THE PROTECTIVE ACTION OF TRYPAN RED AGAINST INFECTION BY A NEUROTROPIC VIRUS*

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Study of the factors that may increase or decrease the susceptibility of animals to poliomyelitis virus has recently engaged the attention of a number of investigators. Foster *et al.* (1, 2) have shown that animals on a limited food intake or on a thiamin-deficient diet exhibit moderately increased resistance to intracerebral inoculation of the Armstrong virus. Lichstein *et al.* (3), on the other hand, found that restriction of the intake of pantothenic acid had no effect on the resistance of mice to this virus, but did increase resistance to infection by Theiler's virus. The injection of human convalescent serum intraabdominally has been found to provide some protection to mice against the Armstrong virus (4). Antagonisms between viruses by the so called "interference" reaction, have also been investigated (5, 6).

An interesting case of increased susceptibility to poliomyelitis has been reported by Sandler (7). Rabbits, following insulin hypoglycemia, were found susceptible to poliomyelitis whereas they ordinarily are resistant to this disease.

In studying the effect of certain dyes injected intraperitoneally, we have found that mice and cotton rats, which have been treated with trypan red and certain related dyes become more resistant to infection by a poliomyelitis-like virus. The protective action of the dye is evident when the virus is given intraperitoneally, but is not apparent or only slightly so when this virus or the Armstrong virus is given intracerebrally.

Methods

Animals.—The mice used in these experiments were of the ABC strain and were from the colony of Dr. John J. Bittner, University of Minnesota. They weighed between 11 and 16 gm. and were fed purina fox chow.

The cotton rats (*Sigmodon hispidus hispidus*) were obtained from the Michigan Department of Health, Bureau of Laboratories, Lansing, Michigan, and weighed 175 to 200 gm.

Treatment of Animals with Dye.—Unless otherwise specified, the mice were injected intraperitoneally with 0.1 ml. of 1 per cent solution of the dye in distilled water on 3 successive days. The cotton rats were treated in the same way except that the dye was injected in 0.5 ml. doses. The control animals received no treatment prior to inoculation, with one exception (Table II), but received an identical inoculation of virus. In each experiment the animals were pooled and then selected at random for the control group and dye treatment respectively.

The intraperitoneal injection of the mice was done without anesthesia, but the cotton rats were given a light ether anesthesia.

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Viruses and Inoculation.—Two strains of viruses were used in mice, the MM mouse virus (8) obtained from Dr. C. W. Jungeblut, Columbia University, and the Lansing or Armstrong poliomyelitis virus (9) obtained from Dr. Carl Ecklung, University of Minnesota.

The virus preparations were made from mouse cord and were ground in 9 volumes of physiological saline and centrifuged. The preparation was preserved on dry ice and the same batch was used throughout the investigation. 0.1 ml. was used for intraperitoneal injection of the mice (without anesthesia) and 0.5 ml. for the cotton rats (with ether anesthesia). For the intracerebral inoculation of mice, 0.03 ml. of the virus suspension was used and the mice were given ether anesthesia. Inoculations were done the day following the last dye injection unless noted in the tables.

Criterion of Infection.—The animals were inspected twice daily. Animals which died within 24 hours following the injection of virus were not considered to have died of virus infection and therefore were not included in the data. All animals which subsequently became sick, paralyzed, or were found dead were considered to have succumbed to the virus and were counted as infected animals. Observation was usually continued for 3 weeks; the majority of the animals became infected within 14 days. With the MM virus a considerable number of animals died with encephalitic symptoms without showing a paralysis of the limbs. Most of the mice receiving the Lansing virus presented clear evidences of paralysis.

EXPERIMENTAL

The MM virus is very potent when injected intraperitoneally and has proved to be a very satisfactory virus for the purpose of these tests. It, therefore, was used in most of the experiments. The Lansing virus, on the other hand, does not infect by the peripheral route and could only be tested by a less satisfactory method; *i.e.*, by intracerebral inoculation.

