CHEMOTHERAPEUTIC ACTION OF DYES IN TYPHUS INFECTION OF MICE

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(Received November 1, 1945)

Several papers published during 1944 revealed that the rickettsiae of typhus fever are susceptible to attack by chemotherapeutic agents. Such activity has been shown in experimentally infected mice by Andrewes, King, van den Ende and Walker (1944), using *p*-sulphonamidobenzamidine (V147) and the corresponding amidoxime (V 186); by Yeomans, Snyder, Murray, Zarafonetis and Ecker (1944), using *p*-aminobenzoic acid; by Moragues, Pinkerton and Greiff (1944) with penicillin; by Peterson (1944) with forbisen and toluidine-blue and by Kikuth and Schilling (1944) with methylene-blue. No good results in experimental animals, other than mice, have been reported. The compounds, V147 and V186 and penicillin have proved unpromising in human typhus (van den Ende, Stuart-Harris, Fulton and Niven, 1946).

This paper records the results of extending Peterson's observations on toluidine-blue by studying the action of other dyes, particularly those chemically related to it.

TECHNIQUE

STUDIES OF *in vivo* ACTION IN MICE

Most of the workers, other than ourselves, mentioned above, have used the intraperitoneal route for infecting the mice in their studies. This has the disadvantage that it is available only for murine typhus, not for epidemic strains; nor is it so well adapted for quantitative comparison of drugs as the intranasal technique we employed. This method we have continued to use. Suspensions of rickettsiae, kept at -76°C. , will maintain their potency unchanged for many months, and suitable dilutions inoculated intranasally into mice will produce isolated foci on the lungs. The foci can be counted with satisfactorily reproducible results, and the reduction in count used to judge the effect of drugs. Groups of six mice were used for each drug, with an appropriate control group of six mice. Details are given in the earlier paper (Andrewes *et al.* 1944). All intranasal inoculations were carried out in the special inoculation box (van den Ende, 1943), without the aid of which, we are convinced, the intranasal technique will almost certainly lead to accidental laboratory infections.

In routine testing of many chemotherapeutic agents over two years ago we failed to detect any activity on the part of toluidine-blue or methylene-blue. This was doubtless

because these drugs are very poorly tolerated by the intraperitoneal route which we were using. For further work with dyes we used one of three methods:

(a) Drugs were mixed in a known percentage with the moist food-mash. This method was labour-saving, but did not readily disclose the actual amount of drug taken, particularly as sick mice ate poorly.

(b) Drugs were administered into the oesophagus by means of a 1 cc. syringe and wide needle, of which the point had been cut off and the end blunted. This was done, as in our earlier intraperitoneal tests, two hours before infection, twice daily on the two following days, and on the morning of the third following day.

(c) The drugs were given subcutaneously according to the same time schedule as under (b). Many caused local oedematous reactions and consequently each of the six injections was made at a different site.

STUDIES OF *in vitro* ACTION AND TESTS ON RABBITS' SKINS

Peterson reported that toluidine-blue inactivated murine typhus rickettsiae *in vitro*. We accordingly included in our studies tests of such action. Suspensions of murine rickettsiae were held in contact with aqueous solutions of various dyes for 45 minutes at room temperature, and then inoculated in 0.1 cc. quantities intradermally into skins of shaved rabbits. Several dilutions of each of four or more dyes could be tested on the skin of one rabbit. Staining or inflammatory reactions caused by the dyes themselves rarely caused trouble in the dilute solutions in which we used them. Specific raised erythematous lesions, such as have been described by Giroud (1938), appeared within two or three days whenever the rickettsiae had not been inactivated; they reached their maximal intensity after about five days. Since the texture of the rabbits' skin may affect the size and nature of a rickettsial lesion, several control inocula of rickettsiae alone were always made in different positions into the animal's skin. The number of rickettsiae in the inoculum did not materially affect the results of drug tests over a wide range (1:100 to 1:10,000 of stock suspension); so we used throughout a 1:100 dilution of a stock suspension, 0.05 cc. of which, when diluted 1:10⁵, was capable of producing 40 lesions in a mouse's lungs. The time of contact of rickettsiae and dye did not greatly affect the issue, though slightly better inactivation occurred when contact was for one hour at 37° C. Very probably some action of dyes on the organisms continued after the mixture had been injected intradermally.

