# Supporting Role of Vasodilators in Inducing Regressions of the Ehrlich Solid Tumor.* (32250)

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The effectiveness of chemotherapy or immunological procedures, active or passive, depends in part on the localization of anti-tumor agents in the tumor-bearing tissue. The absence of inflammation in neoplastic tissue not secondarily infected or necrotic denies such tissue the protective role of increased blood vessel permeability which would aid in the localization of immune gamma globulins, cells such as lymphocytes with anti-tumor properties, or chemotherapeutic agents.

This paper presents evidence that one procedure, the use of a long-acting vasodilator, pentaerythritol tetranitrate (PETN), one of a number of procedures we have investigated, does increase the localization in solid Ehrlich mouse carcinoma of a blood vessel permeability measuring dye, Evans blue. Additional evidence suggests that PETN increases the localization of anti Ehrlich antibodies prepared in rabbits as shown by a decreased rate of growth, prolonged survival of the mice or actual tumor regression in mice treated with anti Ehrlich serum and PETN.

These results present the first demonstration of a favorable response of mice with an established Ehrlich solid tumor that we have obtained in a long search for adjuvant drugs or procedures to aid anti-tumor serum in inducing a slowing of growth or regression of the tumor.

*Materials and methods.* Four- to 5-week-old, male, albino mice, 16-20 g, were inoculated subcutaneously in the left flank with 0.2 ml of a 10% (approximately $8 \times 10^6$ cells) suspension of washed Ehrlich ascites tumor cells. After 7-12 days, the mice were shaved and the tumors were measured with calipers, taking the greatest measure of length, breadth, and thickness of the tumor. The 3 measurements were multiplied together to estimate the tumor volume, in $cm^3$. Tumors smaller than 0.8 $cm^3$ or larger than 3.0 $cm^3$ were not used. The mice were distributed into the various groups according to tumor size, to minimize variations due to the rates of growth of the individual tumors and to permit observation of such effects if they existed. The mice were weighed on each day of injection and all inocula were 0.1 ml of the given preparation per 20 g body weight with the exception of Evans blue dye (0.2 ml per 20 g weight).

*Dye localization tests.* Mice selected were injected i.p. with a 1% solution of Evans blue dye (EB) in 0.85% NaCl (saline), daily for 3 days. The vasoactive agents were injected 1 or more times per day for 3 days, the first daily dose given 2 hours after the dye. Control animals received EB and saline, the diluent used for the drugs. On the day following the last injections, the tumors were measured again. Mice in which tumors had decreased in size were discarded. Remaining mice were sacrificed without anesthesia and the tumors were excised. The fibrinous exudate was peeled from the surface of the tumors and the lymph nodes were removed. Each tumor was weighed immediately to the nearest 0.5 mg and either was placed in acid at once for digestion or was frozen on dry ice, stored at −26°C and extracted at a later date. The methods for acid digestion of the tissue and benzalkonium chloride (Zephiran® chloride)-chloroform extraction of the dye were those reported by Beach and Steinetz(1) with little modification (Whatman #40 filter paper was required to obtain a clear chloroform extract and the results were read in a Coleman Universal spectrophotometer, Model 14, at 600 mμ). Two tumors from untreated mice were digested and extracted with each test to serve as standards and controls. Comparisons of the average values obtained from treated and control groups from each experiment were made using Student's "t" test of significance.

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*Regression tests.* The mice were treated daily (6 or 7 days/wk) with vasoactive drugs and antitumor preparations until most of the mice died or the tumors regressed. Deviations from this plan are noted below. Tumors were measured at least once a week to follow their rates of growth. Survival times of the mice and the incidence of complete regression of the tumor were also observed.

