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Lethal Synergy between Sarcoma-180/TG Cells and Vesicular Stomatitis Virus in Mice[1] (39105)

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A widely used technique (1) to increase the yield of virus antibody from immunized mice consists of stimulating ascites with tumor cells and collecting the fluid at some point thereafter. Employing this technique to produce vesicular stomatitis virus (VSV) hyperimmune ascitic fluid in adult imice, an interaction between virus and sarcoma-180/TG (S-180/TG) cells occurred under certain conditions, which resulted in extensive mortality. In this report we characterize the interaction, which we term lethal synergy, and present evidence on its mechanism of action.

Materials and methods. Mice. Female NAMRU (2) or COBS (Charles River Breeders, Wilmington, Mass.) strains of mice were used at 10–12 weeks of age in most experiments.

S-180/TG cells. The origin, preparation, and use of these cells in mice have been described (3). With these conditions, mice became fully ascitic after 3 weeks and survived for 5 weeks or longer.

Virus. The New Jersey and Indiana serotypes of VSV were used. The identity of each virus was confirmed with typing sera from Yale University, New Haven, Conn. The viruses were prepared for use by passage either in primary chick embryo cell cultures (CE), or in the brains of 3–5-day-old NAMRU mice (MB).

VSV inactivation. Where indicated, VSV was inactivated by beta-propiolactone (Fellows Testagar Company, Detroit, Mich.) (4).

VSV assay. Virus was quantitated either by plaque assay using CE cells, or by intraperitoneal (ip) LD_{50} determinations using 3–5-day-old mice ($SMLD_{50}$). The overlay medium for CE cells consisted of 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution (HBSS) plus 1% washed agar. Plaques were resolved with 1:5000 neutral red after incubation at 37°C for 2 days.

Serology. Ascites from mice were immediately centrifuged to remove cells. Complement fixing (CF) and neutralizing antibodies to VSV in the supernate were then determined (5, 6).

Biochemicals. The following chemicals were used: histamine diphosphate monohydrate, serotonin creatinine sulfate complex, L-epinephrine (all from Calbiochem, LaJolla, Ca.), pyrilamine maleate (Robinson Laboratories, San Francisco, Ca.), dibenamine hydrochloride (K and K Laboratories, Plainview, N.Y.), and cortisone acetate ("Cortone," Merck Sharpe and Dohme, West Point, Pa.). All chemicals were used (at nontoxic concentrations as determined in adult mice) in 0.9% NaCl which also contained penicillin (200 units/ml) and streptomycin (200 μg/ml.

Results. Lethal synergy. Table I shows mortality following injections of S-180/TG cells and VSV. Similar data were obtained with either virus serotype. Cells (ca. 5×10^5/animal) were administered by the ip route. Mice inoculated with virus received 0.5 ml each; 0.3 ml ip and the remainder into the deep muscles of the left rear leg (im). Mice exposed to VSV 1 week before S-180/TG cells remained well. When virus and cells were injected simultaneously, mortality was 56%, and this increased to over 90% if the cells preceded virus by 1 week. Thus, the immune status with respect to VSV at the time of S-180/TG cell administration determined whether or not lethal synergy occurred.

The mortality observed was well in advance of ascites development. For example, mice in Expt. 4 (Table I) showed no evidence of ascites when VSV was injected. Within

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TABLE I. MORTALITY IN ADULT MICE FOLLOWING INJECTIONS OF SARCOMA-180/TG CELLS AND VESICULAR STOMATITIS VIRUS

