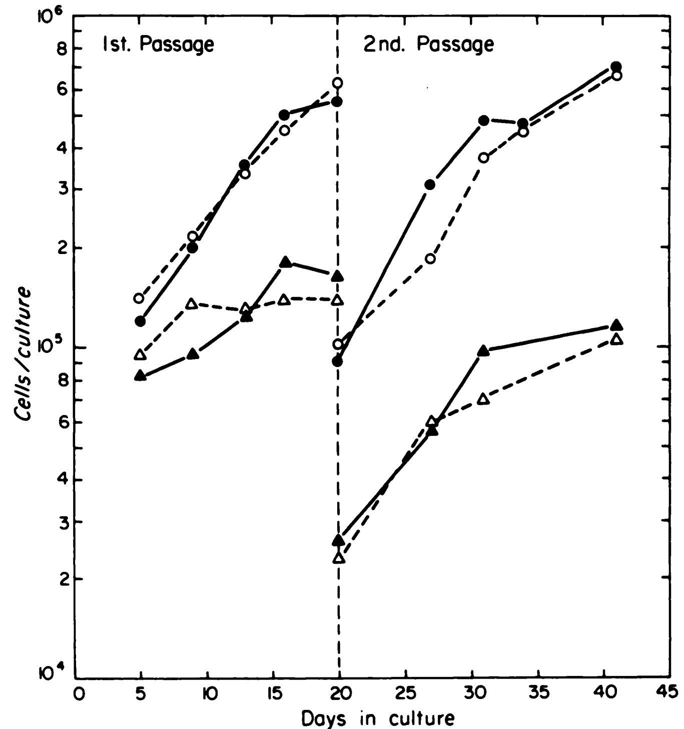

Chart 4. Subculture of retinoblastomas growing on human fibroblasts. Two tumors, RB-267 (○, ●) and RB-247C (△, ▲), were plated either on fibroblasts 356 (●, ▲) or 303 (○, △). Both fibroblasts were obtained from human donors. After growth for 20 days, the nonadherent tumor cells were harvested, and an aliquot was placed on fresh monolayers.

Two observations suggest that the cells obtained after several passages in tissue culture are malignant retinoblastoma tumor cells. First, cells from 3 tumors which were maintained for 7 passages *in vitro* were injected into the eyes of NIH Swiss nude mice and formed tumors. The histology of these tumors was typical of retinoblastomas (Fig. 2). Second, most retinoblastoma tumors have unique chromosome abnormalities. In all cases examined, the abnormalities present in the original tumor have been maintained throughout the culture period without the development of additional abnormalities. The chromosome data will be presented elsewhere.

Twenty retinoblastoma tumors have been tested for growth *in vitro* on fibroblasts previously shown to support the growth of at least 2 retinoblastoma tumors. Thirteen tumor specimens (62%) could be maintained in this way in mass culture. We have not yet been able to clone fibroblast-dependent retinoblastoma tumor cells in semisolid medium. Small clusters form in agar, methylcellulose, and collagen, but the clusters do not continue to grow and we have not been able to establish a line from picked clusters. On the other hand, 2 tumors acquired the ability to grow without fibroblasts, after a period of time on fibroblasts, and these lines could then be cloned.[4] Addition of various factors (nerve growth factor, fibroblast growth factor, epidermal growth factor, retinoids, taurine, hyaluronic acid, fibroblast-conditioned medium, and tumor cell-conditioned medium) has had no effect on the cloning efficiency of retinoblastoma tumor cells. Additions of medium conditioned by mouse