*Preparations of drugs and antisera.* $NaNO_2$, pentaerythritol tetranitrate (PETN),† isosorbide dinitrate (Isordil,® Ives Laboratories), and epinephrine hydrochloride were the vasoactive drugs employed. $NaNO_2$ and epinephrine were dissolved in saline. PETN and Isordil were ground in a mortar and 1 ml of saline was added to the contents of 1 30-mg capsule or to 1 10-mg tablet, respectively. The concentrations of PETN and Isordil are referred to as 30 mg/ml (or 3 mg/0.1 ml) and 10 mg/ml throughout. In addition to the volume displaced by the drugs themselves, each preparation contained inert substances necessary because of the explosive nature of PETN alone. The 0.1 ml of each suspension contained less than the 3 mg and 1 mg ascribed to it below. Since PETN and Isordil were insoluble, a single inoculum was aspirated into the syringe at one time to minimize dosage errors. More uniform suspensions were obtained in 1% carboxymethylcellulose (CMC) than in saline, but in dye tests CMC caused a decrease in the dye localizing in tumors. The decrease was not significant ($P>0.10$) with the protocol of injections used (0.1 ml/day) but was consistent enough to warrant rejection of CMC as a suspending medium. Routes of injection of the drugs were i.m. with change to s.c. if necessary later in the test. All drugs were prepared immediately before use.

Anti Ehrlich carcinoma antisera (IRS) were prepared in New Zealand rabbits by the i.v. and s.c. inoculation of washed, viable, ascitic tumor cells. Serums with titres of 160 to 640 in mouse cytotoxicity tests(2) (employing $1 \times 10^6$ cells/inoculum) were used. The IRS was adsorbed 3 times at 37°C for 30 minutes with washed mouse blood cells (in proportions of 9:1, 9:1, and 1:1). Adsorption of the immune sera did not prevent death after i.v. or i.p. injection. However adsorbed sera could be injected i.m. or s.c. without lethal effects and with minimal tissue damage at the site of injection. Unadsorbed serum produced marked tissue damage at the site of an i.m. or s.c. injection and frequently was lethal.

Normal guinea pig serum (NGPS) was aseptically collected, prepared, pooled, and stored as for complement. Immediately before use, IRS and NGPS were combined in equal volumes and kept in an ice water bath until injected (IRS-NGPS).

Control mice received saline instead of the drugs and the sera.

*Results.* The localization of EB in solid Ehrlich tumors was increased in mice treated with $NaNO_2$, PETN, and Isordil. For purposes of comparison, results obtained with epinephrine hydrochloride, which decreased the amount of EB found in the tissues, are given in Table I.

In regression tests, 5 injections of $NaNO_2$ per day were required to demonstrate even slight inhibition of growth of tumors in mice receiving the antitumor agent, IRS-NGPS. Therefore, the longer-acting nitrates were substituted for $NaNO_2$. Administration of 3 mg PETN daily did not cause loss of weight in tumor-bearing mice, although their hair became ruffled or matted. With larger daily doses of this drug, loss of weight did occur and the average life span of the animals decreased. Although 3 mg of PETN daily was enough to slow the rate of growth of the tumors and to prolong the survival times of the mice when given with IRS-NGPS, the number of tumors which regressed was not different from the various controls. It was found that intermittently increasing the PETN during the first 3 weeks of therapy improved the number of regressions without severely impairing the health of the mice. For example, in a test in which treatment was initiated on the 8th day after the inoculation of the tumor cells, the mice received 2 3-mg PETN injections per day (0 hr, +6 hr) 9 times during the first 21

† Pentritol® Tempules® some of which were kindly supplied by Armour Pharmaceutical Co.

