Abrogation of Tumor Rejection by Trypan Blue

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ABSTRACT

Trypan blue treatment prevented tumor-specific rejection in three animal model systems. These included the spontaneous rejection of tumors of mice (UVT-2051) and guinea pig (line 10 hepatoma) as well as vaccine-induced rejection of a guinea pig tumor (line 10 hepatoma). Secondary immune reactions to line 10 cell challenges were not abolished by trypan blue treatment. Although trypan blue is a potent inhibitor of macrophage cytotoxicity in vitro, the mechanism by which it inhibited tumor-specific rejection has not been established.

INTRODUCTION

The macrophage may be an important component of the tumor rejection response. The support for this inference is derived from the following observations. Macrophages activated in vitro kill tumor cells more readily than they kill normal cells (13). Macrophages from specifically immunized hosts may be cytotoxic for tumor cells in vitro (9). Treatment of animals with a variety of immune stimulants (12) may nonspecifically activate macrophages to kill tumor cells.

These observations suggest but do not prove that macrophage functional activities are requisite for tumor rejection. Direct evidence for the participation of macrophages in tumor rejection immunity would be provided by the failure of tumor rejection in animals in which macrophages were either depleted or functionally inhibited. Analogous approaches involving animals deprived of T-cells as a result of either congenital athymia (8) or a combination of thymectomy, lethal irradiation, and bone marrow reconstitution (7) have supported the role of thymus-dependent lymphocytes in the rejection of Moloney sarcoma. Attempts to selectively ablate macrophages in vivo have involved a variety of agents including silica (24), sodium aurothiomalate (20), antimacrophage serum (14), and trypan blue (12).

The mechanism of action of macrophage inhibitors has not been completely established. Silica (16) and carrageenan (19) directly kill macrophages, but it is not known whether aurothiomalate and trypan blue also kill these cells. Some agents concentrate in lysosomes and inhibit hydrolase activity (1, 10, 16, 22). Recent studies have suggested that transfer of lysosomes from macrophages to target cells was an essential component of macrophage cytotoxicity. Trypan blue-laden lysosomes were ineffective (10).

Information is not available on the specificity or dose-response relationships of the putative macrophage-suppressing agents. Particulate agents such as silica, carrageenan, and aurothiomalate are only transiently available to macrophages since phagocytosis, granuloma formation, and fibrosis subsequently deny macrophage accessibility. An additional problem with silica is availability. Most successful studies have used silica from a single source; commercial preparations are often unsatisfactory. Trypan blue is an especially attractive agent because of its solubility, ready availability, and low cost. Systemic treatment of the host with trypan blue permits the growth of tumor allografts (2). Trypan blue treatment inhibited the nonspecific cytotoxicity in vitro induced by BCG* (10), Corynebacterium parvum, or pyran (21).

The purpose of this report was to determine the effect of trypan blue on 3 animal tumor models in which strong, tumor-specific rejection immunity developed. These included 2 highly antigenic, syngeneic, transplantable tumors: an UV-induced cutaneous tumor (UVT-2051) of mice (17) and the diethylnitrosamine-induced line 1 hepatoma of guinea pigs (23). Both of these tumors grew temporarily in the s.c. space of normal, adult hosts but were eventually rejected. A third model, the line 10 guinea pig hepatoma, was nonantigenic, but animals that received a BCG-irradiated tumor cell vaccine developed strong immunity and rejected a simultaneous, contralateral challenge (3). In all 3 systems, we evaluated the effect of chronic trypan blue treatment on the resistance induced to primary tumor transplants.

MATERIALS AND METHODS

Trypan Blue Preparation. Trypan blue (Direct Blue 14) was purchased from Matheson, Coleman and Bell, Norwood, Ohio. The method originally described by Hibbs (11) was used to reduce the concentration of contaminating salts and other low-molecular-weight impurities. A stock solution of trypan blue (10 mg/ml) was prepared and dialyzed in fresh, glass-distilled water for 48 hr with water replacement twice each day. A red dye usually appeared in the dialysate. The dialysate was lyophilized to dryness, weighed, dissolved to 10 mg/ml in pyrogen-free phosphate-buffered saline (0.68% NaCl, 0.17% NaH2PO4, and 0.02% KH2PO4, pH 7.4), aliquoted, and sterilized in an autoclave.

Animals and Tumors. An UV-induced tumor (UVT-2051) was obtained from Dr. M. Kripke, Frederick Cancer Research Center, Frederick, Md. The tumor was propagated

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by serial trocar passage in thymectomized, irradiated (430 rads, 60Co) male C3H/HeJ mice obtained from the Drug Research and Development Branch, Division of Cancer Therapy, National Cancer Institute, Bethesda, Md. For experiments, trocar fragments (1 × 2 mm) were implanted s.c. into the back. For mice, trypan blue was administered i.p. on the dose schedule of Hibbs (11), with a loading dose of 4 mg/mouse on Day -1, 3 hr before tumor grafts, and 1 mg twice each week thereafter.

