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Effects of Trypan Blue Treatment on the Immune Responses of Mice

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It has been reported that trypan blue treatment decreases the nonspecific resistance of mice to transplanted tumors and inhibits the in vitro cytotoxic activity of activated macrophages. We wished to determine whether this effect of trypan blue could be due to a selective inhibition of certain macrophage functions or whether it reflected a broader form of immunosuppression. We therefore tested the effects of trypan blue on a variety of immunological responses. Treatment of mice with trypan blue delayed their rejection of skin allografts and transplants of a highly antigenic syngeneic ultraviolet light-induced tumor. Trypan blue treatment of either donor or recipient decreased the local graft-versus-host reaction. Filtration of lymph node cells from trypan blue-treated donors on a nylon wool column before use in the graft-versus-host assay abrogated the depressive effect of trypan blue. A transient reduction in the blastogenic response of spleen cells to concanavalin A and lipopolysaccharide mitogens was observed after a single injection of trypan blue, but the response of lymph node cells was unaffected. The depressed response of splenic lymphocytes was not entirely reversed by removal of adherent cells. The primary and secondary hemagglutinin responses to sheep erythrocytes were unaffected in trypan blue-treated mice, and the proportion and phagocytic activity of thioglycolate-induced peritoneal macrophages were also unaltered. We conclude that treatment of mice with trypan blue selectively inhibits certain macrophage functions but, at high doses, it can also inhibit some lymphocyte activities.

Mouse peritoneal macrophages, activated in vivo by chronic BCG or *Toxoplasma* infection, are cytotoxic in vitro to neoplastic target cells. This cytotoxic activity can be inhibited by exposure of the macrophages to nontoxic levels of trypan blue in vitro, before their interaction with the tumor target cells (13). In vivo, the treatment of mice with trypan blue abrogates BCG- and *Toxoplasma*-induced nonspecific resistance to tumor transplants and depresses the rejection response against tumor allografts (14). This azo dye is actively taken up by macrophages, but not by viable cells of the lymphocytic series (26).

Taken together, these observations suggest that the effect of trypan blue in vivo might be to selectively suppress certain macrophage functions without destroying these cells and without altering the activity of lymphocytes. To explore this possibility, we tested the effects of this material both in vivo and in vitro on a variety of immunological reactions. Here, we report the effects of trypan blue treatment on skin allograft rejection, syngeneic tumor immunity, production of hemagglutinins to sheep erythrocytes (SRBC), graft-versus-host (GvH) reactivity, macrophage induction and phagocytosis, and mitogen-induced lymphocyte blastogenesis.

MATERIALS AND METHODS

Mice. C3Hf/Sm, CBA/Sm, and A/Sm mice, 8 to 12 weeks of age, from the University of Utah Inbred Rodent Colony, were used to test skin graft rejection, tumor rejection, and hemagglutinin production. Specific-pathogen-free female C3H/HeN-(MTV$^-$) and [C57BL/6N × C3H/HeN(MTV$^-$)]F_1 mice, hereafter designated C3H$^-$ and B6C3F_1, respectively, from the Frederick Cancer Research Center Animal Production Facility were used in all other experiments.

Trypan blue. Trypan blue (Matheson Scientific Co.) was dialyzed for 48 h against glass-distilled water, lyophilized, and resuspended in sterile water at a concentration of 10 mg/ml. All injections of trypan blue were by the subcutaneous (s.c.) route.

Skin allograft rejection. Full-thickness, circular, abdominal skin grafts (15 mm in diameter) from strain A (H-2^a) female donors were transplanted to the thorax of CBA or C3Hf (H-2^k) recipients by the

method of Billingham and Medawar (3). Plaster casts were removed after 8 days, and the grafts were inspected daily for gross signs of rejection. Complete destruction of the epidermis was used as the rejection end point.

Syngeneic tumor rejection. The tumors were induced in C3Hf mice by chronic ultraviolet irradiation and maintained by serial passage in immunosuppressed (adult thymectomized, whole-body X-irradiated with 450 R) syngeneic recipients. These spindle cell tumors grow only to a limited extent, if at all, in immunocompetent syngeneic recipients (16). The recipients were tested for their ability to reject s.c. implants of 1-mm^3 tumor fragments. The mice were palpated once a week for tumors, and the tumor measurements were recorded.

