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Viruses as an Aid to Cancer Therapy: Regression of Solid and Ascites Tumors in Rodents After Treatment with Bovine Enterovirus

M. W. TAYLOR, B. CORDELL, M. SOUHRADA, AND S. PRATHER

Department of Microbiology, Indiana University, Bloomington, Ind. 47401

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ABSTRACT **Treatment of ascites and solid tumors in mice (Sarcoma-1 and Ehrlich ascites carcinoma) with bovine enterovirus-1 resulted in regression of the tumors without any pathological effect on the animals. Death of mice with lymphatic-leukemia L4946 was delayed after such treatment. The oncolytic specificity of the virus does not appear to involve the production of interferon, but requires specific adsorption of virus to the tumor cells. The specificity of killing extends to cells in culture, since viral-transformed cells and oncogenic cells are susceptible to the virus, in contrast to cells of untransformed lines and cells of primary cultures, which are resistant.**

The possibility of utilizing the specificity of nonvirulent viruses in therapeutic treatment of human cancers is considered.

The use of viruses specific for the destruction of cancer has remained essentially a laboratory curiosity. Many researchers have suggested that it should be possible to utilize biological mechanisms with sufficient specificity to discriminate between a malignant tumor cell and a normal cell in order to control tumor growth. During the last forty years, a few laboratories have investigated this possibility with different viruses (1–3). However, apart from a report (4) of the partial inhibition of a transplantable murine leukemia by M-P virus, most oncolytic viruses have been found to be too lethal for the host for this treatment to be practical.

A few successful instances of oncolysis in man have been reported. However, the results of such experiments are difficult to evaluate since viral treatment followed other therapeutic measures that could have played a part in tumor regression. Wheelock and Dingle (5) achieved a temporary remission of acute leukemia in a human subject by successive inoculation of six different viruses.

In this paper, we present evidence of viral oncolysis in rodents carrying both solid and ascites tumors without any apparent effect on the host animal.

MATERIALS AND METHODS

Virus production

Bovine enterovirus-1 (BEV-1) was grown in cell monolayers of Maden Bovine kidney or mouse L-cells in Eagle's minimal essential medium supplemented with 5% chick serum, streptomycin (100 μg/ml), penicillin (100 units/ml), and fungizone (2.5 μg/ml). High-titer lysates of BEV-1 were produced by infecting monolayers of confluent roller cultures (690 cm^2; about 5×10^8 cells) with virus at a multiplicity of infection of 1–10 plaque-forming units (PFU)/cell in medium without serum. Cultures were rolled for 1 hr at 1 rpm on a Bellco roller apparatus to allow virus adsorption. At that time, fresh medium containing 5% serum was added, and infected cells were incubated 12–15 hr. Bottles were passed through three cycles of freeze and thaw to release virus. The cell debris was removed by centrifugation for 10 min at 3000 rpm. Lysates were stored in small aliquots at −20°C. BEV-1 was assayed by the plaque technique and hemagglutination as previously described (6).

Tumors

Ehrlich ascites tumor and Sarcoma-1 (SA-1), in ascites form, were maintained in the peritoneal cavity of adult Swiss–Webster mice. The tumor cells were transferred intraperitoneally at 8-day intervals. Tumor growth was measured by direct cell count in a hemocytometer, or by measuring the increase in weight of the mouse, which is a reflection of proliferation of tumor cells and increased ascites fluid (3). Solid tumors were established by inoculating known amounts of cells of Sarcoma-1 or Ehrlich ascites tumor into the thighs of mice. Mouse leukemia 4946 was kindly supplied by Dr. J. Simmons, University of Chicago. This tumor was transferred intraperitoneally at 7-day intervals. We have expressed tumor size as an approximation of the palpable surface area (cm^2). Initial measurements demonstrated a clear correlation between the weight of excised tumor and surface-area measurements *in situ*. This type of measurement was necessary in order to follow the fate of individual tumors after treatment.

The adsorption of BEV-1 to mouse primary cells and tumors was measured by assaying virus unadsorbed to cell monolayers or to organ homogenates after 60 min of incubation at 37°C. Unadsorbed virus was measured by plaque assay and hemagglutination. Mengovirus, a host-specific virus for the mouse, was used as a control under the same conditions.

BEV-1 was inactivated by treating virus with ultraviolet light (Mineral Light) for 30 min from 10-cm distance. Inactivation of the virus was demonstrated by its inability to form plaques at a 10,000-fold dilution.