Experiment number	Regimen[a] (days)				Virus dose ($SMLD_{50}$/mouse)	Mice (*n*/strain)	Mortality (%)
	0	7	14	35			
1	V	V	V	C	$10^{5.0}$	280/NAMRU	0
2	V	V, C	C		$10^{6.5}$	280/NAMRU	0
3	V, C	V, C			$10^{5.0}$	50/NAMRU	56
4	C	V			$10^{5.0}$	280/NAMRU	93
5	C	V			$10^{6.5}$	280/NAMRU	90
6	C	V_I[b]	V_I		—	280/NAMRU	0
7a	C	V_I			—	280/NAMRU	0
7b[c]			V, C		$10^{6.5}$	280/NAMRU	35
8	V	V	V		$10^{8.0}$	50/NAMRU	0
9	C	C			—	50/NAMRU	0
10	C	V			$10^{6.5}$	15/COBS	93
11	V				$10^{8.0}$	15/COBS	0
12	C				—	15/COBS	0

[a] Injection sequence on days indicated of VSV(V) and ca. 5×10^5 S-180/TG cells (C)/mouse.
[b] $10^{6.5}$ $SMLD_{50}$VSV inactivated before use with beta-propiolactone.
[c] Animals of Expt. 7a after Day 14.

24 hr mortality was 20%, and most animals died within 48 hr. Similar results were obtained using either MB or CE produced VSV. In addition, when Expt. 4 was repeated with mice that received prior injections of normal mouse brain, CE, or S-180/TG cells subjected to five cycles of freeze–thaw, the results were not altered. Therefore, protection against lethal synergy could not be achieved by preexposing the animals to either virus host cell or S-180/TG cell antigens.

Results markedly different from those of Expt. 4 (Table I) occurred when the time between exposure of the mice to S-180/TG cells and VSV was extended to 21 days. Well developed ascites existed in these animals, yet mortality occurred only after 7–15 days and was reduced to 50% or less, especially in older mice. Survivors usually resorbed their ascites within 2 weeks of VSV injection and remained nonascitic following administration of additional S-180/TG cells or VSV.

Serology. The ascites of 10 mice in Expt. 1 (Table I), where no mortality existed, contained VSV antibody with an average neutralization index of 6×10^3 and a CF titer of 1:64 2 weeks after the injection of S-180/TG cells. In contrast, no CF antibody was detected in the rare survivors of Expt. 4 two weeks after VSV injection, although neutralizing antibody levels were the same for both groups.

Virus replication. VSV was able to grow to high titer in the well developed ascites of mice free of antibody to this virus. Table II shows that most of the VSV injected was cell-associated within 5 min. A high titer was reached at 24 hr which increased significantly during an additional day. After 5 days no VSV was detected in the residual ascites of these animals.

The *in vitro* growth of VSV in S-180/TG cells removed from similar mice is shown in Fig. 1. In order to control pH, the cells were washed after removal from the mouse and diluted before infection. At the end of the growth period, each of 10 normal mice was injected ip with 1 ml of the suspension. No ascites or mortality developed in this group. A similar experiment using S-180/TG cells from mice with ascites containing both neutralizing and CF antibody to VSV reproduced the data in Fig. 1. Therefore, tumor cells from animals which rapidly cleared injected virus with no detectable synthesis were unaltered in their ability to synthesize VSV *in vitro*.

Protection. The rapid onset of mortality

TABLE II. GROWTH OF VESICULAR STOMATITIS VIRUS IN SARCOMA-180/TG CELLS IN VIVO

Time[a]	VSV titer in ascites (PFU/ml)	
	Cells[b]	Fluid
5 min	not done	3×10^3
24 hr	4×10^6	3×10^8
48 hr	1×10^8	5×10^9
5 days	0	0

[a] Each of 10 adult mice injected ip at Time 0 with 2×10^7 PFU of VSV 3 weeks after ascites initiated with S-180/TG cells.

[b] Cells washed three times in HBSS, resuspended to original volume, and disrupted by freeze–thaw before assay.