Hemagglutinin titers. Mice were injected intravenously with 5×10^7 SRBC in 0.5 ml of saline on days 0 and 12. The mice were bled from the tail vein at intervals after each immunization. Sera were collected and stored at −20°C before testing. Serum samples from each animal were assayed individually for hemagglutinating antibodies in microtiter plates. All sera were tested on the same day.

Preparation of lymphoid cells. Axillary, brachial, cervical, inguinal, and mesenteric lymph nodes or spleens were pooled from at least three $C3H^-$ or $B6C3F_1$ donors. Single-cell suspensions were prepared aseptically by forcing the organs through a wire mesh sieve (E-C Apparatus, St. Petersburg, Fla.) in RPMI 1640 medium, filtering the suspensions through a nylon strainer, and centrifuging them at 400 × *g* for 10 min at 4°C. To remove erythrocytes from the spleen cell suspension, the cells were resuspended in 8 ml of Hanks balanced salt solution and placed into centrifuge tubes (17 by 100 mm; no. 2059, Falcon Plastics, Oxnard, Calif.). Five milliliters of lymphocyte separation medium (Litton Bionetics, Inc., Kensington, Md.) was carefully layered underneath the spleen cell suspension, and the tubes were centrifuged at 400 × *g* for 40 min at room temperature. After centrifugation, the lymphocytes at the interface were recovered with a Pasteur pipette, washed in excess RPMI 1640, and pelleted at 400 × *g*. Cell viability was determined by trypan blue exclusion.

In some experiments, the lymphoid cells were partially purified by incubating them on a nylon wool column, according to the method of Julius et al. (15). The eluted cells morphologically resembled small lymphocytes, and approximately 90% of them were positive for theta antigen, as determined by immunofluorescence.

GvH reaction. This was measured by the popliteal lymph node weight gain assay as described for mice by Twist and Barnes (32). A suspension of 0.05 ml of Hanks balanced salt solution with 5×10^6 viable lymph node cells (LNC) from at least 3 mice was injected into the left hind footpads of 6 to 10 $B6C3F_1$ recipients. Seven days later, the popliteal nodes were removed and weighed, and the ratios of the left (injected) versus right (uninjected) nodes were calculated. The effect of trypan blue treatment of the lymphoid cell donor or the $B6C3F_1$ recipient was tested. Control groups consisted of mice receiving identical treatments with trypan blue, but lymphoid cells from $B6C3F_1$ donors.

Macrophage induction and phagocytosis. Peritoneal exudate cells (PEC) were obtained as previously described (24). $C3H^-$ mice were injected intraperitoneally (i.p.) with 3 ml of thioglycolate (Baltimore Biological Laboratories, Baltimore, Md.), and 4 days later, their peritoneal cavities were washed with 10 ml of cold Hanks balanced salt solution containing 2 U of heparin per ml. The cells were centrifuged at 400 × *g* for 5 min and resuspended in complete RPMI 1640 medium (CRPMI) containing 5% heat-inactivated fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine (Grand Island Biological Co., Grand Island, N. Y.). Cell viability was determined by trypan blue exclusion. Approximately 5×10^5 PEC were plated onto glass cover slips, incubated at 37°C for 60 min in a humidifed atmosphere containing 5% CO_2, and refed with CRPMI. After 24 h, phagocytosis was measured by incubating adherent peritoneal cells for 60 min with either India ink (21) or opsonized SRBC. Opsonization was accomplished by incubating the SRBC with a 1:100 dilution of antiserum from mice injected i.p. 5 days earlier with 0.25 ml of 10% washed SRBC. After incubation, the cover slips were washed vigorously in beakers of saline, fixed in methanol, and stained with Wright stain.