Concentration of Virus.—The protective action of trypan red, against infection of mice by MM virus injected intraperitoneally and in varied concentration, is shown in Table I. On the day following the third injection of trypan red the dye-treated animals were split into five groups of 10 animals each and corresponding control groups were set up. Control and dye groups were then inoculated with 0.1 ml. of various concentrations of the cord preparation of virus. It will be noted in Table I that with 10^{-1} concentration of virus all the mice succumbed to the disease in 3 days. With 10^{-3} virus 90 per cent became infected and there was no difference between the two groups of mice except that the dye group survived somewhat longer. However, when the concentration of virus was reduced to 10^{-5} and 10^{-7} there was a difference between the groups. In the 10^{-5} virus groups the onset of the disease was slower in the trypan red group, and in addition only 20 per cent were infected as compared to 60 per cent in the non-treated group. In the 10^{-7} groups none of the dye-treated animals became sick whereas 20 per cent of the control group were infected.

The results of Table I show clearly that trypan red lowers the incidence of infection by MM virus. The protective action of the trypan red can be masked, however, if an overwhelming dose of virus is given.

Table II gives still more convincing proof of the protective action of trypan

red. In this experiment larger groups of animals were tested, and, in order to make the control group more comparable to those that were treated with

TABLE I

The Effect of Trypan Red Dye on the Incidence of Infection of Mice by MM Virus Injected Intraperitoneally and in Varied Concentration

Concentration of virus	10^{-1}		10^{-3}		10^{-5}		10^{-7}	
No. of mice	10	10	10	10	10	10	10	10
Treatment	Dye	None	Dye	None	Dye	None	Dye	None
1–3 days	10	10	2	4	0	1	0	0
3–6 days	0	0	5	5	0	5	0	1
No. infected 6–9 days	0	0	2	0	1	0	0	0
9–12 days	0	0	0	0	0	0	0	0
12–15 days	0	0	0	0	1	0	0	1
Total infected	10	10	9	9	2	6	0	2
Infected, *per cent*	100	100	90	90	20	60	0	20

TABLE II

The Effect of Trypan Red Dye on the Incidence of Infection of Mice by 10^{-5} MM Virus Injected Intraperitoneally

No. of mice	47	50
Treatment	Dye	H_2O*
1–3 days	0	0
3–6 days	1	11
No. infected 6–9 days	0	21
9–15 days	0	3
15–18 days	1	0
Total infected	2	35
Infected, *per cent*	4	70

* Control group was injected with 0.1 ml of distilled water when the corresponding dye group was injected with trypan red.

trypan red, the controls were injected with distilled water in a manner comparable to the injection of dye. All mice were injected with a 10^{-5} virus preparation. Of the 47 mice treated with trypan red only 2 succumbed to the disease, whereas 35 out of 50 became ill in the control group. The probability

that a difference as great as this would occur by chance is 50 billion to 1 when calculated by the method of chi square (10). The protective action of trypan red against the MM neurotropic virus is thus clearly demonstrated.

Concentration of Trypan Red.—The concentration of trypan red which is necessary in order to elicit the protective action was next investigated. The trypan red concentration was varied in two ways; in one, the concentration of trypan red was maintained constant at 1 per cent and the number of 0.1 ml. injections was varied, in the second, the number of injections of trypan red was constant at three 0.1 ml. doses but the concentration of the dye was varied. Virus from a single preparation was injected in all animals at the same time on the 4th day following the first injection of trypan red.

TABLE III

Effect of Number of Injections and Concentration of Trypan Red on the Incidence of Infection of Mice by 10^{-5} MM Virus Injected Intraperitoneally

No. of mice	15	15	15	15	15	15
Dye, *per cent*	0.0	1.0	1.0	1.0	0.5	0.1
No. of dye injections	None	1	2	3	3	3
No. infected 1–3 days	0	0	0	0	0	0
3–6 days	7	3	0	0	0	1
6–9 days	4	5	2	2	0	1
9–12 days	0	0	1	0	1	2
12–21 days	0	0	1	0	0	0
Total infected	11	8	4	2	1	4
Infected, *per cent*	73	53	27	13	7	27

The results in Table III show that with an increasing number of injections of 1 per cent trypan red there was a lowered incidence of infection. With one injection the incidence dropped from 73 to 53 per cent, with two to 27 per cent, and with three to 13 per cent. When the number of injections was maintained constant at three and the concentration of dye was reduced, the protection was good at all three concentrations (last three columns, Table III), though somewhat less, perhaps, when the dye was reduced to 0.1 per cent. It therefore, appears necessary to give repeated injections of the dye, but the concentration can be reduced somewhat below 1.0 per cent.