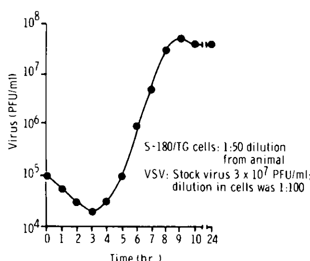

FIG. 1. Growth curve of VSV in S-180/TG cells *in vitro*.

from lethal synergy suggested the possibility that some form of hypersensitive response was initiated by VSV in mice during the early stages of ascites formation. The time interval between exposure to S-180/TG cells and VSV was shortened to determine its effect on mortality. Each of 20 mice was inoculated ip with 2×10^7 washed S-180/TG cells followed in 3 hr by $10^{5.0}$ $SMLD_{50}$ of VSV. All mice survived 1 day, but mortality was complete within 2 days. Lethal synergy was therefore initiated at very early stages of ascites formation. When this experiment was repeated using either S-180/TG cells or VSV treated before use with beta-propiolactone, no mortality occurred.

We next determined if selected chemicals known to modify hypersensitive states produced a significant effect on lethal synergy. These chemicals were used to treat groups of 20 mice individually given ca. 5×10^5 S-180/TG cells 1 week earlier. VSV was injected 3 hr later to initiate lethal synergy. The mortality, shown in Table III, occurred almost completely between the first and second day

TABLE III. ACTION OF SELECTED BIOCHEMICALS ON LETHAL SYNERGY IN ADULT MICE[a]

Biochemical	Dose[b]/ mouse (mg)	Mortality (%)
Serotonin	1.5	100
Histamine	3.0	90
Dibenamine	3.0	40
Pyrilamine	1.5	90
Dibenamine/pyrilamine	3.0/1.5	30
Dibenamine/serotonin	3.0/1.5	70
Epinephrine	0.01	100
Cortisone	0.25	100
None	—	100

[a] Lethal synergy initiated by VSV ($10^{6.5}$ $SMLD_{50}$) in mice administered S-180/TG cells one week earlier.

[b] Injected 3 hr before VSV in 0.5 ml 0.9% NaCl (0.3 ml ip, 0.2 ml im). Each group contained 20 mice.

after virus was given. The capacity of histamine or serotonin to enhance mortality could not be determined since all control mice died. However, in the group given the serotonin antagonist dibenamine, 60% survived, and serotonin was able to reverse this protective effect. In the group given both dibenamine and the histamine antagonist pyrilamine, 70% survived. No marked protective effect was noted when the latter compound was used alone. Control groups included mice treated in the same way with each of the chemicals in Table III, but with the difference that lethal synergy was avoided by withholding S-180/TG cells, VSV, or both. No mortality occurred in these animals.

Survivors from the dibenamine groups in Table III were observed for several months. These mice did not develop ascites, although in rare instances solid tumors did arise in the region of im injection. To further quantitate the influence of dibenamine on lethal synergy and ascites formation, the following experiment was carried out. S-180/TG cells removed from the ascites of mice free of VSV antibody were washed, counted, serially diluted, and immediately injected into untreated mice and corresponding animals given dibenamine 24 hr earlier. Both groups were injected with VSV ca. 3 hr later. Table IV shows that dibenamine again protected

TABLE IV. EFFECT OF DIBENAMINE ON LETHAL SYNERGY AND THE INDUCTION OF ASCITES IN SURVIVING ADULT MICE[a]

S-180/TG cells[b] (number/mouse)	Mortality (%)		Ascites induced (%)	
	Dibenamine[c]	Control	Dibenamine	Control
2×10^7	30	100	0	—
2×10^6	10	40	0	0
2×10^5	0	0	10	60
2×10^4	0	0	10	60
2×10^3	0	0	0	50

[a] Lethal synergy initiated by VSV ($10^{6.5}$ $SMLD_{50}$) in mice administered S-180/TG cells 3 hr earlier.

[b] Washed in HBSS, counted, and diluted for use. Each group contained 20 mice.