Lymphocyte blastogenesis in vitro. Spleen cells and LNC were resuspended in CRPMI with 10 μM mercaptoethanol. A total of 10^5 viable cells in 0.1 ml were plated in quadruplicate into flat-bottomed Microtest II wells (Falcon Plastics, Oxnard, Calif.). Portions of 0.05 ml of various mitogen concentrations in CRPMI were added to the wells. Controls were cultures without mitogen. The mitogens used were concanavalin A (ConA; Calbiochem, La Jolla, Calif.) and lipopolysaccharide W from *E. coli* O55:B5 (LPS; Difco, Detroit, Mich.). The cultures were incubated for 72 h at 37°C in a humidifed atmosphere containing 5% CO_2; 1 μCi of [^{3}H]thymidine (specific activity, 2 Ci/mmol; New England Nuclear, Boston, Mass.) per well was present during the last 16 to 18 h. The cultures were processed using a multiple automatic sample harvester (MASH II, Microbiological Associates, Bethesda, Md.), and the radioactivity was measured in a liquid scintillation spectrometer.

RESULTS

Effect of trypan blue treatment on skin graft and tumor rejection. Several doses and regimens of trypan blue were tested for their ability to delay the rejection of skin allografts across a major histocompatibility barrier. These results are summarized in Table 1. A single dose of 1 mg of trypan blue caused a modest delay in graft rejection, whereas 4 mg nearly doubled the median survival time. Treatment of the graft donor with 4 mg of trypan blue 24 h before grafting did not influence graft survival, even though the grafts had ab-

sorbed enough dye to become dark blue (group 4). Chronic treatment with a low dose of trypan blue also delayed skin graft rejection from a median time of 11.5 days to 15 days.

The effect of trypan blue treatment on the rejection of syngeneic ultraviolet radiation (UV)-induced tumors is shown in Table 2. Groups of C3Hf mice were injected s.c. with various doses of trypan blue or with saline, beginning at the time of tumor implantation. Syngeneic immunosuppressed mice served as positive controls for tumor growth (groups 4 and 7). In the first experiment with tumor no. 2009, 14 of the 15 tumor implants completely regressed in the controls injected with saline (group 1). A single dose of 4 mg of trypan blue significantly delayed tumor rejection (group 3), whereas 0.4 mg had no detectable effect (group 2). In the second experiment, 4 mg of trypan blue on day 0, followed by a maintenance dose of 0.4 mg twice per week, markedly retarded the rejection of tumor no. 2051 (group 6), compared with the saline-treated controls (group 5). However, even with continuous trypan blue treatment, tumor rejection was not completely abolished, and only a few animals died with a progressively growing tumor. Delaying the trypan blue treatment until day 7 after tumor challenge had no effect on tumor rejection (not shown).

The effect of trypan blue treatment on the rejection of tumor no. 2051 in preimmunized hosts is shown in Table 3. Mice were immunized with s.c. implants of either normal skin (control) or tumor tissue and challenged with tumor fragments 4 weeks later. For purposes of comparison, mice given 450 R of whole-body X irradiation were included in this experiment. In sham-immunized mice, treatment with trypan blue (group 2) or X rays (group 3) retarded tumor rejection, compared with controls (group 1). However, neither treatment affected tumor rejection in preimmunized mice (groups 4 and 5), indicating that the second-set rejection response was not detectably altered. However, the combined use of trypan blue and X irradiation (group 6) in preimmunized mice caused a substantial delay in tumor rejection, and four of

TABLE 1. *Effect of trypan blue treatment on skin allograft rejection*

Donor → recipient	Group	Treatment	No. of mice	Rejection time (days), median (range)	P[a]
A → CBA	1	None	6	9 (9–11)	
A → CBA	2	1 mg of trypan blue to recipient, day 0	5	11 (9–22)	<0.05
A → CBA	3	4 mg of trypan blue to recipient, day 0	6	17.5 (13–23)	0.001
A → CBA	4	4 mg of trypan blue to graft donor, −24 h	6	10 (9–12)	>0.05
A → C3Hf	5	Saline three times per week for 4 weeks	8	11.5 (10–15)	
A → C3Hf	6	0.1 mg of trypan blue three times per week for 4 weeks	7	15 (13–19)	0.005

[a] P, Probability of no difference between control and treated groups, determined by a one-tailed Mann-Whitney U test.