TABLE I. Effect of Several Vasoactive Drugs on Amount of Evans Blue Dye Recovered from Subcutaneous Ehrlich Mouse Tumors.

| Treatment | | | Evans blue in tumor | | | | |
|---|---|---|---|---|---|---|---|
| | | | Treated mice | | Control mice | | |
| Agent | No. of injections of agent | Dose ($\mu$g/0.1 ml)* | No. of mice | $\mu$g EB/mg tumor | No. of mice | $\mu$g EB/mg tumor | P† |
| $NaNO_2$ | 3 | 1.5 | 5 | .54 | 5 | .43 | >.10 |
| " | " | 15 | 7 | .51 | 9 | .45 | >.10 |
| " | " | 150 | 6 | .44 | 6 | .43 | >.10 |
| " | " | 1500 | 4 | .64 | 6 | .39 | <.05 |
| " | 4‡ | 1500 | 12 | .75 | 6 | .36 | <.001 |
| PETN | 3 | 3000 | 9 | .76 | 7 | .56 | <.002 |
| Isordil | " | 1000 | 7 | .49 | 6 | .41 | <.01 |
| Epinephrine | 14 | 0.5 | 10 | .60 | 12 | .75 | <.05 |
| " | " | 0.05 | 10 | .63 | 12 | .75 | <.10 |

* 0.1 ml/20 g body wt injected.
† Determined by Student's "t" test.
‡ 4 injections/2 days; 0.4 ml EB/20 g wt/day.

days of therapy and a single 3-mg PETN injection (0 hr) on the intervening days and on each day 22 through 44. At +2 hr, 0.1 ml of IRS-NGPS was injected daily. (This schedule is referred to as 6 mg/3 mg below.) A number of experiments have been done over a period of several years with some modification of the protocol described. All experiments support the findings of a more inclusive final experiment, the results of which are reported in this paper. Fig. 1 shows that the average tumor growth rate was lower in the mice treated with both PETN and IRS-NGPS than in the several control groups of mice. Curves are not continued past 36 days since deaths caused great irregularity after this time. All the mice which died had large tumors. Table II indicates that survival time was prolonged and that 60% of the tumors regressed in the test group. A second tumor in the IRS-NGPS and saline group also had regressed by the 44th day, but grew again. Tumors were absent in all survivors at 100 days. The weekly average weights of the several groups of mice are presented in Fig. 2. The decrease in weight in the test group in the 3rd and 4th weeks probably was due to differences in tumor sizes.

TABLE II. Effect of Treatment with IRS-NGPS and PETN on Regression of S.C. Ehrlich Carcinomas and on Survival of Mice After 50 Days of Tumor Growth.

| Treatment | No. of mice | No. of mice surviving on day 50 | No. of tumors regressed |
|---|---|---|---|
| PETN, IRS-NGPS | 10 | 7 | 6 |
| IRS-NGPS | 9* | 4 | 1 |
| PETN | 10 | 2 | 2 |
| NGPS | 10 | 4 | 3 |
| Saline | 10 | 3 | 2 |

* 10th mouse died with intramuscular and ascitic tumors on day 20.

Treatment with 3 mg PETN/day decreased the tumor-growth rate and prolonged the average survival time of the mice by about 10 days, but the % of regression was not different from the controls (0-20%). Fig. 3 permits comparison of the average sizes of the tumors at various times during treatment with 3 mg, 2.5 mg, and 2 mg PETN per day with the 6 mg/3 mg schedule results from the previous experiment. Regression and survival data are in Table III.

As far as duration of treatment with PETN and IRS-NGPS is concerned, about 5 weeks was the minimum required for regression. This period of time prevented reappearance of tumors which had regressed (were no longer palpable). Tumors which had grown to large size by this time were unaffected by further treatment. With shorter terms of treatment, slowly growing or regressing tumors and those which apparently had regressed completely overcame the inhibition after treatment was discontinued and the average growth rate soon approached that of the saline-treated controls, Fig. 4. Final percentage of regression was not different in test and control groups. These mice received 6 mg/3 mg PETN schedule for

