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Tissue Culture and Animal Studies with an Oncolytic Bovine Enterovirus (Bovine Enterovirus 1)[1]

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SUMMARY

Treatment with bovine enterovirus 1 (BEV-1) in microtest plates of mouse cells derived from Sarcoma 180, Ehrlich ascites, A755, methylcholanthrene-induced epithelial carcinoma, and an L-cell line causes highly significant decreases (18 to 98%) in viable cells, whereas cells derived from spleen, lung, thymus, liver, and kidney of normal mice showed no significant change after exposure to virus. Application of the method to cells derived from human tumors revealed decreases of 0 to 51% whereas a variety of normal tissues responded by decreases from 0 to 39%. However over one-half the human tumor preparations responded with decreases greater than 17% whereas only 4 of 22 normal preparations showed declines of that magnitude.

The susceptibility of rabbit and mouse kidney cells to BEV did not change over 3 (mouse) to 12 (rabbit) passages in culture. However the "normal" cells of the continuous lines Vero, BSC-1, and LLC-RK$_1$ were susceptible to the virus in both the microtest plate and plaque assays.

Treatment of normal mice, rabbits, and dogs with BEV-1 by intracranial injection led to no clinical abnormalities, nor could virus be recovered from the brain. Some inconstant changes were noted on pathological examination. Mice bearing Sarcoma 180 and treated by i.p. injection of BEV-1 showed only mild splenic hyperplasia early in the course of treatment.

INTRODUCTION

Periodically, viruses of one type or another are proposed as therapeutic tools. Most viruses tested have failed to measure up to the expectations of their proponents and oncologists are properly skeptical of claims made on behalf of these agents (7, 11). While there is need for caution, proposed oncolytic agents should not be summarily dismissed, as a number of viruses produce useful regressions (1, 4, 8, 9, 14, 15).

BEV-1[3] has been investigated by Taylor *et al.* (10, 12, 13) and by Casto *et al.* (2, 3, 5, 16). Taylor *et al.* (12, 13) found the virus lytic for both solid and ascites tumors of the mouse. The virus produced a cytopathic effect in an astrocytoma and several glioblastomas derived from humans but did not affect either a normal brain or a mengioma cell line (10). We have, utilizing a new technique, extended the observations of Taylor's group to other types of tumors and to a variety of cells derived from normal tissues. The effect of BEV on intact animals has also been explored.

MATERIALS AND METHODS

Virus

BEV-1 was obtained from Dr. M. Taylor and propagated in L-cells as described (13). Clarified lysates, produced by freezing, thawing, and centrifugation of infected cells, were stored at −20°.

Tissue Culture Systems

Cells were maintained in Auto-Pow MEM (Flow Laboratories, Rockville, Md.): 5% inactivated FCS. The medium was supplemented with 20 mM glutamine, 30 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (pH 7.2), 0.13 M sodium bicarbonate, 100 units penicillin per ml, and 100 units streptomycin per ml.

BSC-1 and LLC-RK$_1$ cells were from stocks maintained in the laboratory. L-cells were obtained from Dr. Taylor and Vero cells were from the American Type Culture Collection (No. CCL 81).

Microtest Plate System. Cell suspensions were diluted to 1×10^4 cells per ml in MEM:20 FCS. Uninfected L-cell lysate or virus suspension at 20 to 100 PFU/cell was added to the cell suspension and 200 cells (20 µl) were transferred to each of 7 wells of a microtest plate (Falcon Plastics, Cockeysville, Md.). After 24 hr the media were carefully aspirated from the wells and replaced by a 0.05% solution of trypan blue. The number of unstained (living) cells was recorded. In most experiments 2 additional rows of 6 wells, each containing L-cells plus lysate or L-cells plus BEV-1 were included as controls. The mean difference in survival between virus-treated and control cells was computed and the significance of the differences was determined with the *t* test.

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[3] The abbreviations used are: BEV-1, bovine enterovirus 1; MEM, Eagle's minimum essential medium; FCS, fetal calf serum; 20 FCS, 20% FCS; PFU, plaque-forming unit.