RESULTS

Mouse Tests.—We readily confirmed Peterson's finding that toluidine-blue had a chemotherapeutic action when given mixed with the food. Table I shows that it wholly suppressed lung lesions when given in a 1.5 per cent concentration, that 0.5 per cent was less effective and 0.1 per cent useless. Oral administration with a syringe suppressed lesions wholly when 5 mg. were given on six occasions; results were less striking when the dose was reduced. The drug given subcutaneously in doses of 6 × 1 mg. or 3 × 2 mg. was also completely effective; these doses, however, produced considerable oedematous swellings at the sites of inoculation. We confirmed our earlier findings that toluidine-blue was ineffective when given in the maximal dose which was tolerated intraperitoneally (1 mg.).

Table I shows that methylene-blue was equally effective. Possibly it was a little more so, but the differences were not significant. Similar results were obtained in a feeding test with medicinally pure zinc-free methylene-blue. These experiments were carried out with murine typhus (Wilmington strain). In two

experiments toluidine-blue was shown to act also in epidemic typhus infection, though rather less effectively. Six doses of 5 mg. *per os* reduced the lung spot-count from 56 to 14.

Of other dyes tested only three (selenium-methylene-blue, new methylene-blue and 3-diethylamino-7-di-*n*-butylaminophenazthionium chloride) had any

TABLE I

CHEMOTHERAPEUTIC EFFECT OF METHYLENE-BLUE ANALOGUES AGAINST MURINE TYPHUS IN MICE

Drug	How given	Dose	Lung Spot Count (average of 6 mice)	Lung Spot Count in un-inoculated mice (average of 6)
Toluidine-blue	Mixed with food	1.5%	0	34
" "	" " "	0.5%	2	44
" "	" " "	0.5%	4.5	28
" "	" " "	0.1%	28	28
Toluidine-blue	<i>Per os</i> (syringe) twice daily (6 doses in all)	5 mg.	0	42
" "	" " " <i>Per os</i> (syringe) once daily (3 doses in all)	2.5 mg.	1.5	42
" "		2 mg.	13	47
" "		5 mg.	2	27
Toluidine-blue	Subcut. 6 doses	1 mg.	0	24
" "	" 3 "	2 mg.	0	27
Methylene-blue	Mixed with food	1.5%	0	44
" "	" " "	0.5%	0	28
" "	" " "	0.1%	10	28
" "	" " "	0.1%	17	24
Methylene-blue	<i>Per os</i> (syringe) (6 doses in all)	2 mg.	12	47
Methylene-blue	Subcut. 6 doses	1 mg.	0	24
" "	" 3 "	0.5 mg.	1	30
New-methylene-blue	Subcut. 6 doses	1 mg.	2	45
3-Diethylamino-7-di- <i>n</i> - butylaminophenaz- thionium chloride hydrochloride	Subcut. 3 doses	1 mg.	0	12
" "	" 3 "	0.25 mg.	20	30
Selenium - methylene - blue	Subcut. 3 doses	2 mg.	3.5	30
" "	" 3 "	0.5 mg.	14	30

action at all *in vivo*. The first two of these were apparently slightly less active than, the third about equal to, methylene and toluidine-blues. With the other dyes, no significant differences occurred between the spot-counts of treated and control mice. They were given at their maximal tolerated dose as shown in