In this connection it should be mentioned that when equal volumes of 10^{-1} virus preparation and 1 per cent trypan red were incubated together at 37° for 36 hours, and then the virus preparation was diluted to 10^{-5} and injected in mice, no effect of trypan red was observed. Likewise if the trypan red and

virus were injected simultaneously there was no effect of the dye. It therefore seems certain that there is some reaction produced within the animal body by the dye or by the body on the dye which is essential in order to elicit the protective action.

Duration of the Effect of the Dye.—The duration of the protective action of trypan red was determined and the results are shown in Table IV. The mice were given three injections of trypan red and then at intervals control animals and dye-treated animals were removed from the corresponding groups and

TABLE IV

Duration of the Protective Action of Trypan Red against Infection of Mice by MM Virus Injected Intraperitoneally

Time after dye injections before inoculation, *days*	1		8		16		22		29	
Concentration of virus	10^{-5}		10^{-5}		10^{-5}		10^{-4}		10^{-3}	
No. of mice	10	10	10	10	9	6	9	9	11	9
Treatment	Dye	None	Dye	None	Dye	None	Dye	None	Dye	None
1–3 days*	0	1	0	0	0	0	0	0	0	0
3–6 days	0	9	0	7	0	3	0	3	2	7
No. infected 6–9 days	1	—	0	3	0	2	0	2	3	2
9–15 days	0	—	0	—	1	0	0	1	0	0
15–21 days	1	—	0	—	0	0	0	0	1	0
Total infected	2	10	0	10	1	5	0	6	6	9
Infected, *per cent*	20	100	0	100	11	83	0	67	54	100

* Days after inoculation with the virus.

inoculated. Some difficulty was encountered in this experiment, since the resistance of the mice to infection apparently increased with age, and there was some uncertainty in selecting a virus concentration which would infect but yet not obscure the protective action of the dye. It will be recalled, as shown in Table I, that the virus concentration must be properly balanced before protection can be demonstrated.

With the above limitations considered it is clear that there was protection for at least 29 days. How much the protection changed with time is somewhat difficult to judge, because the infective dose of the virus was not the same at all times. For example, at 29 days the inoculation was done with 10^{-3} virus and 100 per cent of the control mice died, whereas in the experiment at

22 days 10^{-4} virus was used and only 67 per cent of the controls died. Obviously the same per cent of protection by the dye is not to be expected in the two experiments.

Effect of Different Dyes.—To determine whether or not uniform results could be obtained with different samples of trypan red, three different products were tested. The data in Table V show that protection was obtained with each sample of dye. Dye 1 was the sample of trypan red used in all other experiments of this investigation.

TABLE V

Test of Different Samples of Trypan Red for Protective Action against Infection of Mice by MM Virus Injected Intraperitoneally*

Concentration of virus	10^{-5}				10^{-6}			
No. of mice	17	16	11	16	17	16	10	16
Treatment	Dye (1)	Dye (2)	Dye (3)	None	Dye (1)	Dye (2)	Dye (3)	None
No. infected 1–3 days	0	0	0	0	0	0	0	0
3–6 days	2	5	4	7	1	2	0	7
6–9 days	2	1	1	4	1	0	1	3
9–15 days	1	1	0	2	1	1	0	2
15–21 days	1	0	0	0	0	0	0	0
Total infected	6	7	5	12	3	3	1	12
Infected, *per cent*	35	44	45	75	18	19	10	75

* Dye (1), Lot 2806 National Aniline and Chemical Co., Henry Heil Chemical Co.
Dye (2), Grübler and Co., Leipzig.
Dye (3), Lot 10775 National Aniline Division, Allied Chemical and Dye Corp.