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Suspension Cultures. Bone marrows and some tumors were maintained in MEM:20 FCS at 4×10^4 cells per ml. They were infected at 100 PFU/cell and aliquots were removed at intervals for staining with trypan blue and counting.

Tumors and Normal Tissues

Human tissues were obtained at operation and immediately transported to the laboratory in Puck's Saline A. The cells were dissociated by mincing with scissors and stirring in a trypsinizing flask with 5 mM (ethylenedinitrilo) tetraacetic acid:0.25% trypsin. The single cells were suspended in the MEM described above, containing 20 FCS (MEM:20 FCS).

Tissues and tumors were obtained from freshly killed animals and treated in the same manner. Primary cultures were derived by plating in 60-mm plastic dishes or 75 sq cm bottles and maintained as described above.

Bone marrow was obtained by aspiration. The marrow was suspended in MEM:20 FCS.

Animal Inoculation Experiments

Mice. Normal random-bred Swiss mice were given inoculations intracerebrally of approximately 10^8 PFU of BEV-1 in 0.02 ml within 2 days of birth. The animals were killed by cervical fracture and the brains and selected organs were removed for examination for virus (brain) or histological examination (brain and organs).

Young adult Swiss mice bearing Sarcoma 180 were given inoculations i.p. of BEV-1. The animals were observed frequently and killed and autopsied at various times after injection of virus.

Rabbits. New Zealand white rabbits were given injections i.p. of about 10^8 PFU of BEV-1 within 3 days of birth. The animals were observed clinically until sacrifice and autopsy. Brains were examined histologically and cultured for virus.

Dogs. Several dogs bearing tumors of various types were donated for these experiments. The tumors were biopsied and 2 or 3 days later 10^8 PFU of BEV was injected into the tumor. The animals were observed for at least 1 week and changes in gross clinical condition or tumor size were recorded. In addition, 1 litter of 4 puppies was given inoculations intracerebrally on the 3rd day of life.

Virus Recovery. Brains of injection-treated animals for BEV-1 assay were homogenized in sterile phosphate-buffered 0.9% NaCl solutions and clarified at low speed, and the supernatant fluid was plated on a monolayer of L-cells. These were observed for cytopathic effects. None was ever noted.

RESULTS

Differential Effects of BEV-1 Observed on Normal and Tumor Cells Derived from the Mouse: Microtest Plate System

BEV-1 treatment of L-cells caused a decrease in survival of 83 to 98%. Decreases in survival of cells derived from other malignant tissues ranged from 18 to 66%. All these decreases were significant ($p \leq 0.01$). On the other hand, cells derived from most normal tissues showed either no changes after BEV-1 treatment or decreases of 1 to 10%. Addition of antiserum prevented the action of BEV-1. A cutoff point of 17% decrease in survival would differentiate all these normal from malignant tissues. These results are presented in Table 1, and the effect of virus is portrayed in Fig. 1.

Differential Effects of BEV-1 Observed on Normal and Tumor Cells Derived from Humans

Microtest Plate System. Twenty-two preparations derived from 9 different types of normal tissue and 18 preparations from malignant tissue were tested (Table 2). In the majority of instances treatment with BEV-1 was associated with decreased viability of cells at 24 hr in both the normal and malignant tissues. In the case of cells derived from normals, the decrease was significant ($p \leq 0.05$) in 7 instances (32% of the specimens) but a decrease greater than 17%, the cutoff point for mice, was noted in only 4 (18%). In 1 of these latter instances the difference dropped from 25.9 to 4.6% on repetition of the test, and we suspect mislabeling of the 1st specimen. In contrast to the normals, 11 (65%) of the 18 malignant tissues exhibited decreases greater than 17%, and in 10 instances statistically significant decreases were obtained. Two benign tumors showed decreases of 17 and 37% but the result with the breast fibroadenoma alone was statistically significant.