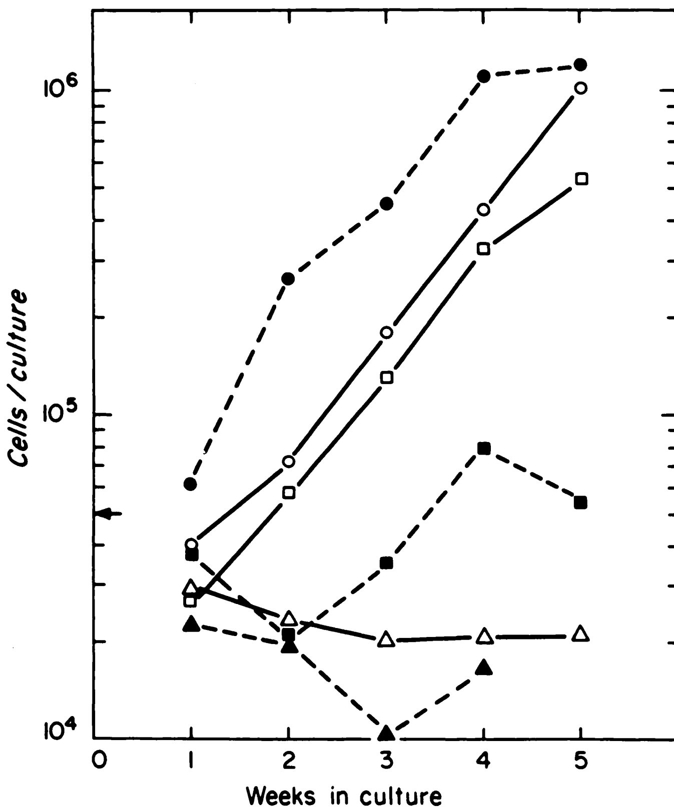

Chart 3. Heterogeneity of growth potential of different tumors. Three different tumors, RB-355 (●, ○), RB-328 (■, □), and RB-385 (▲, △), were plated on human fibroblasts 356 (●, ■, ▲) or mouse fibroblasts [C3H10T½ (○, □, △)].

[4] W. Holmes, unpublished observations.

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spleen cells, insulin, and 2-mercaptoethanol have slightly improved growth rates of retinoblastoma tumors on fibroblasts.

DISCUSSION

Several investigators have reported that feeder layers or conditioned medium enhances the growth of human tumors. Hamburger and Salmon (9) initially used conditioned medium to clone human tumor stem cells. They and their coworkers have also used feeder layers of human RBC (10), and Buick *et al.* (3) found that adherent monocytic cells obtained from the tumor itself enhance the clonogenicity of tumor cells. In early studies, Aaronson *et al.* (1) used human fibroblasts to grow mass cultures of human sarcoma cells. In their hands the best feeder layers were obtained from adult fibroblasts passaged 20 to 30 times. Although we have not investigated the influence of either age or passage number, our initial impression is that neither parameter is critical to the ability of fibroblasts to support the growth of mass cultures of retinoblastomas. More recently, Epstein and Kaplan (7) used fibroblast feeder layers to clone human malignant lymphomas. They also found that only selected lines of fibroblasts gave good growth in cell culture. In their hands, fibroblasts from pediatric donors produced the best monolayers. Since most of our samples were obtained from pediatric donors, we cannot rigorously evaluate the efficiency of fibroblasts from adults. However, the ability of some mouse fibroblasts to work equally well suggests that there will not be a strong dependence on human fibroblasts for supporting growth of retinoblastomas.

As mentioned above, Albert *et al.* showed photographs of primary cultures of retinoblastomas which appeared to have tumor cells growing on autologous fibroblasts (2). Although they interpreted the adherent layer to be tumor cells, they may, in fact, have observed the growth of retinoblastoma tumor cells on feeder layers of fibroblasts. However, in contrast to our experiments, they found that when tumor cells were placed on an adherent layer of WI-38 human fibroblasts, the fibroblasts beneath and around the tumor cells were destroyed. We have observed disorientation of the typical swirling pattern of fibroblasts, but we have never observed destruction of the fibroblasts around the tumor.

Other cell types have been tested for feeder activity, but only fibroblasts have reproducibly supported tumor growth. The retina is adjacent to the pigment epithelium which may provide nutrients to the retina. We have grown monolayers of human pigment epithelial cells (4) and tested them as feeder layers; however, in preliminary tests, pigment epithelium appeared highly toxic to retinoblastoma tumor cells. Because one of the purported functions of the pigment epithelium is to phagocytose debris from retinal cells, this toxicity is perhaps not surprising. Bone marrow is a common site of metastasis for retinoblastomas (6), and for this reason we have tested adherent cells from mouse bone marrow. These monolayers support the growth of hemopoietic stem cells (5). While they provide some support for the growth of human retinoblastomas, they are not as good as fibroblasts, and we have not carefully characterized bone marrow feeder layers.