TABLE 2. *Effect of trypan blue treatment on the rejection of syngeneic UV-induced tumors*

Tumor no.	Group	Treatment	Tumor incidence[a] in week:				
			1	2	3	4	5
2009	1	Saline, day 0	15/15	15/15	2/15	1/15	1/15
	2	0.4 mg of trypan blue, day 0	9/10	9/10	5/10	2/10	2/10
	3	4.0 mg of trypan blue, day 0	10/10	10/10	9/10[b]	6/10[c]	5/10[d]
	4	Thymectomy + 450 R	18/20	18/20	18/20	17/20	17/20
2051	5	Saline, two times per week	10/10	1/10	1/10	0/10	0/10
	6	4.0 mg of trypan blue, day 0; 0.4 mg twice a week thereafter	10/10	10/10[b]	10/10[b]	7/10[c]	3/10
	7	Thymectomy + 450 R	5/5	5/5	5/5	5/5	5/5

[a] Number of tumor-bearing mice per number challenged.
[b] $P < 0.001$ versus saline controls.
[c] $P < 0.01$ versus saline controls.
[d] $P < 0.02$ versus saline controls, chi-square test.

the mice eventually died from progressive tumor growth.

Effect of trypan blue treatment on antibody production. In this experiment, three groups of C3Hf mice were immunized intravenously with 5×10^7 SRBC on days 0 and 12. Twenty-four hours before this first immunization, one group was given 4 mg of trypan blue s.c. followed by 0.4 mg twice per week, beginning in the second week. Another group was given the same regimen of trypan blue beginning 24 h before the second SRBC immunization. A third group received 0.5 ml of saline s.c. twice per week. As illustrated in Table 4, trypan blue treatment had no effect on either the primary or the secondary hemagglutinin response against SRBC.

To test its effect directly on the antigen-antibody reaction, trypan blue was added in vitro to the sera dilutions. The highest concentration tested, 0.16 mg/ml, did not alter the antibody titers, indicating that this material does not interfere with antibody binding or agglutination.

Effect of trypan blue treatment on the local GvH reaction. Donor mice were treated with trypan blue at several intervals before their lymph node cells were used to induce a local GvH reaction; recipient mice were treated either before, or before and after, the initiation of a GvH reaction. The control groups received F_1 cells, rather than parental ($C3H^-$) cells.

The combined results of two independent experiments are shown in Table 5. Trypan blue treatment of donors or recipients in the control groups ($B6C3F_1 \rightarrow B6C3F_1$) had little or no effect on the popliteal lymph node weight ratios. A threefold increase in the weight of the

TABLE 3. *Effect of trypan blue treatment on the second-set rejection of a syngeneic UV-induced tumor*

Group	Immunizing tissue	Treatment	Tumor incidence[a] in week:			
			1	2	3	4
1	Skin	None	10/10	0/10	0/10	0/10
2	Skin	4 mg of trypan blue, day −1; 1 mg twice per week s.c. thereafter	10/10	6/10[b]	5/10[b]	4/10[c]
3	Skin	450 R, day −1	3/10	8/10[d]	7/10[b]	7/10[b]
4	Tumor no. 2051	4 mg of trypan blue, day −1; 1 mg twice per week s.c. thereafter	0/9	0/9	0/8	0/8
5	Tumor no. 2051	450 R, day −1	0/9	0/9	0/9	0/9
6	Tumor no. 2051	4 mg of trypan blue + 450 R, day −1; 1 mg of trypan blue twice per week s.c. thereafter	5/9	6/9[b]	6/9[b]	6/9[b]

[a] Number of tumor-bearing mice per number challenged with tumor no. 2051.
[b] $P < 0.01$ versus group 1.
[c] $P < 0.05$ versus group 1, chi-square test.
[d] $P < 0.001$ versus group 1.

TABLE 4. *Effect of trypan blue treatment on the hemagglutinin response to SRBC*

Treatment[b]	No. of mice	Hemagglutinin titers[a]					
		Primary response on day:			Secondary response on day:		
		5	7	10	14	17	21
Saline	10	10 (9–11)	9 (8–10)	9 (7–10)	9 (8–11)	12 (10–13)	11 (10–13)
4 mg of trypan blue, day −1; 0.4 mg twice per week thereafter	10	10 (9–12)	9 (8–10)	10 (9–11)	10 (9–10)	12 (9–13)	11 (9–13)
4 mg of trypan blue, day +11; 0.4 mg twice per week thereafter	10	10 (8–11)	9 (7–9)	9 (8–9)	9 (8–11)	12 (10–13)	12 (10–13)

[a] Log_2 of reciprocal of highest serum dilution showing complete agglutination. Median (range) of serum titers from individual mice.
[b] All groups were immunized with 5×10^7 SRBC intravenously on days 0 and 12.

nodes was produced by injection of lymphoid cells from untreated donors, indicating a strong local GvH reaction. This reaction was diminished by pretreatment of the donors with trypan blue before collection of their lymphoid cells. A dramatic reduction of the reaction was obtained when the recipients were given trypan blue 1 day before, and 5 days after, cell transfer. This reduction was most likely due to the injection of trypan blue just before the peak of the local reaction (day +5), since a single injection on day −1 had no effect on the response.