Line 1 and line 10 diethylnitrosamine-induced guinea pig hepatomas were provided by Dr. B. Zbar, National Cancer Institute. Both tumors were propagated in ascites form by serial passage in syngeneic, male Sewall-Wright strain 2 guinea pigs obtained from the Frederick Cancer Research Center. For experiments with line 1 hepatoma, 0.1 ml of washed, ascites-derived cell suspension containing 3 × 10⁶ dye-excluding cells was injected s.c. The third model was the line 10 guinea pig hepatoma which was nonantigenic by the usual immunization methods. Animals received a challenge injection of 1 × 10⁶ dye-excluding line 10 cells in 0.1 ml which was given i.d. on the right flank. A vaccine composed of 1.5 × 10⁶ irradiated (12,000 rads, 60Co) line 10 cells and 6 × 10⁶ viable units of BCG (TMC 1029; Trudeau Institute, Saranac Lake, N. Y.) in 0.1 ml was simultaneously administered on the left flank. Tumors were measured in 3 diameters, indurated DH reactions were measured in 2 dimensions with calipers (4), and the geometric mean diameter was calculated. Trypan blue was given s.c. at dose levels of 15 to 30 mg/kg body weight. This treatment was less than the maintenance dose given to the mice (50 mg/kg) since the mouse dose schedule was severely toxic for guinea pigs.

RESULTS

Growth of UVT-2051 Tumor in Immunodepressed Mice. A total of 4 independent experiments were performed, each including untreated, young adult mice as well as mice immunodepressed by different strategies including newborn (<24 hr old) hosts, adults receiving 300 rads, adults treated with thymectomy and 450 rads, or adults treated with trypan blue. UVT-2051 tumors grew progressively in less than 10% of untreated, adult mice (Table 1). Tumors reached an average size of approximately 1 cm about 2 weeks after transplantation and were completely gone 2 to 3 weeks later. In contrast, tumors grew in 67% of newborn mice, in 60% of 300-rad-treated mice, and in almost 89% of thymectomized-irradiated mice. Trypan blue permitted tumor growth in 76% of treated mice. Prior to rejection, the tumor growth rates were not different in immunodepressed versus untreated hosts.

Growth of Line 1 Guinea Pig Hepatoma in Untreated or Trypan Blue-treated Hosts. A total of 13 untreated guinea pigs were injected s.c. with 3 × 10⁶ ascites-derived line 1 cells. Seven of these, which received no other treatment, developed a maximum tumor diameter of about 1.5 cm 2 weeks after injection (Chart 1). All tumors were rejected within 30 to 40 days after transplantation. The remaining 6 guinea pigs received both the line 1 tumor cells and trypan blue (30 mg/kg on Day 0 and twice per week for the duration of the experiment). There was 1 early death, and tumors grew progressively in the rest. Prior to tumor rejec-

Table 1

<table>
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<th>Experiment</th>
<th>Untreated</th>
<th>Newborn</th>
<th>300 rads</th>
<th>Thymectomy + 450 rads</th>
<th>Trypan blue</th>
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<td></td>
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<td></td>
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<tr>
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<td>10/15 (67)</td>
<td>6/10 (60)</td>
<td>25/28 (89)</td>
<td>11/14 (76)</td>
</tr>
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a All treated groups differ significantly (p < 0.025; Fisher's exact probability test) from respective untreated control groups.

b Numbers in parentheses, percentage.
tion, the growth rates of tumors in untreated and trypan blue-treated hosts were not different.

Growth of Line 10 Guinea Pig Hepatoma in Untreated, Vaccine-treated, and Vaccine- and Trypan Blue-treated Hosts. Tumors in untreated guinea pigs (Group A) grew progressively, but tumors were completely suppressed in 9 of 10 vaccine-treated animals (Group B) (Chart 2). Trypan blue-treated guinea pigs did not benefit from the vaccine (Group C); 8 of 8 animals surviving more than 25 days developed progressively growing tumors. Trypan blue was given at a loading dose of 100 mg/kg on Day 0 and a maintenance dose of 15 mg/kg twice each week. Two animals died at the outset of the experiment prior to the development of tumor, possibly of trypan blue toxicity.

We also studied the response to trypan blue treatment of established immunity in 30 guinea pigs cured previously by vaccine treatment of line 10 tumors (Chart 3). The "control" group contained previously untreated animals. "Immune" animals had been cured of line 10 tumors 6 months previously and were unstimulated in the interim. "Boosted" animals were similarly cured of line 10 tumors but had received injections of $1 \times 10^8$ living line 10 cells 2 weeks prior to challenge. No tumors developed from these injections during 9 weeks of observation, confirming the immunity of these hosts. Trypan blue was given to "immune" and "boosted" animals on the same schedule and from the same preparative batch as in the previous experiment. The treatment was started 1 week before tumor cell challenge. The initial dose proved toxic since it killed 6 of 10 guinea pigs in each of the 2 treated groups. This toxicity was more severe than that observed in the first experiment; we have no explanation for this difference. Challenge sites received $1 \times 10^6$ line 10 cells i.d. Animals immune to line 10 tumors developed significant DH reactions compared to untreated controls (Chart 3, left), both at 24 ($p < 0.05$; Student's $t$ test) and 48 ($p < 0.025$) hr. Trypan blue treatment did not alter the size of the reactions (Chart 3, center). The previously boosted animals developed more intense reactions than did the unboosted animals ($p < 0.05$) (Chart 3, left). Trypan blue treatment of boosted animals prevented augmentation of the reactions but permitted DH responses (Chart 3, right) comparable to those seen in unboosted animals. Despite the reduction in hypersensitivity, trypan blue did not permit line 10 tumor growth to emerge from the reaction site.