The significance of the slow growth rate of retinoblastomas is unclear. Although the long doubling time may be an intrinsic property of this tumor, several observations suggest that suboptimal culture conditions contribute significantly to the slow growth rates. Approximately 38% of tumors fail to grow *in vitro*, yet 95% will grow i.o. in nude mice (8). None of the fresh tumors form clones under the current conditions, but established retinoblastoma lines such as Y79 (15) and WERI RBI (13) readily form colonies under the same growth conditions.[5] The observation that some tumor cells will grow in liquid culture when they are on fibroblasts, but not when they are immobilized in semisolid medium above a feeder layer of fibroblasts, suggests either that direct contact with fibroblasts is required or that the factors released by fibroblasts are unstable and do not diffuse over long distances.

The growth of retinoblastomas in mass culture allows analysis of chromosome changes in tumors and studies on the differentiative potential of these slow-growing tumors. Nevertheless, the primary objectives in this field must be to obtain conditions which allow the clonogenic growth of retinoblastoma tumor cells. On the basis of our results, we predict that clonal growth of retinoblastomas will require a combination of conditions involving a feeder layer, complex nutrient media, and growth factors.

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[5] B. L. Gallie, W. Holmes, and R. A. Phillips, unpublished observations.

Fig. 1. Photograph of tumor RB-369A growing on fibroblast 356. Note that the tumor cells grow in tight clumps loosely attached to the fibroblasts. *Bar*, 100 μm.

Fig. 2. Photomicrograph of tumor RB-267 grown in nude mouse eye after 6 months in tissue culture on fibroblasts. Mouse cornea (*C*), iris (*I*), and lens (*L*) are evident with tumor (*RB*) in the anterior chamber of the eye. *Bar*, 100 μm.

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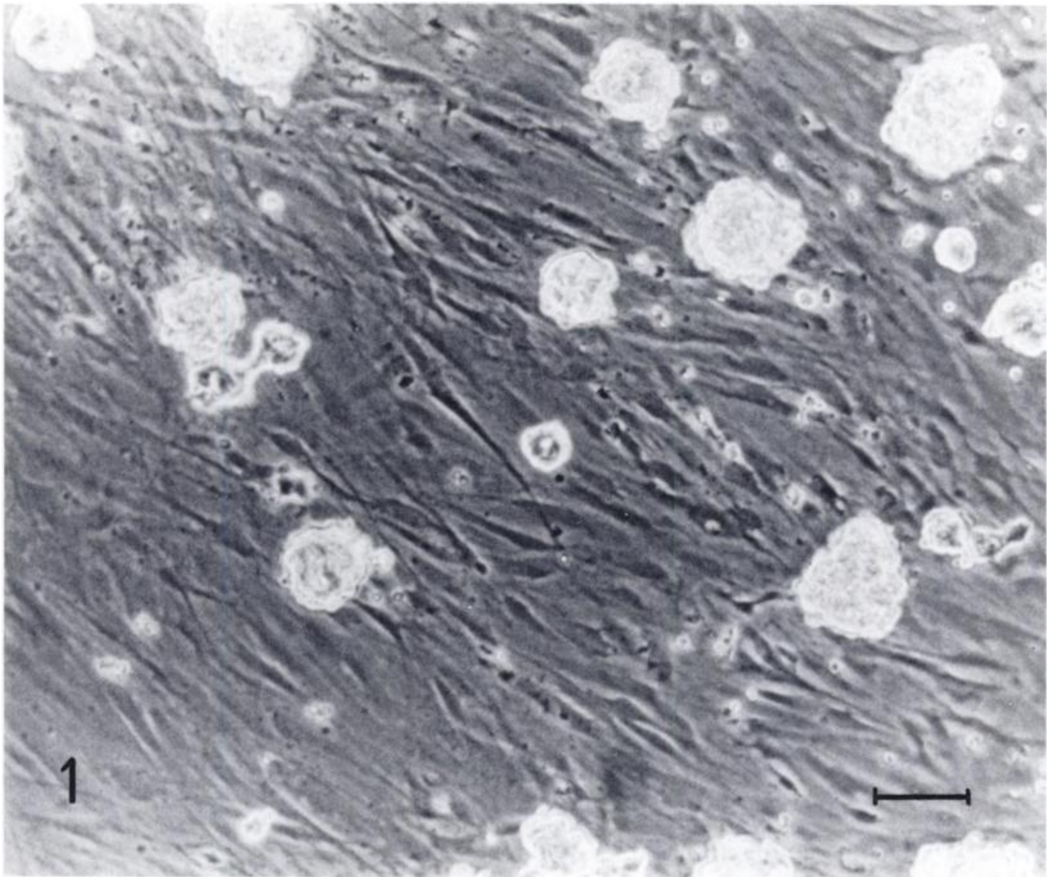