TABLE II
DETAILS OF TESTS OF DYES AGAINST TYPHUS IN RABBITS AND MICE

Group to which dye belongs	Dye	Route of inoculation and dose (m.t.d.) used for mouse tests—in mg. unless otherwise stated	Activity in mouse test (see Table I)	Effective dilution <i>in vitro</i> as shown by rabbit test
Acridines	Proflavine Acriflavine 2: 7-Diaminoacridine 2: 8-Tetramethyldiamino-10-methyl-acridinium methosulphate Atebrin	0.37 i.p. 0.06 i.p. 8 i.p. no test 2 s.c.; also tested 0.5%	0	Ineffective at 1:2,000
Oxazines	Brilliant-cresyl-blue Cresyl-fast-violet 2B Capri-blue G.O.N. Nile-blue B	2 i.p. 0.5 s.c. 0.2 s.c. 0.5 s.c.	0	
Phenazines	Janus-green Neutral-red Safranine	0.05 i.p. 1 s.c.; also tested 0.1% in diet 0.5 s.c.	0	
Azo-dyes	Trypan-red Trypan-blue Congo-red	0.5 s.c. 0.25 s.c. 1 s.c.	0	
Thiazines	Thionine Thiodiphenylamine	2-4 s.c. also tested 0.5% in diet 1% in diet	0	
Pyronine	Pyronine B	No test	0	1: 5,000
Phenazine	Methylene-violet 3RA	0.25 s.c.	0	1: 25,000
Thiazine	1-Methyl-3-diethylamino-7 (mono)- <i>n</i> -butylamino-P*	0.25 s.c.	0	1: 4,000
Oxazine	Meldola-blue	0.1 s.c.	0	1: 50,000
Thiazine	Methylene-blue	1 s.c. (also tested in diet)	+	1: 500,000
"	Toluidine-blue	1-2 s.c. (also tested in diet)	+	1: 500,000
"	Methylene-green	1 s.c.	0	1: 125,000
"	1-Methyl-3-diethylamino-7-di- <i>n</i> -butylamino-P*	0.25	0	1: 500,000
"	1-Methyl-3-diethylamino-7-di- <i>n</i> -propylamino-P*	0.25	0	1: 500,000
"	1-Methyl-3: 7-bis (diethylamino)-P*	0.25	0	1: 500,000
"	New-methylene-blue	1 s.c.	+	1: 500,000
"	3-Diethylamino-7-di- <i>n</i> -butylamino-P*	1 s.c.	+	1: 500,000
Selenazine	Selenium-methylene-blue	2 s.c.	+	1: 500,000

*P=phenazthionium chloride hydrochloride.

Table II—mostly subcutaneously. Forbisen, which Peterson reported to have some activity, was by our technique weakly active. When given by mouth in 6×25 mg. doses, it reduced the average spot-count from 26 to 5 in one test, and from 47 to 17 in another.

Rabbit Tests.—The last column of Table II shows the final concentration of dye which would inactivate rickettsiae *in vitro* when tested as described earlier. It is apparent that a few drugs, those of the last section in the table, are very active in the *in vitro* tests, their effective concentrations being about 250 times less than that of most of the dyes tested. All but four of the drugs we examined could by this test be readily classified as either “very active” or “useless.”

It will be noticed that three of the thiazines were as active as methylene-blue in the rabbit, but were not active in the mouse test; this is presumably because they were more toxic and could only be given in 0.25 mg. doses.

DISCUSSION

All the dyes in Table II, with the exception of the azo-dyes represented by trypan-red, trypan-blue and congo-red are similarly constituted and belong to the class of vital stains. They fall into the ortho-quinonoid group of dyes to which Ehrlich attached special significance. Presumably they are all taken up to a greater or lesser extent by the rickettsiae, but one half of them, as shown by the *in vitro* test, are comparatively harmless. The other half inactivate the rickettsiae for the most part at relatively high dilutions. In the mouse test, where one is dealing with a more complex chemotherapeutical system, the dyes have not only to be tolerated by the host but have to enter the cells harbouring the rickettsiae. Nevertheless the two tests do show a rough correlation.

The results in the absence of further evidence are capable of more than one interpretation, but an acceptable view of the chemotherapeutic process would be to suppose that the vital dyes are adsorbed or absorbed by the rickettsiae in either form of the test and the specific fixation of the dye interferes with some vital function necessary for reproduction of the parasite.

The action of the active thiazines seems unlikely to be necessarily dependent on a photodynamic effect, since dye introduced subcutaneously or *per os* is effective on rickettsiae within the lungs.

Comparison of the results of the mouse tests and rabbit tests shows that the latter may have some value as a screening method before resort to the direct test in mice, if allowance is made for toxicity. The five compounds effective in mice are amongst those which are most active in the rabbit test. Such correspondence as there is between the two tests must not be taken as necessarily applying to compounds chemically unrelated to these dyes: the drugs V147 and V186 and related compounds, which are more effective *in vivo* than any of the dyes tested (Andrewes *et al.*, 1944), fail to show any *in vitro* killing power when mixtures are tested in the rabbit's skin.

SUMMARY

Dyes have been tested for activity against typhus rickettsiae (a) by holding mixtures of organisms and dye in contact *in vitro* and inoculating into the rabbit's skin to test whether infectivity has been destroyed and (b) by a direct *in vivo* chemotherapeutic test in mice. The results of the two tests, so far as dyes were concerned, were closely parallel. Activity was found only in thiazines and in one selenazine.

We extend our thanks to Mrs. Frances Hamilton for her help with some of the inoculations and to Drs. L. Hellermann and O. L. Peterson, and to Imperial Chemical (Pharmaceuticals), Ltd., for their kindness in supplying us with compounds for test.

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