Suspension Cultures. There were no differences from untreated control values in 4 cases of acute lymphoblastic leukemia in relapse or 4 normal marrows. Five solid tumors were tested in suspension culture. Of these, 2 showed decreases of 20 and 35% ($p \leq 0.05$). Normal tissues other than marrow did survive in suspension and this approach was abandoned.

Effect of Cell Passage on Susceptibility to BEV-1

Fibroblasts derived from human skin showed no discernible difference in susceptibility to BEV-1 (as determined in microtest plates) with passage (Table 2). Plaque assays were attempted on 2 of these with negative results.

Cells derived from the rabbit, mouse, and dog were tested in the microtest plate system with the results shown in Table 3. These cells, not susceptible in primary culture, did not develop sensitivity to the virus despite repeated passage (up to 12 in the case of rabbit kidney). Similarly, plaque assays attempted on these cells were uniformly negative.

The results with continuous cell lines derived from normal monkey kidney (Vero, BSC-1) and normal rabbit kidney (LLC-RK_1) were quite different. Virus plaqued on all 3, and the cells were very susceptible in the microtest plate system. Each of these normal lines was examined by Dr. Catherine Palmer and found to be polyploid.

Transformation Experiments. Six plastic Petri dishes (60 mm, 1×10^6 cells) and 3 plastic bottles (25 sq cm, 1.5×10^6 cells) of skin fibroblasts were treated with 100 PFU of

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Table 1

Effect of BEV on mouse cell viability

Single cell suspensions were prepared with trypsin:(ethylenedinitrilo)tetraacetic acid, except for ascites cells. BEV-1 was added at 100 PFU/cell. Cell viability was assessed at 24 hr by exclusion of trypan blue.

Cell type	Av. no. living cells ± S.D.		% decrease
	BEV-treated	Control	
A. Normal			
Spleen	28.2 ± 4.2	25.3 ± 4.4	
Lung	22.7 ± 2.6	23.8 ± 4.5	4.6
Thymus	41.3 ± 3.9	35.2 ± 6.8	
Liver	19.0 ± 2.3	21.2 ± 3.1	10.4
Kidney (1)	64.3 ± 9.9	61.2 ± 8.1	
(2)	31.3 ± 6.5	32.0 ± 6.8	2.2
(3)	42.3 ± 7.4	42.8 ± 8.2	1.2
B. Malignant			
L-cells (1)	12.3 ± 2.9	82.7 ± 26.3	85.8[a]
(2)	9.8 ± 14.8	57.7 ± 5.9	82.9[a]
(3)	2.3 ± 1.5	32.7 ± 4.4	92.8[a]
(4)	3.7 ± 2.0	54.0 ± 9.3	93.3[a]
(5)	1.3 ± 1.5	57.0 ± 7.7	97.9[a]
L-cells + BEV antiserum	65.2 ± 14.2	57.0 ± 7.7	
Ehrlich ascites (1)	36.3 ± 2.2	54.0 ± 6.4	17.7[a]
(2)	58.8 ± 3.9	84.5 ± 8.5	30.4[a]
Sarcoma 180	76.5 ± 14.9	221.8 ± 22.4	65.6[a]
A755	46.7 ± 10.3	63.0 ± 6.4	25.9[a]
Methylcholanthrene-induced epithelial carcinoma	46.0 ± 4.2	78.2 ± 3.2	41.7[a]

[a] $p \leq 0.01$.

BEV-1 per cell for 30 min. MEM:15% inactivated FCS was added and changed once or twice a week. After 1 month with no apparent transformation, the cells were trypsinized and transferred to plastic flasks. The cells attached but did not divide well. After another month the cells were trypsinized and transferred to 0.3% agar in MEM:20 FCS. After 1 week all the cells were dead.