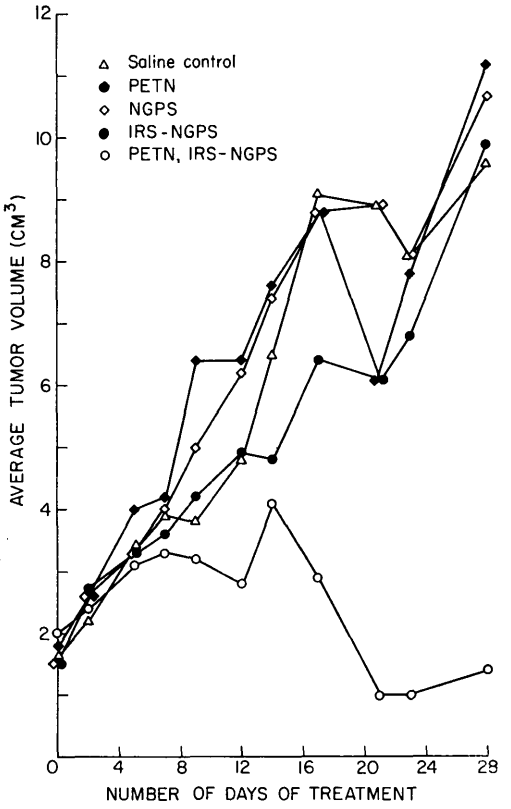


FIG. 1. Effect of PETN and IRS-NGPS on growth of Ehrlich carcinoma.

the full term of therapy; the antitumor agent was IRS-NGPS.

NGPS was injected with IRS since normal guinea pig serum is required for the destruction of Ehrlich cells by IRS *in vitro*(3). NGPS had no antitumor effect itself, as shown in Fig. 1, nor did it reduce the inhibitory action of IRS. There seemed to be a slight tendency toward enhancement of regression with the mixture IRS and NGPS when compared to IRS alone, but the increase was not significant. Further investigation is in progress.

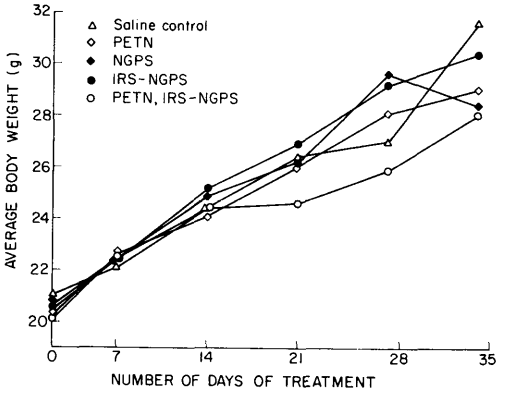


FIG. 2. Average weights of mice treated with IRS-NGPS and PETN (6 mg/3 mg schedule) and of control mice.

Results of regression tests with Isordil (2 mg daily, i.m.) and IRS-NGPS were similar to those with PETN, 3 mg daily, and the sera. Periodic increases in dosage have not been tested. Such a schedule may enhance the % of regressions as was observed with PETN.

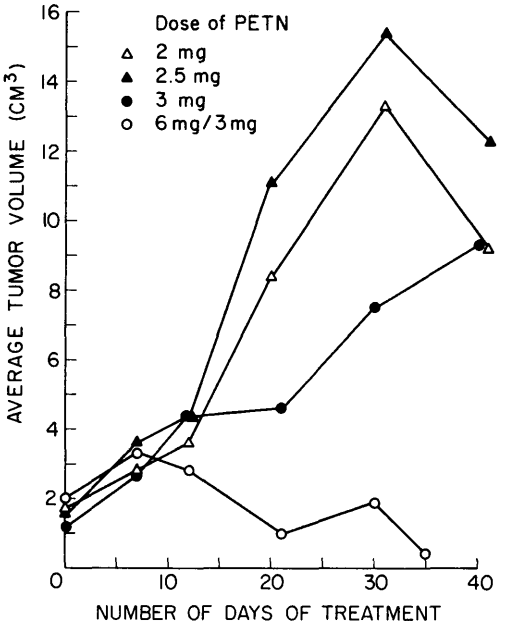


FIG. 3. Effect of IRS-NGPS on Ehrlich tumor in mice treated with 4 different amounts of PETN/day.