[c] Ten mice/group were given dibenamine (3 mg/mouse) day before use; the rest remained untreated (control).

mice from lethal synergy, which could be initiated by VSV in untreated animals receiving as few as 2×10^6 S-180/TG cells 3 hr earlier.

Survivors of this experiment also were observed for 3 months. Table IV shows that after 4–6 weeks some developed ascites, and almost all these mice were from the untreated group. Dibenamine, therefore, appeared to suppress ascites formation in those mice that were given S-180/TG cells in numbers below the threshold that produced lethal synergy. It was without effect in suppressing ascites formation in animals not given VSV.

Discussion. Earlier work by Lindenmann (7, 8) and others (9, 10, 11) demonstrated that certain viruses produced oncolysis in mice bearing ascitic tumors. These animals, or normals immunized with the lysates, did not develop ascites when challenged later with viable tumor cells of the same line. However, normals immunized with virus-free tumor material did develop ascites when challenged in this way. It has been proposed (12) that such viruses expose tumor-specific antigens that are masked or suppressed (13) in intact or homogenized tumor cells.

We worked with a sarcoma-180 cell originated and later utilized by Sartorelli (14). This line generated large volumes of ascites in mice before causing mortality. In contrast, other S-180 or Ehrlich ascites carcinoma cells used in most of the studies cited above killed more quickly. By utilizing S-180/TG cells under conditions that did not kill animals within 5 weeks, we were able to distinguish two types of response of tumor-bearing mice to VSV. Conventional oncolysis occurred in fully ascitic mice that had been given S-180/TG cells some 3 weeks before virus. In this case, VSV adsorbed and replicated to high titer in the tumor cells. Lethality had a delayed onset, usually after 7–15 days, and many mice survived. The second type of response occurred in mice given S-180/TG cells 3 hr to 1 week before VSV exposure. Almost all mice died, usually on the first or second day. When S-180/TG cells were administered 3 hr before VSV, 100% of the animals died within 2 days. If the interval between inoculation of sarcoma cells and VSV was extended to 1 week, some animals survived, and not all deaths occurred within 2 days. This suggested that some difference in mouse response occurred as the period between S-180/TG and virus exposure was extended.

The second type of response was termed lethal synergy. The rapid onset of mortality under these conditions led us to postulate that it was caused by hypersensitivity. Hypersensitivity reactions at tumor sites have been demonstrated with chemical inducers (15). In addition, the suggestion has been made (16) that the response of mice to tumor lines of high virulence can mimic anaphylactic shock. Tumor-bearing mice have altered sensitivities to vasoactive substaneces such as endotoxins (17), and variations in response also can occur in the presence of other substances which modify the host-defense system (18, 19).

We observed that mice treated with the serotonin antagonist dibenamine showed a marked reduction in mortality from lethal synergy. This protective effect was reversed by serotonin. The ability of dibenamine to antagonize the effects of serotonin on the vascular damage and edema resulting from hypersensitivity reactions has been demonstrated (20). In addition, VSV has been shown (21) to activate certain hypersensitivity states which also could be antagonized by adrenergic blocking agents similar to dibenamine. A relationship has been estab-

lished (22) between sarcoma-180 and Ehrlich carcinoma. Cells of the latter type were found in the heart, blood vessels, and meninges within 1 week of ip administration (23). In addition, such cells varied markedly during ascites formation in their concentrations of polyamines which interacted adversely with the host (24). Lethal synergy may result from VSV-induced lysis of S-180/TG cells that then release into the blood of sensitized animals sufficient amounts of adrenergic stimulating substances to cause death.

Summary. An interaction between sarcoma-180/TG cells and vesicular stomatitis virus in adult mice resulted in the rapid onset of extensive mortality. This interaction, termed lethal synergy, occurred only at early stages of ascites induction in animals with no prior virus contact. A significant sparing effect conferred by the serotonin antagonist dibenamine was reversed by the administration of serotonin. The cause of death was not determined, but a mechanism involving hypersensitivity is indicated.

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