Animal Experiments

Normal Mice. Three litters of 13, 8, and 11 mice were given injections intracerebrally at Age 1 or 2 days of 0.01 to 0.02 ml containing approximately 10^8 PFU of BEV-1. The mice were observed clinically and individual mice were killed over a period of 6 to 90 days. Brains were examined histologically or extracted in an attempt to recover virus or both. No abnormal behavior or signs of illness were ever observed. The brains of the 1st 2 litters, obtained between 6 and 60 days, were grossly and microscopically normal except for several collections of small cells, apparently lymphocytes, in the cortex and along a vessel in 2 instances. The brains from the 3rd litter were collected on either Day 60 or 90. The 6 brains of the 1st group were all entirely normal whereas those from the mice killed at 90 days after injection showed a number of small collections of glial cells in the cortex of the ventral surface. These were also apparent in uninjected control mice and are of unknown significance. No virus was recovered from the 10 brains tested (Ages 7 to 20 days).

Mice Bearing Ascites Tumor Sarcoma 180. Although healthy mice are apparently not harmed by (intracranial) injection of BEV, it is conceivable that mice with advanced cancers might respond differently to the agent. To test this possibility mice bearing Sarcoma 180 were treated with BEV and examined at intervals. Eight young mice were given inoculations of a suspension of ascites cells and 1 week later the injection-treated animals all had enlarged abdomens. Each mouse was given injections i.p. of 10^8 PFU (0.2 ml) of BEV-1. Single animals were killed at 1, 3, 5, 6, 8, and 25 days and the kidneys, lungs, heart, spleen, and liver were fixed and examined histologically. By Day 8 most tumor cells were gone. The only other change noted was some hyperplasia of the spleen. The last animal developed purulent peritonitis, probably as a result of infection at the time of injection. The virus itself seems to have produced little or no change other than lysis of the tumor cells. This is in accord with the clinical observations of Taylor *et al.* (12), who have observed that mice cured of Sarcoma 180 are clinically healthy 1 year after treatment.

Normal Rabbits. Two litters of 5 and 6 rabbits were given injections intracranially on the 6th day with 10^8 PFU in a volume of 0.05 ml. Two animals were killed on the 10th day and the remainder on the 63rd day. At the time of death all animals and their mothers were clinically normal. The brains were removed and examined histologically. One of the 8 animals examined after 2 months showed some xanthochromic staining of the left hemisphere. This was apparently the result of insertion of the needle. This animal also had microscopic evidence of diffuse, interstitial pneumonitis with lymphocytic and reticuloendothelial cell infiltration. Kidney, liver, spleen, and intestines were normal. The remainder of the brains were unremarkable and a 2nd animal

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Table 2
Effect of BEV on viability of human cells

See legend to Table 1.