A preliminary investigation has been made with a number of dyes other than trypan red to determine whether they effect a reduction in the incidence of infection by MM virus. Three basic dyes and eleven acid dyes were tested. One of the basic dyes, safranin O, was toxic and consequently could not be tested with the virus. The dyes were injected intraperitoneally on 3 successive days, using 0.1 ml. of a 1 per cent solution, and on the 4th day the virus was injected. Of the nine dyes tested only Congo red, brilliant vital red, and trypan red were definitely effective in reducing the incidence of the disease (Table VI). Of these three the trypan red was the most effective. The slightly lowered incidence observed with some of the other dyes cannot be considered significant because of the small number of animals involved in the test. It is interesting that the three dyes which were definitely effective are all acid dyes and are

structurally similar. However, trypan blue, which likewise is an acid dye and is very similar in structure to trypan red, was not effective.

Protective Action of Trypan Red in Cotton Rats.—Some difficulty was encountered in attempting to demonstrate the protective action of trypan red against MM virus in cotton rats. This is believed to have arisen from two factors, one, the improper selection of virus concentration, and two, failure to allow a sufficient interval of time after the third injection of trypan red before injection of the virus. In two experiments, in which 10^{-5} and 10^{-6} virus were used, one in which there was a 1 day interval after the last dye injection and the other in which there was a 7 day interval, there was no difference between the

TABLE VI

Effect of Different Dyes on the Incidence of Infection of Mice by MM Virus Injected Intraperitoneally

Dye*	No. of mice	No. infected				Total infected	
		Days				No.	Per cent
		1–3	3–6	6–9	9–21		
None	10	0	10	0	0	10	100
Bismarck brown (basic)	11	1	10	1	0	11	100
Neutral red (basic)	11	0	9	0	0	9	82
Alizarin red (acid)	11	0	10	1	0	11	100
Trypan blue (acid)	11	0	10	1	0	11	100
Acid fuchsin (acid)	11	0	5	2	2	9	82
Superchrome violet B (acid)	11	0	4	4	0	8	73
Congo red (acid)	11	0	3	3	0	6	55
Brilliant vital red (acid)	20	0	0	3	7	10	50
Trypan red (acid)	10	0	1	1	1	3	30

* 0.1 ml. of 1 per cent solution of the dye given intraperitoneally on 3 successive days.

dye-treated and control groups. In the successful experiments shown in Table VII the concentration of virus was reduced to 10^{-7} and the interval before virus injection was 7 and 14 days. The dye-treated animals seemed somewhat lethargic, and primarily for this reason virus injection was delayed in order to allow recovery from the effects of the dye. Furthermore, the results with mice in Table IV indicated that the protection would be at least as great after an 8 day as after a 1 day interval. The results, though not showing as marked a protection as in the mice, do demonstrate that trypan red can reduce the incidence of infection in cotton rats under appropriate experimental conditions. The probability that the difference is due to chance, is 1 in 16 by chi square calculation. It is possible that a greater deviation between the dye-treated and control groups could be obtained by more careful balancing of conditions.

Intracerebral Injection of the MM Virus and the Effect of Trypan Red.—When the MM virus was injected intracerebrally in mice, trypan red was not found to provide any protection from infection. In fact the incidence of infection was somewhat higher in the dye-treated animals than in their control group. Typical results are shown in Table VIII. Evaluation of the data by the chi square method indicates that the observed difference is not significant. The odds are only 3 to 1 that the difference is not due to chance.

Intracerebral Injection of Lansing Virus and the Effect of Trypan Red.—Since the Lansing virus does not infect mice when injected by peripheral routes, the effect of trypan red could only be tested with this virus by intracerebral inocu-

TABLE VII

Effect of Trypan Red on the Incidence of Infection of Cotton Rats Injected with 10^{-7} MM Virus Intraperitoneally

	Group I*		Group II*	
No. of cotton rats	5	6	14	15
Treatment	Dye	None	Dye	None
No. infected 1–3 days	0	0	0	0
3–5 days	1	4	2	7
5–7 days	0	0	3	1
7–12 days	0	0	0	1
12–21 days	0	0	0	0
Total infected	1	4	5	9
Infected, *per cent*	20	67	36	60