Cell lines

Cells, grown as monolayers, used in this study were: *human*, HeLa, KB, and Hep-2, all derived from human cancerous tissue; *bovine*, Maden Bovine kidney; *mouse*, macrophages, L-

Abbreviations: BEV-1, Bovine enterovirus-1; SA-1, Sarcoma-1.

cell fibroblasts, 3T3, and 3T3 transformed by polyoma virus; *chinese hamster* lung cells and *chinese hamster* ovary cells.

Histology procedure

The histological materials were fixed in 5% formaldehyde for a minimum of 8–10 hr, then processed through alcohol and embedded in paraffin. They were cut at 6 μm and stained with hematoxylin and eosin. The smears were air-dried and fixed and stained with Wright's stain.

RESULTS

We have previously reported that Ehrlich ascites tumor and Sarcoma-1 grown in the peritoneal cavity of adult mice regressed rapidly after treatment with BEV-1 (6). No tumor cells were detectable by microscopic examination in such mice 48 hr after treatment. In control animals (no tumors, treated with BEV-1) no adverse effect on the animals was noted; such animals appear to be healthy, with sleek fur and normal weight gain. "Cured" mice have been retained as long as 1 year without recurrence of the tumor.

Table 1 illustrates the effect of BEV-1 on solid tumors of Sarcoma-1. Mice were injected intramuscularly with 10^8 washed Sarcoma-1 cells. Within 48 hr, a visible swelling was noted on the thigh. At this time, approximately 10^8 PFU of BEV-1 were inoculated into the tumor. Tumor development was measured at 3- to 4-day intervals after virus injection. The data in Table 1 demonstrate that treated mice had significantly smaller tumors within 1 week of BEV-1 inoculation as compared to controls, and that such tumors were arrested in growth. Control animals began to die at about 14 days, whereas treated tumors slowly regressed (1.65 cm^2 by day 21); treated animals have been kept for 6 months after complete tumor regression without recurrence of the tumor.

In order to examine the effect of time of inoculation with BEV-1 after SA-1 injection, 10^5 SA-1 cells were transplanted into 35 adult mice. At the same time, and on the next 4 consecutive days, 10^8 BEV-1 were injected into the site of the tumor in different groups of animals. 12 days after tumor transplantation, the size of the tumor was measured. All of the control animals (10) had palpable tumors; the average size of the tumor was 2.96 ± 0.55 cm^2. In animals injected with BEV-1 on the same day as SA-1 transplantation, only one animal out of five had a palpable tumor (size 1.26 cm^2). This tumor grew to 1.5 cm^2 by day 17, regressed to 0.84 cm^2 by day 20, and was not palpable by day 24. Mice inoculated with BEV-1 on day 5 had tumors on day 12(1.39 ± 0.76 cm^2). However, by day 20, these tumors had decreased in size to 1.02 ± 0.25 cm^2, and by day 24 a small tumor was found in only one of the mice. The number of tumors found depended on the time of BEV-treatment. These data are summarized in Table 2. In control animals, the tumors reached their maximum size on day 17 (3.68 ± 1.02 cm^2) and had regressed by day 20, at which time the mice began to die.

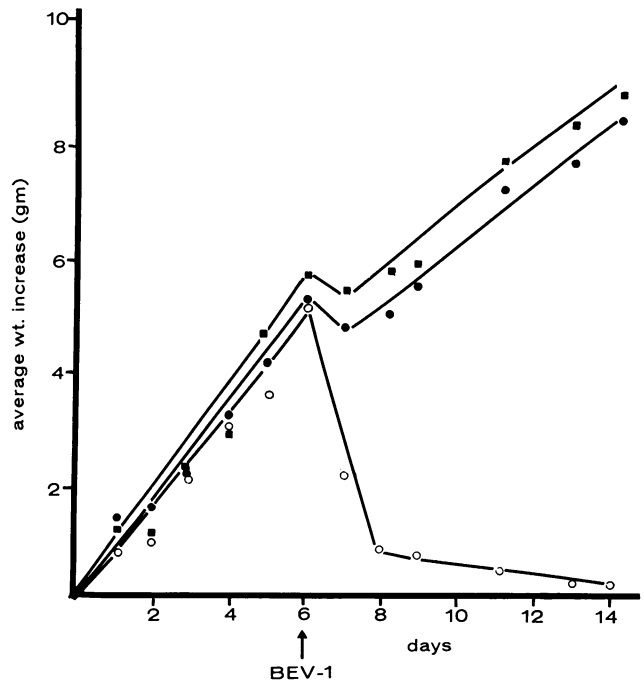

FIG. 1. Effect of UV radiation inactivation of virus on oncolysis. ■—■, no virus added; ●—●, UV-treated lysate; O—O, active virus. Tumor growth measured as increase in weight of the mouse (groups of 10 mice).