Cell type	Av. no. living cells ± S.D.		% decrease
	BEV-treated	Control	
A. Normal			
Skin (1)	26.7 ± 1.8	28.8 ± 2.3	7.3[a]
(2)	36.2 ± 7.3	37.8 ± 6.4	4.4
(3)	29.0 ± 3.1	33.8 ± 6.7	14.2
(4)	32.8 ± 4.4	31.2 ± 6.1	
(5)	22.3 ± 2.3	30.2 ± 7.8	25.9[b]
	67.0 ± 11.5	70.2 ± 7.3	4.6
(6)	18.3 ± 3.1	19.7 ± 1.9	6.8
Liver (1)	43.7 ± 2.7	47.2 ± 2.9	7.4[a]
(2)	15.8 ± 1.9	17.2 ± 2.6	8.2
Lung (1)	60.7 ± 8.7	57.5 ± 12.3	
(2)	17.8 ± 1.5	20.2 ± 4.5	11.9
(3)	102.8 ± 11.4	90.8 ± 13.4	
Spleen (1)	26.7 ± 2.2	36.0 ± 2.6	25.8[b]
(2)	52.5 ± 3.5	86.3 ± 4.5	39.2[b]
(3)	88.2 ± 9.9	82.7 ± 12.8	
Breast (1)	20.5 ± 2.3	22.3 ± 5.4	8.5
(2)	93.2 ± 9.0	95.0 ± 5.2	1.9
Salivary gland (1)	53.0 ± 8.4	52.0 ± 3.9	
(2)	40.5 ± 5.8	44.3 ± 4.8	8.6
Colon	36.2 ± 3.4	38.0 ± 7.6	4.8
Ileum	32.8 ± 3.1	37.5 ± 3.1	12.5[a]
Tonsil	22.2 ± 6.1	27.3 ± 8.0	18.6[a]
B. Tumors			
1. Epidermoid carcinomas			
Tongue (1)	38.8 ± 5.0	60.3 ± 4.8	35.6[b]
(2)	33.7 ± 8.2	37.5 ± 5.2	10.0
Larynx	20.3 ± 3.7	22.3 ± 2.3	10.3
Throat	24.8 ± 3.9	48.7 ± 6.4	49.0[b]
Lung (1)	112.0 ± 13.8	108.8 ± 12.8	
(2)	64.7 ± 6.2	72.3 ± 4.8	10.5[a]
Mouth (1)	49.2 ± 10.0	60.3 ± 14.6	18.4
(2)	68.3 ± 23.4	71.2 ± 28.9	4.1
2. Adenocarcinomas			
Pleura	50.3 ± 6.7	71.0 ± 2.0	29.2[b]
Small intestine	18.5 ± 3.6	36.3 ± 4.8	49.0[b]
Colon	21.7 ± 4.6	26.8 ± 3.8	19.0
3. Miscellaneous			
Breast (ductal)	58.7 ± 8.3	58.7 ± 8.2	0
Breast (medullary)	17.5 ± 5.7	35.8 ± 6.7	51.1[b]
Lung (undifferentiated)	22.2 ± 5.3	40.7 ± 2.0	45.4[b]
Liver (undifferentiated)	68.8 ± 8.3	79.3 ± 5.1	18.3[a]
Kidney (renal cell)	35.2 ± 4.5	38.2 ± 4.8	7.9
Bladder (transitional cell)	69.2 ± 5.0	85.0 ± 7.8	18.6[b]
Skin (sarcoma)	16.2 ± 2.3	21.3 ± 2.8	23.6[b]
4. Benign			
Parotid (mixed)	39.8 ± 3.9	48.2 ± 11.3	17.4
Breast (fibroadenoma)	13.8 ± 3.0	21.8 ± 4.0	36.7[b]

[a] $p \leq 0.05$.
[b] $p \leq 0.01$.

submitted for complete autopsy revealed no gross or microscopic abnormalities.

Seven of the brains (2 from the animals sacrificed at 10 days and the remainder from those killed at 63 days) were extracted for virus, but none was found.

Normal Dogs. Four puppies were given injections intracranically of 2×10^7 PFU (0.02 ml) of BEV-1 on the 3rd day of life. The mother did not nurse them and 2 died 3 days later and were discarded. One died on the 10th day of life. The brain was grossly normal but on microscopic examination some minimal focal subacute meningitis was seen. The brain did not yield virus. The remaining animal was sacrificed on the 11th day. That brain was entirely normal.

Tumor-bearing Dogs. Although 6 dogs were received and given inoculations of virus, only 2 of the tumors were suitable for evaluation. The remainder was either benign or showed too much cartilaginous metaplasia to permit evaluation of change in the tumor. All the animals were observed clinically for at least 1 week. No abnormalities in

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behavior, appetite, or stool were noted. The 2 evaluated tumors were a myxoma of the head and a seminoma. The former showed no response to 2 weekly injections whereas the latter regressed somewhat for 1 week and then continued to enlarge despite a 2nd injection. Cells from the myxoma did not respond to BEV-1 in the microtest plate assay (61 *versus* 66 living cells). The seminoma showed a decrease (46 *versus* 68 living cells). Interestingly, when the tumor was removed a month after the last injection it was resistant to BEV-1 *in vitro* (70 *versus* 74 living cells).