**DISCUSSION**

This study demonstrates the effects of trypan blue administration on tumor-specific rejection in vivo. Tumor rejection immunity is a complex process in which many components of the lymphoreticular system interact, but the principal effector cells appear to be T-cells and macrophages. Prevention of tumor rejection by T-cell depletion supports the conclusion that they are essential to rejection (7, 8). Similar studies with macrophage depletion have been uncommon. Trypan blue has depressed nonspecific cytotoxicity of macrophages in vitro (10) and prevented the suppression of tumor growth induced by allograft rejection or by nonspecific immunostimulants such as BCG (11), pyran, or C. parvum (21). The experiments reported here were
designed to determine whether immunologically mediated tumor rejection could be prevented by trypan blue treatment.

Kripke first demonstrated that transplants of a highly antigenic, UV-induced cutaneous tumor of mice (UVT-2051) were rejected in normal syngeneic hosts but grew progressively in mice immunodepressed by thymectomy and irradiation (17) or by trypan blue (18). We directly compared the immunodepressive effects of immaturity, treatment with 300 rads, or treatment with thymectomy and 450 rads with the effects of trypan blue. Trypan blue proved to be almost as effective as thymectomy and irradiation and was superior to 300 rads or immaturity in providing environments that permitted growth of this highly antigenic tumor. Our results confirm Kripke's (18) observations.

Previous studies with the guinea pig line 1 hepatoma demonstrated temporary growth in i.d. sites (6), but it was eventually rejected. The spontaneously cured animals manifested specific, strong, cutaneous DH responses to challenge and were resistant to subsequent growth of tumor cells (6). In our studies, trypan blue completely prevented the spontaneous rejection of s.c. transplants of line 1 hepatoma.

In the line 10 hepatoma system, we found that trypan blue abrogated the curative effects of a vaccine containing BCG and tumor cells. Cutaneous DH reactions of line 10 tumor-cured guinea pigs were not abolished by trypan blue, but the enhanced reactivity of recently boosted animals did not develop. Trypan blue did not permit progressive growth of the viable tumor cells injected into immunized animals. This result was especially striking in view of the near lethal doses of trypan blue that were used.

The mechanism by which trypan blue permitted these transplantable tumors to escape immunological rejection was not established. Several possibilities exist. The dye could directly stimulate the growth of tumor cells, producing a rate of tumor cell production greatly exceeding the rate of destruction or growth inhibition. This explanation does not seem likely since the growth rates of line 1 or UVT-2051 tumors in trypan blue-treated hosts did not differ from that of tumors growing in untreated hosts prior to rejection. Further, trypan blue did not stimulate tumor cell growth in challenged animals previously cured of line 10 tumors. In other experiments in our laboratory, trypan blue did not accelerate i.m. line 1 tumor growth in immununized hosts. Line 1 tumors are not spontaneously rejected when grown in muscle, although tumors in this site immunize and can be rejected by an adequately immunized host (6).

Another possible mechanism for trypan blue effects is a generalized toxicity that interferes with tumor inhibition. We have found a high incidence of acute lethality associated with immunodepressive dosage of trypan blue (15). Dose-response relationships have not yet been systematically studied in any tumor model. Toxicity does not appear to be a sufficient explanation for these observations; the greatest toxicity was found in the experiment in which trypan blue abolished neither cutaneous hypersensitivity nor tumor immunity. In our studies, gross and microscopic examination of animals dying early did not reveal an obvious cause of death. Late mortality in chronically treated animals was often associated with infections, especially pneumonitis and enteritis, and could be attributable to immunodepression.

In all 3 systems studied, tumor rejection was inhibited in trypan blue-treated hosts. Immunodepression is the most likely explanation for the effects observed here. Failure to eliminate completely the secondary responses of animals previously cured of line 10 hepatoma does not exclude immunodepression as the trypan blue mechanism. For example, secondary immune responses are more radioresistant than primary responses (5). The complete nature of trypan blue interference with immunity is not known. Our experiments were not designed to identify the relative trypan blue sensitivity of the afferent and efferent arms of the immune response. It is not clear whether trypan blue affects the specific components of the tumor rejection or only suppresses the nonspecific, presumably macrophage-mediated component. The latter possibility is consistent with previous findings (11, 21).

The experiments reported here clearly demonstrate that in 3 tumor systems primary tumor rejection immunity was trypan blue sensitive. The proper interpretation of this observation awaits a comprehensive survey of the effects of trypan blue on major components of the immune response, particularly effects on functional activities of leukocytes other than macrophages.

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