No recurrence of tumor growth has been noted in the "cured" animals. However, such animals were still sensitive to a further inoculum of 10^6 SA-1 cells. Similar findings were found when solid Ehrlich ascites tumor was used in place of SA-1.

Intraperitoneal transfer of Leukemia 4946 leads to death of inoculated Swiss–Webster mice within 7–8 days. On treating such animals with BEV-1, 2 days after the transfer of Leukemia 4946, we have noted a 48-hr delay in their death. However, in all cases, death does occur. We believe that these re-

TABLE 1. *Treatment of solid SA-1 tumors with BEV-1*

Treatment (days)	Approx. area of tumors (cm^2)	
	Control	Treated
9	3.92 ± 0.58 (10)	2.13 ± 0.69 (10)
14	4.95 ± 0.98 (9)*	1.92 ± 0.69 (10)

Mice were injected intramuscularly with 1 × 10^8 SA-1 cells. 2 days later, BEV-1 (about 10^8 PFU) was added. Measurements of tumors were made 9 and 14 days after tumor inoculation. $P < 0.01$, as calculated by Student's t test. Values given as means ± SD (no. of animals in parentheses).

* (1 animal dead).

TABLE 2. *Number of mice with solid sarcomas at different times after inoculation with BEV-1*

Times of addition of BEV-1	Number of mice with tumors		
	Day 12	Day 17	Day 24
Control—no BEV	10/10	10/10	9/9
Day 1	1/5	1/5	0/5
Day 2	3/5	3/5	2/5
Day 3	3/5	2/5	1/5
Day 4	4/5	5/5	1/5
Day 5	5/5	5/5	1/5

Mice were injected intramuscularly with 10^5 washed SA-1 cells, followed by treatment with 10^8 BEV-1 on different days.