DISCUSSION

The tests conducted in microtest plates resulted in a clear distinction between the response of normal and malignant cells of murine origin to treatment with BEV-1. The difference in survival of the 2 classes of cells was usually marked and in our experience to date there has been no overlap. For practical purposes a percentage decrease of 17 divides normal from malignant cells. However, we have avoided lymphomas, which Taylor *et al.* (12) showed to be resistant to BEV-1. The spectrum of tumors examined is not wide but includes 2 solid as well as 2 ascites tumors and 1 of the former was chemically induced.

The results obtained with human tissues were mixed. Whereas the bulk of the 22 normal preparations tested showed only minor changes after treatment with BEV-1, 4 exhibited marked decreases. Even after allowance is made for the 1 probable error, in 3 (14%) of the preparations a decrease of over 18% in survival attended treatment with virus. On the other hand, 11 (61%) of the preparations derived from malignant tissues responded to BEV-1 with a drop in survival of more than 18%.

Evaluating the results obtained with single cell preparations derived from solid tumors is difficult, particularly when their origin is spontaneous. Even when, as here, care is taken to obtain tissue that appears grossly malignant, cells of normal origin (fibroblasts, endothelial and blood cells, etc.) may be admixed. The data from both the murine and human experiments indicate that these cells are resistant and that their presence would serve to lower the apparent response of the "malignant" tissues. This would cause the difference between control and treated preparations to be less than it would be if only tumor cells were present. The objection is less in the case of ascites tumors, as these are largely homogeneous. The best has been made of the situation by obtaining tissue that appeared grossly malignant at operation and confirming its essential homogeneity by pathological examination. It can be concluded safely that treatment of single cell suspensions with BEV-1 in microtest plates under the conditions described will clearly distinguish those normal murine and malignant tumors that were tested and in general will serve to differentiate normal from tumor tissue in man. The method does not work with bone marrow (and indeed, as the results with spleen and tonsil reported in Table 2 show, may not really be suited for any tissue of lymphoid or hematopoietic nature). However, in consonance with the results of Taylor *et al.* (12) with mouse leukemia L4946, we have found suspensions of both normal and leukemic marrow resistant to killings by the virus.

While the microtest plate method may differentiate between normal and malignant tissues, it may not serve as a true indicator of susceptibility of tumors *in situ* to lysis by the virus (11). A broad spectrum of transplantable mouse tumors are susceptible *in vitro*, and the *in vivo* results with Ehrlich ascites and Sarcoma 180 have been confirmed in this laboratory (M. E. Hodes and S. Morgan, unpublished data). Treatment of 2 spontaneous tumors of dogs showed some correlations of susceptibility *in vitro* and *in vivo*, although for many reasons (duration and dose, mode of administration, etc.) the results were not conclusive.

The experiments in intact animals comprised clinical observations on mice and dogs treated for tumors and pathological examination of animals killed at various times after inoculation with BEV-1. Particular emphasis was placed

Table 3

Effect of cell passage on susceptibility to BEV

Cells were carried as monolayers in plastic flasks or Petri dishes and passed with the aid of trypsin:(ethylenedinitrilo)tetraacetic acid when confluence was reached. Rabbit and mouse kidney cells were derived from fresh tissue (see legend to Table 1).

Cell type	Passage	Av. no. living cells ± S.D.		% decrease	Plaque assay
		BEV-treated	Control		
Rabbit kidney	0	61.2 ± 7.1	68.7 ± 9.9	10.9	0
	3	147.0 ± 27.1	143.8 ± 19.3		0
	5	70.8 ± 6.7	72.3 ± 4.0	2.1	0
	12	69.5 ± 7.1	70.5 ± 10.3	1.4	
Mouse kidney	0	74.5 ± 10.6	76.2 ± 12.2	2.2	0
	2	82.5 ± 6.3	82.5 ± 7.3	0	0
	3	44.7 ± 4.9	45.8 ± 7.7	2.4	
Vero	Continuous	2.3 ± 2.2	64.8 ± 11.4	97.0[a]	+
BSC-1	Continuous	30.2 ± 11.0	66.2 ± 13.5	54.5[a]	+
$LLC\text{-}RK_1$	Continuous	56.0 ± 6.8	70.7 ± 12.1	20.8[b]	+

[a] $p \leq 0.01$.
[b] $p \leq 0.05$.