To determine whether the inhibitory effect of trypan blue treatment was mediated via adherent cells or nonadherent lymphocytes, the LNC from control and trypan blue-treated mice were partially purified by nylon wool filtration before injection. Filtration of the LNC from untreated $C3H^-$ or $B6C3F_1$ mice did not affect their ability to induce a local GvH reaction (Table 6). Again, some depression in GvH reactivity was noted with LNC from trypan blue-treated donors. However, nylon wool filtration of the cells before their injection into $B6C3F_1$ recipients fully restored their ability to induce a GvH response.

Effect of trypan blue treatment on the induction of PEC. Groups of five $C3H^-$ mice were given 3 ml of thioglycolate i.p., and their PEC were collected 5 days later. The mice also received an s.c. injection of 4 mg of trypan blue on days −1, −4, or −7 before harvesting of PEC. The number of PEC and the differential cell count were determined for each individual animal. The number of PEC was substantially higher than that of control mice in the group given trypan blue 7 days before PEC collection; the other treatment groups were unaffected (Table 7). The proportions of the various cell types were not altered (Table 7), nor were the number and activity of phagocytic cells affected by the trypan blue injections (not shown).

Effect of trypan blue on lymphocyte blastogenesis. The last experiments tested the effect of trypan blue on the response of spleen and lymph node cells to mitogen-induced blastogenesis. The results, reported in Table 8, show that the injection of 4 mg of trypan blue either 1 or 4 days before testing led to pronounced suppression of the spleen cell response to both ConA and LPS. In contrast, mitogen-induced blastogenesis of LNC was not suppressed by in vivo treatment of the donors with trypan blue.

To determine which subpopulation of spleen cells was being affected by the trypan blue treatment, the spleen cells were fractionated by passage over a nylon wool column before in vitro stimulation with ConA. Table 9 illustrates one of several such experiments using doses of ConA ranging from 0.25 to 4.00 μg of ConA per ml. Again, the response of unfractionated spleen cells from trypan blue-treated mice was lower than that of cells from saline-treated control mice at all doses of ConA. The response of spleen cells from all treatment groups to doses of ConA greater than 0.5 μg/ml increased after nylon wool filtration. The depressed response of cells from mice given try-

TABLE 6. *Effect of nylon wool filtration of LNC before use in the local GvH assay*

LNC donor	Trypan blue treatment[a] on day	Popliteal node wt ratio (injected/uninjected)	
		Unfiltered	Nylon wool filtered
$C3H^-$	None	4.40 ± 0.42[b]	4.20 ± 0.47[b]
	−1	3.23 ± 0.19[c]	5.21 ± 0.45
	−4	3.01 ± 0.31[c]	4.00 ± 0.49
	−7	3.83 ± 0.37	4.55 ± 0.46
$B6C3F_1$	None	1.27 ± 0.04	1.49 ± 0.12

[a] Trypan blue, 4 mg s.c.
[b] Mean ± standard error of four to five recipients.
[c] $P < 0.05$ versus untreated control.

TABLE 5. *Effect of trypan blue treatment of donor or recipient on the local GvH reaction*

Treatment	Popliteal node wt ratio (injected/uninjected)	
	$C3H^- \rightarrow B6C3F_1$	$B6C3F_1 \rightarrow B6C3F_1$
None	4.22 ± 0.26[a]	1.36 ± 0.06[a]
Donor: 4 mg of trypan blue s.c. on day −1	3.43 ± 0.21[b]	1.23 ± 0.03
Donor: 4 mg of trypan blue s.c. on day −4	3.60 ± 0.28	1.66 ± 0.10[b]
Donor: 4 mg of trypan blue s.c. on day −7	3.05 ± 0.11[c]	1.22 ± 0.02
Recipient: 4 mg of trypan blue s.c. on day −1	3.96 ± 0.19	1.19 ± 0.12
Recipient: 4 mg of trypan blue s.c. on day −1 and 1 mg on day +5	2.31 ± 0.40[c]	1.33 ± 0.14

[a] Mean ± standard error of 6 to 10 recipients; all statistically significant differences are indicated by superscript letters.
[b] $P < 0.05$ versus untreated control (two-tailed Student's t test).
[c] $P < 0.005$ versus untreated control (two-tailed Student's t test).