Within the range of tumor volumes used, the size of the tumor at the beginning of the test did not seem to be related to the final outcome (enlargement of the tumor and death or regression) in any of the groups, treated or control.

*Discussion.* The regression of established, subcutaneous Ehrlich carcinomas was obtained consistently in about 60% of the mice treated daily for 5 weeks or longer with mouse blood cell-adsorbed, rabbit, anti-Ehrlich tumor antiserum, normal guinea pig serum, and pentaerythritol tetranitrate. Since Evans blue dye localization increased in the tumors of mice treated with PETN and since none of the

TABLE III. Effect of Amount of PETN/Day on Regression of Tumor and Survival of Mice also Receiving IRS-NGPS.

| PETN (mg/20 g wt) | No. of mice | Age of tumors at start (days) | No. days at least 50% of mice survived | No. tumors regressed |
|---|---|---|---|---|
| 6/3 alternating | 10 | 8 | ≧100 | 6 |
| 3 | 10 | 7 | 59 | 0 |
| 2.5 | 10 | 11 | 45 | 2 |
| 2 | 10 | 11 | 48 | 0 |

agents alone caused regression of the tumors, regression apparently resulted from improved penetration of antibodies into the tumor tissue.

Studies of the anatomic and physiologic properties and reactions of tumor blood vascular supplies have been summarized by Urbach(4), Day(5), and Gullino(6). The possibility that Ehrlich tumor blood vessels might be resistant to drugs capable of eliciting responses, direct or indirect, in blood vessels supplying normal tissues was obvious. In search for a procedure which could alter permeability of tumor blood vessels to macromolecules, nontoxic doses of all but a few of the vasoactive drugs tested did not provoke a response detected by the localization of Evans blue dye. Possible causes of this may be (a) due to their characteristics, the tumor blood vessels were incapable of reacting; (b) the dye localization method was not capable of detecting the response; (c) the mode of action of sodium nitrite and the nitrates (on the arteriolar bed presumably) is the one which is required. Probably all 3 are important. Since the tumor tissue was not perfused, there was no indication whether the permeability of the vessel walls actually was increased and the dye passed into the interstitial fluid of the tumor tissue or the blood vessels simply were dilated to a greater extent and, therefore, contained more dye. Possibly both situations were present.

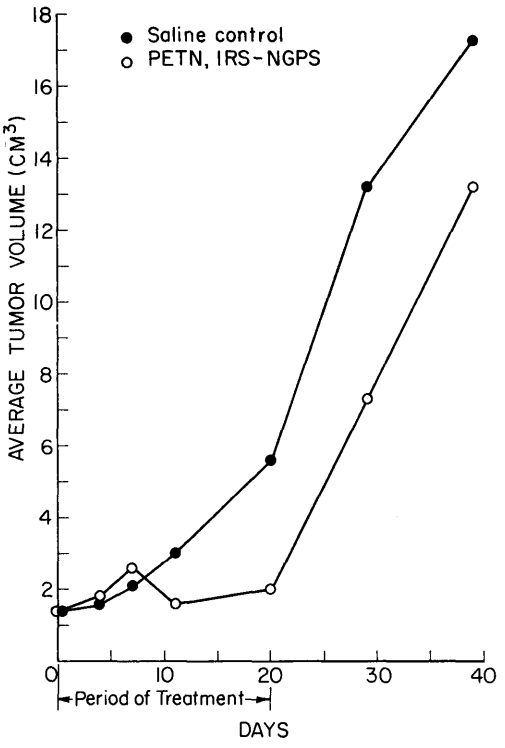


FIG. 4. Effect of withdrawal of treatment of Ehrlich tumor with PETN (6 mg/3 mg) and IRS-NGPS after 20 days.