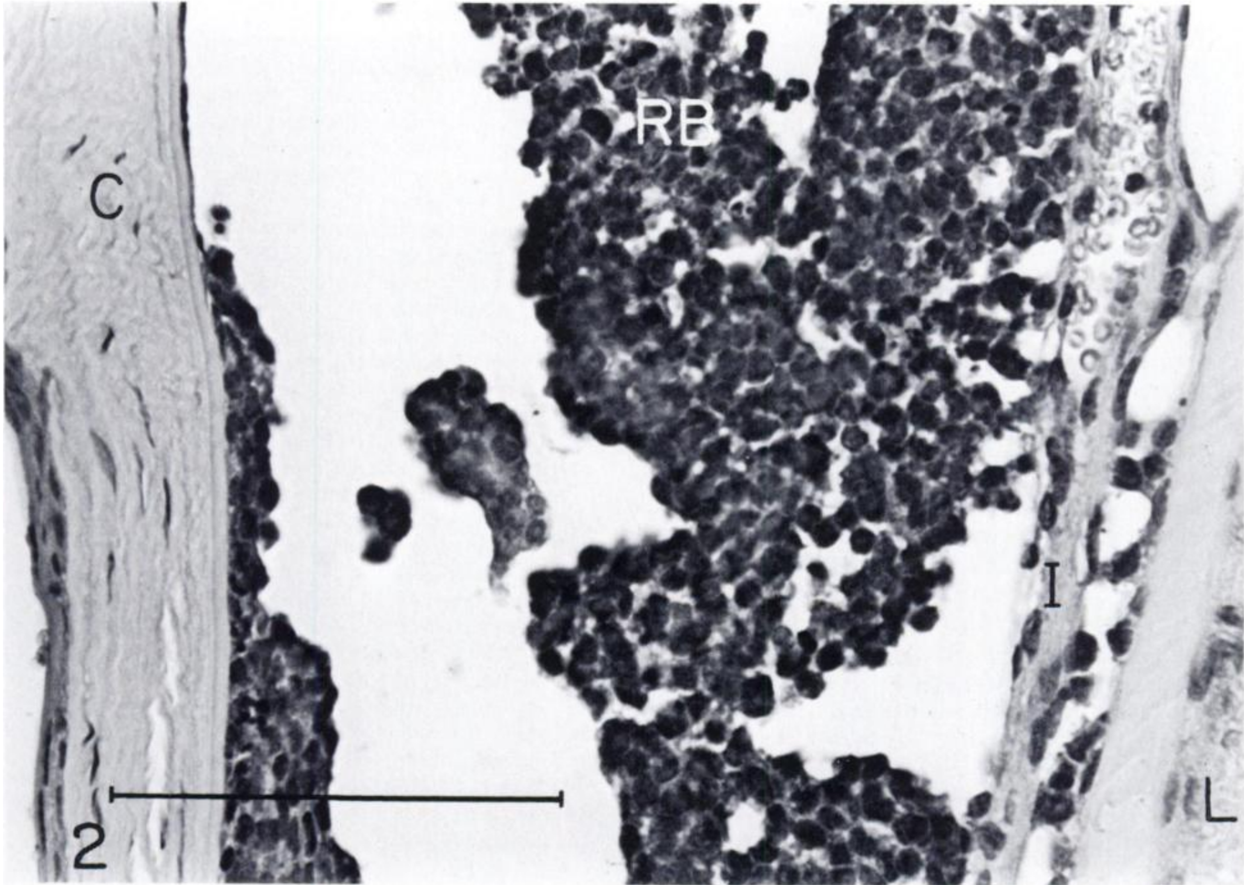

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