TABLE 7. *Effect of trypan blue treatment on induction of PEC*

Trypan blue treatment[a] on day	No. of PEC/mouse[b]	Macrophages (%)	Lymphocytes (%)	Polymorphonuclear leukocytes (%)
None	$3.92 \pm 0.40 \times 10^7$	94.8 ± 1.3	3.0 ± 0.7	2.2 ± 0.7
−1	$3.51 \pm 0.19 \times 10^7$	95.4 ± 1.1	2.4 ± 0.2	2.2 ± 1.0
−4	$4.03 \pm 0.28 \times 10^7$	92.0 ± 2.3	3.6 ± 0.9	3.7 ± 1.7
−7	$7.18 \pm 0.32 \times 10^{7c}$	95.6 ± 1.1	3.2 ± 0.9	1.2 ± 0.4

[a] Trypan blue, 4 mg s.c.
[b] Mean ± standard error from five mice.
[c] $P < 0.001$ versus untreated control (two-tailed Student's t test).

TABLE 8. *Effect of in vivo trypan blue treatment on lymphocyte blastogenesis in vitro*

Source of lymphoid cells	Treatment	Avg cpm[a]		
		Control	+ ConA[b]	+ LPS[c]
Spleen	Untreated	5,400	41,040	25,330
	4 mg of trypan blue on day			
	−1	4,150	19,880[d]	18,140[d]
	−4	4,080	14,400[d]	15,590[d]
	−7	7,300	40,980	23,540
Lymph nodes	Untreated	300	27,060	1,120
	4 mg of trypan blue on day			
	−1	410	32,960	1,300
	−4	690	31,120	3,100
	−7	420	28,580	1,120

[a] Average counts per minute of quadruplicate cultures. Standard deviations did not exceed 15% of the mean.
[b] ConA: 1.0 μg per ml.
[c] LPS: 10 μg per ml.
[d] $P < 0.001$ versus untreated group.

TABLE 9. *Effect of nylon wool filtration of spleen cells from trypan blue-treated mice on ConA-induced blastogenesis*

Spleen cells	ConA (μg/ml)	Control	Trypan blue treatment[a] of spleen cell donors on day:		
			−1	−4	−7
Unfiltered	4.00	7,080 ± 900	3,870 ± 280	4,010 ± 310	11,380 ± 1,280
	2.00	49,980 ± 2,720	26,280 ± 3,440	5,750 ± 360	24,160 ± 2,450
	1.00	37,530 ± 5,480	15,220 ± 2,410	4,100 ± 700	14,340 ± 1,770
	0.50	16,450 ± 2,540	2,340 ± 870	1,240 ± 40	7,060 ± 700
	0.25	2,760 ± 160	640 ± 130	960 ± 210	2,700 ± 300
	0	210 ± 160	400 ± 80	190 ± 90	1,960 ± 980
Nylon wool filtered	4.00	13,440 ± 1,290	13,500 ± 1,590	13,440 ± 770	20,260 ± 1,870
	2.00	100,000 ± 13,140	86,300 ± 4,480	52,090 ± 5,230	95,460 ± 7,880
	1.00	52,020 ± 4,230	33,260 ± 3,210	15,660 ± 2,600	41,100 ± 6,460
	0.50	6,170 ± 430	2,640 ± 380	1,940 ± 20	5,510 ± 1,100
	0.25	490 ± 120	230 ± 20	550 ± 140	840 ± 130
	0	120 ± 50	170 ± 10	150 ± 20	160 ± 30

[a] Trypan blue, 4 mg s.c.
[b] Counts per minute ± standard deviation of quadruplicate cultures labeled with [^{3}H]thymidine.

pan blue 1 or 4 days earlier was less pronounced after nylon wool filtration, and the reactivity of filtered cells to ConA returned to a normal level by 7 days after trypan blue injection.