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on changes after intracerebral inoculation. No constant pathological findings were evident in the experiments.

Anergy is known to accompany advanced malignant disease (6) and fatalities have followed viral therapy in man (11, 15). For these reasons mice with advanced (Sarcoma 180) disease were treated and subjected to pathological examination. Except for 1 mouse that developed purulent peritonitis, probably as a result of injection, nothing remarkable was found. It had previously been reported that mice can live at least 1 year after cure with BEV-1 (12). The mice, rabbits, and dogs reported here also showed no ill effects from viral treatment after periods of 1 to 3 months.

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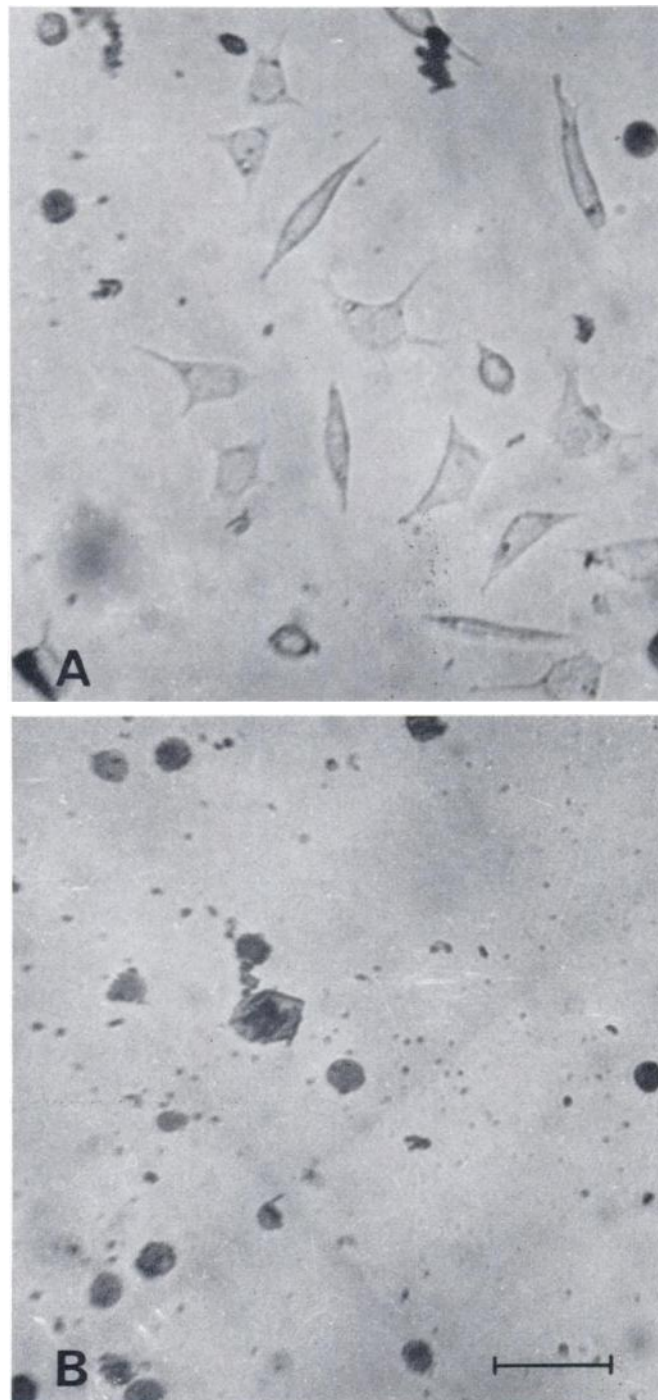

Fig. 1. L-cells 24 hr after treatment of trypsinized cell suspension with lysate of uninfected L-cells (A) and BEV-1 (B). The high-power views are of a portion of a well of a microtest plate after aspiration of medium and staining with trypan blue. *Bar*, 100 μm.

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