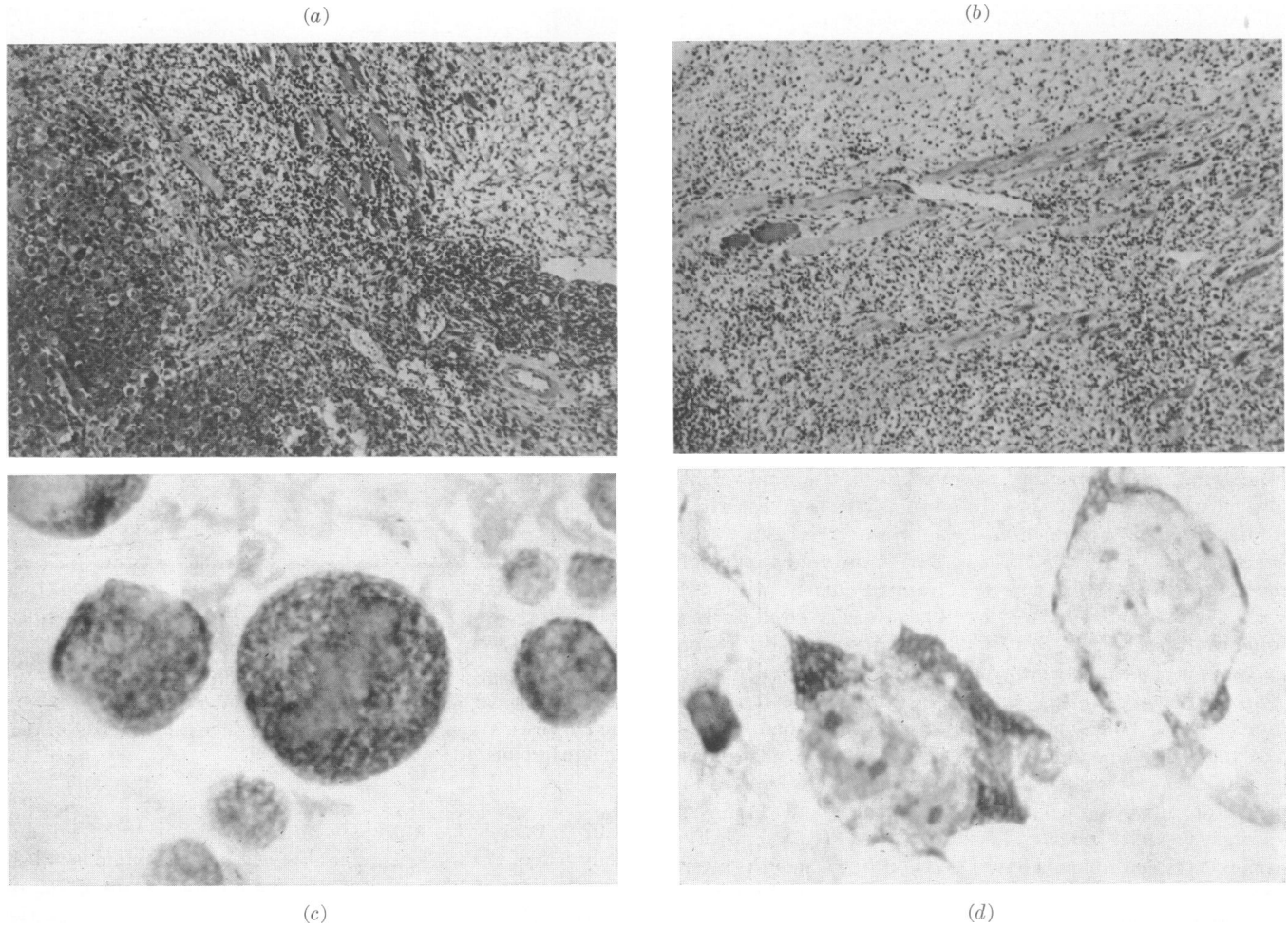

FIG. 2. (*a*) Cross section through the thigh area, 5 days after Sarcoma-1 injection. Striated muscle has undergone extensive necrosis; note on *left side* of plate, cells possessing attributes of malignancy (numerous mitotic figures, giant cells, pyknotic nucleoli). (*b*) Cross section of treated mice (48 hr after BEV-1), area of necrosis much smaller. Polymorphonuclear leukocytic component more pronounced. No visible tumor cells. In other sections, individual tumor cells undergoing necrosis (lysis). (*c*) Untreated Ehrlich ascites tumor cells from peritoneal cavity. (*d*) Ehrlich ascites cells, 12 hr after virus treatment (*in vivo*). Note extensive vacuolation and nuclear destruction. Magnification, (*a*), (*b*) ×100; (*c*), (*d*) ×900.

sults are negative, although we are currently investigating the reason for the delay in killing.

It was of interest to examine whether tumor regression was due to the direct cytolytic effect of the virus, or to an indirect effect in which the presence of viral protein or RNA was sufficient. It has been suggested that oncolysis may be due to interferon production in the tumor cells (5). Many animal cancers have been shown to have a viral etiology, so that infection of the tumor cell by a second virus (BEV-1) could lead to interferon production. This interferon would then act to interfere with the growth of the tumor cell. Since foreign nucleic acid is sufficient to induce interferon synthesis, ultraviolet-irradiated virus is able to induce synthesis of interferon (7). Since inactivated virus is incapable of replication, we could determine whether tumor regression is due to direct cytolysis (as a result of viral replication) or is a consequence of interferon stimulation.

A BEV-1 viral lysate was treated with UV radiation to 100% lethality, as measured by plaque assay. When inactivated virus was injected intraperitoneally into mice carrying Ehrlich ascites tumor or Sarcoma-1 in the peritoneal cavity, no regression of the tumor occurred. These animals showed a weight increase (reflecting tumor growth), as did the untreated controls (Fig. 1). However, active virus from the same lysate gave a typical oncolytic pattern, i.e., rapid decrease in weight, suggesting direct cytolysis as the mechanism of tumor regression.

In a further test of this mechanism of tumor regression, tumors of animals treated with virus were studied histologically. The results of this investigation show that there are almost no tumor cells present in the peritoneal cavity of treated animals, and that the few remaining tumor cells are undergoing cytolysis. In the treated solid tumor, there is less necrosis of muscle tissue than in controls, and the tumor appears to be undergoing degenerative changes. Groups of cells appear to be surrounded by polymorphonuclear leukocytes, a phenomenon absent in untreated animals [Fig. 2 (*a–d*)].