Finally, 10-fold dilutions of trypan blue were added to spleen or LNC cultures in vitro to determine the direct effect of this substance on the blastogenic response to ConA. The trypan blue was present during the entire 72-h culture period. At trypan blue concentrations of up to 10 μg/ml, the ConA response was unaffected. At concentrations of 100 μg/ml and greater, the

response was decreased to less than 10% of that of control cultures containing no trypan blue (data not shown).

DISCUSSION

Based on recent studies showing that trypan blue inhibits the cytotoxic effector function of activated macrophages in vitro (13) and in vivo (14), it seemed possible that the action of trypan blue might be specific for macrophages. In this report we examined the effect of trypan blue on several immune responses in mice to ascertain whether its effect in vivo was due solely to the alteration of macrophages or included direct or indirect effects on the functions of lymphocytic cells. The results demonstrate that systemic trypan blue treatment interferes with skin allograft and syngeneic tumor rejection, the local GvH reaction, and the blastogenic response of spleen cells in vitro. It does not alter antibody production, the number and phagocytic capacity of induced peritoneal macrophages, or the blastogenic response of lymph node cells. Furthermore, histopathological examination of the lymphoid organs by light microscopy revealed no alterations in trypan blue-treated mice, except for dye-laden macrophages in the draining lymph nodes 1 to 3 days after s.c. injection of trypan blue.

The modest, yet significant, delay in skin allograft rejection confirms the results of Brent and Medawar (5) and could be due to the effects of trypan blue on macrophages. It has been shown that peritoneal macrophages from mice grafted with allogeneic skin or injected with allogeneic tumor cells are cytotoxic in vitro against target cells from the donor strain and accelerate the rejection of skin allografts when passively transferred to normal syngeneic recipients (8, 28, 35). In addition, there are several reports that describe an infiltrate of cells in skin allografts that morphologically and histochemically resemble macrophages (10, 11, 28, 33). These findings are consistent with earlier studies in mice demonstrating that trypan blue interferes with resistance to tumor allografts (21, 30).

Trypan blue treatment delays the primary rejection of highly antigenic syngeneic UV-induced tumors. Based on the finding that macrophages play an important role in tumor immunity (for review, see reference 19), this observation could also stem from the effect of trypan blue on macrophages. The effect of trypan blue on tumor rejection in this syngeneic system was not as dramatic as that reported for the destruction of L1210 lymphoma cells in allogeneic CBA mice (14). This discrepancy may be due to several factors: (i) The rapid growth rate of the L1210 cells (mice die 14 to 21 days after tumor grafting) could make a modest suppression of host resistance by trypan blue appear highly effective, whereas the more slowly growing UV-induced tumors might be controlled by host lymphocytes, even though the cytotoxic activity of macrophages is inhibited by trypan blue. Alternatively, the paralysis of macrophage cytotoxic function induced by trypan blue in vivo may be only partial, and the residual cytotoxic capacity of macrophages in trypan blue-treated mice may be sufficient to eventually overcome the more slowly growing UV-induced tumors. (ii) Macrophages may play a more important role in the rejection of tumor allografts than in the rejection of syngeneic tumors. (iii) L1210 cells may be more susceptible than a solid tumor implant to killing by macrophages.

Trypan blue treatment had no detectable effect on the rejection of a syngeneic UV-induced tumor in preimmunized mice. This may be due either to quantitative or qualitative differences among the cells involved in the first- versus the second-set rejection reactions, or to both, since these are not mutually exclusive possibilities. Further experiments are required to settle this issue. However, in earlier studies, trypan blue treatment suppressed the second-set rejection of tumor allografts, rejection of the L1210 lymphoma in allogeneic CBA mice (14), and rejection of the allogeneic Brown-Pearce carcinoma in rabbits (30).

The abrogation of second-set tumor rejection by combined treatment with trypan blue and X irradiation, but not by either treatment alone, is subject to a number of interpretations. The most intriguing possibility is that these two treatments affect different cell populations (presumably, macrophages and lymphocytes) and act synergistically to suppress tissue rejection. However, this possibility is based on the assumptions that trypan blue does not inactivate memory cells (lymphocytes), and that there are subpopulations of macrophages and thymus-derived lymphocytes that are radioresistant (6, 7, 16).