* Group I was inoculated 7 days and Group II 13 days after the last dye injection.

lation. In contrast to the results with the MM virus, with which no protection was found against intracerebral inoculation, a slightly lowered incidence of infection in the trypan red–treated animals was found with the Lansing virus. The results presented in Table IX are typical. The lowered incidence of infection was observed with each concentration of virus tested. When calculated by the chi square method the probability is 30 to 1 that the difference is not due to chance. Whether or not the reducton in infection is due to a generalized alteration in the condition of the animal rather than to a specific action of the dye is not known. Since the dye-treated mice do not grow as rapidly as the untreated mice it is possible that a condition similar to that studied by Foster *et al.* (1) may exist, in which the incidence of poliomyelitis is reduced because of inadequate food intake. However, we do not believe that this condition can be completely responsible for the very good protection

TABLE VIII

The Effect of Trypan Red on the Incidence of Infection of Mice by MM Virus Injected Intracerebrally

Concentration of virus	10^{-5}		10^{-6}		10^{-7}	
No. of mice	15	15	15	15	15	15
Treatment	Dye	None	Dye	None	Dye	None
No. infected 1–3 days	7	4	5	2	1	1
3–6 days	3	5	2	3	0	0
6–9 days	0	2	1	0	0	0
9–15 days	3	0	0	0	0	0
15–18 days	0	0	0	0	0	0
Total infected	13	11	8	5	1	1
Infected, *per cent*	87	73	53	33	7	7

TABLE IX

The Effect of Trypan Red on Incidence of Infection of Mice by Lansing Virus Injected Intracerebrally

Concentration of virus	$10^{-1.7}$		$10^{-2.7}$		10^{-3}	
No. of mice	16	20	19	20	14	20
Treatment	Dye	None	Dye	None	Dye	None
No. infected 1–3 days	2	2	0	2	0	1
3–6 days	3	4	2	3	2	0
6–9 days	5	5	1	3	2	3
9–12 days	2	6	4	3	0	5
12–15 days	1	2	1	2	0	3
15–18 days	0	1	1	2	2	0
18–21 days	1	0	2	1	1	1
Total infected	14	20	11	16	7	13
Infected, *per cent*	87	100	58	80	50	65

that is obtained when MM virus inoculation is intraperitoneal. In this case a more specific action seems indicated.

Experiments with Monkeys.—Although the MM virus induces histopathological alterations similar to those found in poliomyelitis, it differs in some respects from the classical poliomyelitis virus. Its classification is thus somewhat uncertain. The question arises as to whether or not trypan red will

provide any protection in typical poliomyelitis. The methods used with the Lansing strain of virus were not satisfactory because infection other than by intracerebral inoculation was not possible. Particularly in view of the increasing opinion that the poliomyelitis virus may invade *via* the gastrointestinal route, it would be interesting to know whether or not trypan red raises the resistance to poliomyelities with infection by this route. However, at the present time it has not been possible to conduct such an experiment because of the lack of a suitable host. The investigations of Howe and Bodian (11) with chimpanzees suggest that this animal might meet the requirements of

TABLE X

The Effect of Trypan Red on the Incidence of Infection of Rhesus Monkeys by McK Virus Injected Intraperitoneally and in Varied Concentrations

Concentration of virus	10^{-1}		10^{-2}		10^{-3}	
No. of monkeys	2	2	2	2	2	2
Treatment	Dye	None	Dye	None	Dye	None
No. infected 1–3 days	0	0	0	0	0	0
3–6 days	2	1	0	0	0	0
6–9 days	—	1	0	0	0	0
9–13 days	—	—	0	0	0	0
13–21 days	—	—	0	0	0	0
Total infected	2	2	0	0	0	0
Infected, *per cent*	100	100	0	0	0	0

such an experiment but we are not prepared, at present at least, to carry on an investigation with chimpanzees.

One experiment was attempted with *rhesus* monkeys.

Twelve monkeys were used in the experiment, of which six were treated with trypan red. Three injections of 5 ml. of 1 per cent trypan red were made intraperitoneally on alternate days. Eight days after the last dye injection the monkeys were inoculated intraperitoneally with 20 ml. of a cord suspension from a monkey paralyzed with McK virus. The virus was received from Dr. J. F. Kessel of the University of Southern California.