The specificity of the virus for the tumor cell could be due to either an external or internal barrier to viral infection of the normal host cell. Reasons for this could be: (*a*) lack of ability of BEV-1 to adsorb to nonmalignant host cells; (*b*) inability of BEV-1 to replicate in the normal host cell; or (*c*) no damage to the normal host cell during BEV replication.

When primary mouse embryo cell cultures or primary mouse

kidney cell cultures were infected with BEV-1, no cytopathic effect was noted. When virus adsorption was measured on these cells, after 1 hr at 37°C, less than 1% of the added virus was on the cells. On the other hand, adsorption to Ehrlich ascites tumor cells was very efficient. McLaren *et al.* (8) have shown that the kinetics of viral adsorption are the same for intact cells and for cellular debris resulting from freeze–thaw cycles. Mouse kidneys (organs) and Ehrlich ascites cells were either homogenized with a Dounce homogenizer or passed through cycles of freeze–thaw. The cellular debris was centrifuged, and the pelleted material was resuspended and tested for its ability to adsorb BEV-1. As is shown in Fig. 2, BEV-1 did not adsorb to the kidney homogenate, although there was efficient adsorption of this preparation to an Ehrlich ascites tumor homogenate. To exclude the possibility that all viral receptor sites on the kidney have been damaged, mengovirus adsorption to both homogenized tissues was measured. This occurred as expected.

The specificity of BEV-1 infection was further tested by examining whether any correlation could be found between the cytopathic effect of BEV-1 on cells in culture and known oncogenic cell lines. Table 3 lists the cell lines tested. No such effect was noted on mouse cell line 3T3 (nononcogenic), although two polyoma-transformed cell lines were sensitive to the virus. Note that three human oncogenic cell lines, HeLa, KB, and Hep-2, are very sensitive to virus infection. Adsorption was also tested and, in the cases where no cytopathic effect was observed, the virus was found incapable of irreversible attachment. In those cell lines exhibiting cytopathic effect, adsorption (determined by titering the inoculating supernatant after the 1-hr adsorption period) was 90–95% complete.

DISCUSSION

This paper presents evidence that a virus that is not virulent for a specific host can be used therapeutically to treat either an ascites tumor or a solid tumor. In this particular case, we have used BEV-1, a bovine enterovirus apparently harmless to rodents. In culture, the virus forms large plaques on bovine kidney and L-cell monolayers within 48 hr; its eclipse time is short, about 1–1.5 hr after infection, and it matures 6 hr after infection. It is this short maturation time, concomitant with rapid cell killing, that makes BEV-1 an extremely efficient virus for oncolysis. The utilization of a virus with specificity toward neoplastic cells allows us to control and treat various physiological responses of only these cells. It should be possible to screen nonvirulent viruses for their tissue (or tumor) specificity and to use these viruses against early stages of tumor development. The data in this paper demonstrate that cytopathic effect on one cell type need not be related to the general virulence of the virus. It should be possible and practical to select or adapt viruses to different tumors for therapeutic usage. Of course, an obvious danger in such a treatment would be the occasional virus mutant that is more virulent to the host than is the original strain.

The basis of the specificity of BEV-1 appears to lie in the host cell membrane, i.e., adsorption specificity. It is unlikely that this specificity is a unique one, e.g., a specific receptor site, but rather it is probably a general interaction between tumor membrane and virus. Membranes of tumor cells are known to be different from those of normal cells and to be more negatively charged. Burger (9) has reported that an agglutinin from wheat-germ interacts with tumor-specific surface sites. These sites contain *N*-acetylglucosamine and sialic acid. A nonspecific interaction may occur between viral protein and some "uncovered" areas of the membrane. Our observation that viral-transformed 3T3 cells, but not the parental 3T3,

TABLE 3. *Response of various cell types to infection with BEV-1*

Cell type	Origin	Cytopathic effect
Maden kidney	bovine	++
Primary	bovine	++
L-cells	mouse	++
Macrophage	mouse	—
3T3	mouse	—
3T3 Py 3	polyoma-transformed 3T3	++
3T3 Py 6	polyoma-transformed 3T3	++
Primary	mouse	—
Chinese hamster (DON)	hamster lung	++
Chinese hamster ovary	hamster ovary	—
KB	human	++
HeLa	human	++
Hep-2	human	++