The reduction in the local GvH reaction after trypan blue treatment indicates that both the donor and recipient components of the GvH reaction were affected. In light of the recent evidence that macrophages of the recipient are recruited to lymphoid organs during a GvH reaction (4), it is most likely that the injection of trypan blue into the recipients just before the peak of the response affects the participation of host macrophages, thereby decreasing the enlargement of the popliteal node. However, the

depressive effect of trypan blue treatment of the LNC donors on their ability to mediate a GvH reaction could have been due to an effect on either lymphocytes or macrophages or both. The experiment using nylon wool filtration to remove adherent cells suggests that the T cells that mediate the GvH reaction are not altered by trypan blue treatment. The depressive effect of trypan blue treatment on the reactivity of donor LNC could be due to inactivation of macrophages that would otherwise contribute to the reaction (1), to the induction of a suppressive adherent cell, or to an increase in the percentage of macrophages in the lymph nodes, which simply dilute the effector T cells.

The lymphocyte blastogenesis studies indicate that systemic trypan blue treatment has no effect on the responsiveness of LNC to either LPS or ConA. This suggests that deoxyribonucleic acid synthesis, and presumably cell division, is not impaired in either T or B lymphocytes from trypan blue-treated mice. However, the responses of splenic lymphocytes to ConA and LPS were depressed, at least transiently, after trypan blue injection. This depression was reduced slightly by nylon wool filtration of the cells before mitogen stimulation, but the response to ConA was still markedly reduced in mice treated with trypan blue 4 days earlier. The discrepancy between the effects of trypan blue on splenic and lymph node lymphocytes could be due to differences in the local concentration of the dye in these organs. This possibility is supported by the experiment in which trypan blue was added directly to lymphocyte cultures in vitro. There was a dose-dependent inhibition of the blastogenic response to ConA that was not associated with lymphocyte toxicity. Whether or not macrophages participate in the response of murine lymphocytes to mitogens is not clear. The reports of others on this question are contradictory (9, 12, 18, 20, 34), and our study does not favor either point of view. From the experiment with LNC, we can conclude only that either macrophages do not participate in the response, or that trypan blue does not affect their contribution to the reaction.

Finally, trypan blue had no effect on the induction of PEC, on the phagocytic capacity of PEC, or on the primary or secondary antibody responses of mice to SRBC. In the mouse, the response to SRBC has been characterized as a T cell-dependent immune response resulting in the production of antibody (23, 31). Furthermore, the response in vitro seems to require the cooperation between adherent (presumably macrophage) and nonadherent cell populations (24). It is believed that the role of macrophages is to process antigen for subsequent presentation to other lymphoid cells (2, 26). In our study, it appears that trypan blue, in the dose range tested, does not block antigen processing by macrophages, nor does it interfere with the activity of certain immunocompetent lymphocytes, including helper T cells, plasma cells, and their precursors.

We conclude that the primary site of action of trypan blue is on certain macrophage functions, manifested in vivo by the rejection of skin allografts and syngeneic tumor implants. These experiments are consistent with the results of in vitro studies demonstrating that trypan blue inhibits the cytotoxic effector function of activated macrophages (13). However, we cannot rule out the possibility that trypan blue is inhibiting macrophage participation in the afferent arm of these responses in vivo, even though antigen processing for antibody formation to SRBC was unimpaired. The function of host macrophages in the local GvH reaction, although poorly understood, also appears to be impaired by trypan blue treatment. The finding that antibody synthesis, GvH reactivity of nonadherent lymphocytes, and mitogen responsiveness of lymph node cells are unimpaired by doses of trypan blue that affect certain macrophage functions suggests that this dye acts selectively on macrophages. However, it is not specific for macrophages, since at high concentrations lymphocyte blastogenesis also may be reduced. At doses of trypan blue that interfere with the cytotoxic activity of macrophages, the dye is not toxic to PEC nor does it alter the ability of macrophages to phagocytize and to process antigen. This selective action of trypan blue on certain macrophage functions makes it a useful addition to the cytotoxic substances, such as silica and anti-macrophage serum, currently used to abolish macrophage activity.

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