Results from the experiments with monkeys are shown in Table X. Of those monkeys receiving the 10^{-1} inoculation one monkey of the dye-treated group became paralyzed in the left hind leg on the 6th day and died on the 8th day; the other monkey developed a paresis of the front legs on the 5th day and eventually recovered. In the control group one monkey was prostrate on

the 5th day and died on the 6th day; the other monkey became weak on the 9th day but eventually recovered. Each of these four monkeys had an elevated temperature prior to the onset of their physical symptoms. The remaining monkeys which received the 10^{-2} and 10^{-3} dilutions of cord in both the trypan red and control groups did not show any signs of paralysis or weakness and in no case was an elevated temperature observed.

These results in essence show that when two monkeys were given less than 10 minimal infective doses of virus intraperitoneally, there was no protection by the dye. Although this present test is inadequate and it is not known whether partial protection could have been shown by using a larger series of animals, it is our belief that the present type of experiment is of doubtful value for such a test. The protective action of trypan red probably cannot be adequately tested in monkeys until conditions are obtained more comparable to those of the mouse experiments; *i.e.*, the virus should be infective in monkeys in a fairly uniform manner when inoculated by a peripheral route and in high dilution.

DISCUSSION

It is not the purpose of the present report to provide information concerning the mechanism or mode of action of trypan red, but rather to report the results and largely leave the interpretation for future investigations.

The recent investigation of Aird (12) and Aird and Straight (13) is interesting in this connection. These authors found by comparison of the distribution of cocaine in untreated cats and cats injected intraperitoneally with trypan red that the cocaine in the cerebral cortex of the cats receiving trypan red was lowered by 31 per cent and in the cerebrospinal fluid by 40 per cent, while the concentration in the blood remained essentially the same (13). Furthermore trypan red was found to give effective protection in mice against the convulsive effects of cocaine. The evidence, thus, seems quite conclusive that trypan red alters the distribution of certain materials between the blood and the central nervous system.

It might be inferred from these observations that the altered permeability between the blood and central nervous system may prevent the virus from entering the nervous system. On the other hand, it is apparent that a change in permeability probably reflects a fundamental change in the physiology and biochemistry of the tissues. This change, in itself, may be the factor which is the basis of the action of trypan red rather than an actual prevention of entrance of the virus. The fact that the animals were not effectively protected from infection, when the virus was introduced directly into the nervous system, is not considered significant evidence against this view, because the concentration of virus in the nervous tissue is probably quite different in this case than when inoculation is by a peripheral route.

Direct evidence that trypan red may have another action than alteration of permeability between the blood and central nervous system is indicated by the fact that trypan red is toxic for trypanosomes and has been used as a protectve measure in such infections (14). In this infection it seems doubtful that the protective action of the dye is by an alteration in the permeability of the barrier to the central nervous system. At any rate it is not apparent why such an alteration in the permeability would lead to an increased resistance against trypanosomes.

SUMMARY

Trypan red, when injected intraperitoneally into mice, has been found greatly to lower the incidence of the infection of mice inoculated intraperitoneally with the neurotropic MM virus. The protective action of the dye is overcome if the virus is inoculated in too high concentration. The lowered incidence of infection was observed in mice inoculated with virus for as long as 29 days after the last dye injection. Of a number of dyes tested, trypan red, brilliant vital red, and Congo red were found effective.

In cotton rats inoculated intraperitoneally with MM virus, trypan red was likewise found to lower the incidence of infection.

With monkeys and a typical poliomyelitis virus no protection was observed against the virus inoculated intraperitoneally. The latter experiment is considered to have been inadequate for a critical test of the effect of trypan red on poliomyelitis infection.

When either the MM virus or Lansing virus were inoculated intracerebrally into mice, the effect of the dye on incidence of infection was small. In the case of the Lansing virus the difference was statistically significant, however.

The possible relation of alteration in the permeability of the barrier between the blood and the central nervous system as a cause of the effect of trypan red is discussed.

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