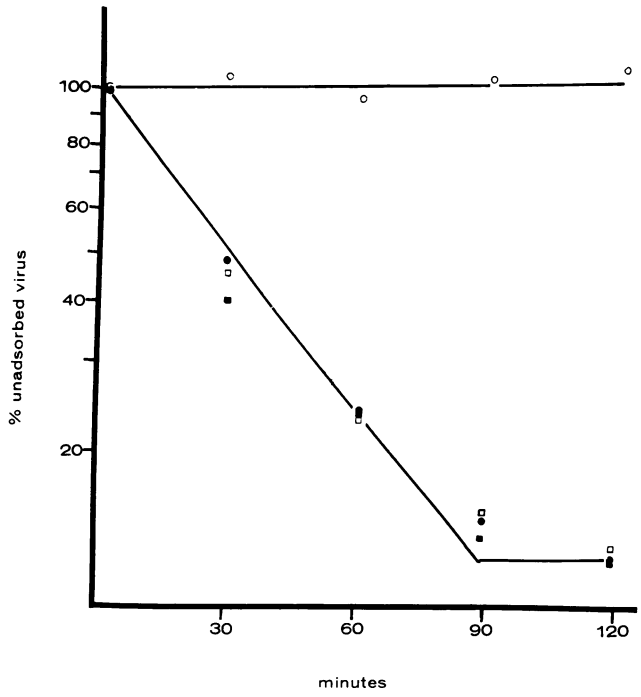

FIG. 3. Test of adsorption of Bovine Enterovirus-1 to Ehrlich ascites tumor (EAT) debris and mouse kidney (organ) debris. BEV-1, at a multiplicity of 20, was added to cell debris from both homogenized mouse kidney and EAT, at a concentration comparable to 2×10^6 cells/ml. Samples of 1 ml were removed at 30-min intervals and centrifuged at 3000 rpm for 10 min. Supernatants were assayed for unadsorbed virus; the number of unadsorbed viruses was determined by both plaque assay and hemagglutination. A control with a mouse-specific virus, mengovirus, was also performed. O—O, BEV-1-kidney; ●—●, BEV-1—EAT; □—□, Mengo—kidney; ■—■ Mengo—EAT.

are sensitive to BEV-1 suggests that such alterations may be a general characteristic of many tumors.

Preliminary experiments with hepatoma 3924 in rats show a partial regression of tumor growth. A possible reason for the absence of complete regression of some of the tumors might be a host-developed immunity to BEV-1. This might be overcome by utilizing antigenically different viruses. For example, four distinct serotypes of BEV-1 are known (10). Sequential infection with each one might overcome immunity problems. Furthermore, treatment with virus might be used after surgical removal of the tumor to eradicate any remaining or metastasizing cells. Nonvirulent viruses might also be used in conjunction with immunosuppressive drugs.

It is not to be expected that all tumor cells will be sensitive to any one group of virus. The lack of killing of L4946 cells could be due to resistance of these cells to BEV-1. Other nonvirulent viruses should be screened on various tumors to examine their host specificity.

Oncolytic viruses might also play a role in diagnostic techniques. If adsorption of the virus is specific to neoplastic tissue, tagged BEV-1 (fluorescent) might be used to detect cancerous growth.

It is our hope that these observations may be extended to different human cancers and to *in vivo* therapeutic processes.

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1. Moore, A. E., *Annu. Rev. Microbiol.*, **8**, 393 (1954).
2. Southam, C. M., *Trans. N.Y Acad. Sci.*, Sec. II, **22**, 657 (1960).
3. Lindenman, J., and P. A. Klein, *Recent Results in Cancer Research* (1967), Vol. 9.
4. Molomut, N., and M. Padnos, *Nature*, **208**, 948 (1965).
5. Wheelock, E. F., and J. H. Dingle, *New Engl. J. Med.*, **271**, 645 (1964).
6. Taylor, M. W., J. N. Davidson, C. Land, and R. Wall, *J. Nat. Cancer Inst.*, **44**, 515 (1970).
7. Henle, W., and G. Hendle, *J. Exp. Med.*, **85**, 347–364 (1946).
8. McLaren, L. C., J. J. Holland, and J. T. Syvertan, *J. Exp. Med.*, **109**, 475–485 (1959).
9. Burger, M. M., *Proc. Nat. Acad. Sci. USA*, **62**, 994 (1969).
10. Barya, M. A., T. Moll, and D. Mattson, *Amer. J. Vet. Res.*, **28**, 1283 (1967).