The need for long-term treatment can be explained by the findings of Goldacre and Sylvén(7) that in necrotic areas of tumors small numbers of viable cells persist. Host defense mechanisms evidently were unable to eliminate such viable cells; early discontinuation of treatment permitted them to multiply again; and the tumor increased in size at a rate similar to that of control tumors.

Although the heterologous antiserum employed still contained some antinormal tissue antibodies after adsorption, these appeared to have no drastically destructive effect on the mice if injection was by the intramuscular or subcutaneous routes. Studies are planned in which mice bearing spontaneous tumors will be immunized with preparations from their own tumors and treated with PETN, since autologous immunization is the only system in which immunologic therapy of tumors is expected to be useful.

In work in progress, similar high regression rates, 60-80%, have been noted in rabbits

bearing the V2 transplantable tumor treated with the vasodilator PETN alone. Because of the longer survival time of the animals there was a greater opportunity for the development of cytotoxic antibodies. We are now developing evidence to verify or deny this assumption.

*Summary.* 1. When mice bearing established, solid Ehrlich carcinomas were treated with pentaerythritol tetranitrate, there was an increase in the localization of Evans blue dye in the tumors. Epinephrine caused a decrease in the dye recovered from the tumors. 2. When mice bearing solid Ehrlich tumors were treated with pentaerythritol tetranitrate, heterologous antitumor serum (adsorbed) and normal guinea pig serum, 60% of the tumors regressed if treatment was continued for 5 weeks or longer. The administration of guinea pig serum may have been unnecessary.

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1. Beach, V. L., Steinetz, B. G., J. Pharmacol. Exp. Therap., 1961, v131, 400.
2. Paradise, L. J., Nungester, W. J., Cancer Res., 1966, v26, 110.
3. Hiramoto, R. N., Nungester, W. J., ibid., 1958, v18, 27.
4. Urbach, F., Nat. Cancer Inst. Monograph No. 10, 1963, p539.
5. Gullino, P. M., Progr. Exp. Tumor Res., 1966, v8, 1.
6. Day, E. D., ibid., 1964, v4, 57.
7. Goldacre, R. J., Sylvén, B., Brit. J. Cancer, 1962, v16, 306.

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## Influence of Vitamin D and Starvation on Cartilage Glycogen and Serum Calcium and Phosphate. (32251)

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The presence of glycogen in the cartilage has been known for over a century(1); however, its exact role in calcification of cartilage is not yet understood. Glycogen breakdown in cartilage is suggested as (i) being involved in the synthesis of phosphoric esters which serve as substrates for alkaline phosphatase producing a local increase in phosphate concentration(2), (ii) being concerned with the formation or alteration of the cartilage matrix (3), (iii) providing energy for the eventual calcification of the cartilage(4). Histochemical methods have indicated that glycogen is present in large concentrations in the cells of the tibial epiphyseal cartilage which have most recently hypertrophied and that it suddenly decreased just prior to their calcification (5,6). Histochemical methods are useful in determining the distribution of glycogen in the various zones of the epiphyseal cartilage plate, but they are semi-quantitative. Quantitative studies have not been reported on the glycogen content of the rachitic rat cartilage at various stages of healing induced by vitamin D administration or by starvation; hence the present study was carried out. This report also deals with the utilization of glucose by rachitic cartilage following vitamin D treatment and attempts to correlate these changes with evidence of healing and alterations in serum calcium and inorganic phosphorus following vitamin D treatment or starvation.

*Methods and materials.* Weanling albino rats of Holtzman strain, weighing about 60 g, were fed a modified Schneider-Steenbock rachitogenic diet(7) having normal content of calcium (545 mg per 100 g diet) and a low phosphorus content (22 mg/100 g diet). At the end of 2 weeks of the regime the animals were distributed into 3 groups. Animals in Group 1 acted as rachitic controls. Animals in Group 2 were administered *per os* a single large dose of vitamin $D_2$ in oil (4000 I.U.) and were sacrificed seriatim at 24, 48, 72 and 96 hours following vitamin D administration. Animals in Group 3 were starved for