THE MOUSE-PROTECTIVE TEST AS A MEANS OF DETERMINING THE INHIBITORY EFFECT OF CHEMICALS ON VIBRIO CHOLERA

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McKenzie and co-workers (1948) have reported on the results of screening a number of chemicals by *in vitro* methods for possible activity against gram-negative bacteria including *Vibrio cholera*. The present report concerns itself with the testing of fifty-seven chemicals selected from table 3 of McKenzie's report.

The strain of *Vibrio cholera* used in our study is the same strain (#34-A-3) which the National Institute of Health employs for the testing of *Vibrio cholera* vaccine and was supplied to us by the Lederle biological testing section.

In a comparison of mucin and peptone water suspensions of 12 hour broth culture, we found that 0.5 ml doses of culture in 5 per cent mucin killed mice at dilutions of 10^{-0}, 10^{-1}, 10^{-2}, and 10^{-3}, but not at 10^{-4}; 0.5 ml doses of the same culture in peptone water killed mice only at the 10^{-0} dilution. These doses of culture were injected intraäbdominally in 3-week-old mice. To extend the range of virulence, we employed the six-hour growth from blood agar slants instead of broth cultures. The growth was suspended in peptone water and standardized turbidimetrically. The standardized suspensions contained about 2 billion organisms per ml by plate count (2.34, 1.87, and 2.21 billion in three tests), and 0.5 ml doses of suspension killed mice at dilutions with 5 per cent mucin of 10^{-4}, 10^{-5}, and 10^{-6}, but not at 10^{-7} (three tests).

The infecting dose used for our tests of chemicals was 0.5 ml of the 10^{-3} dilution of standardized suspension in 5 per cent mucin. Active chemicals were also tested against 0.5 ml of the 10^{-2} dilution of culture.

Both the culture and the chemicals were injected as 0.5 ml volumes into the peritoneal cavity of the mice. This was considered to be the most precise method available. In preliminary experiments with groups of 40 mice, 0.2 mg doses of 2-sulfanilamidopyrimidine were injected at four-hour intervals beginning 5, 4, 2, and 1 hours after the culture dose. These treatments protected 17 per cent (5 hr), 18 per cent (4 hr), 35 per cent (2 hr), and 68 per cent (1 hr). In one experiment, 1.0 mg doses of 2-sulfanilamidopyrimidine were given beginning four hours after the culture dose and 72 per cent of 25 mice were protected.

Because of the limited supply of many of the chemicals, we selected 0.2 mg as the standard dose for our tests, and a schedule of 6 injections at four-hour intervals beginning 1 hour after the culture dose. In a preliminary experiment, when a single dose of 0.2 mg of 2-sulfanilamidopyrimidine was injected intraperitoneally immediately after injecting culture, all the mice died as rapidly as the untreated mice. This experiment showed that there was no *in vitro* in-

hibition of *Vibrio cholera* in the peritoneal cavity at the dosage of chemical and culture used.

The chemicals were prepared for test by the following procedure. Forty-milligram amounts were weighed on the analytical balance and placed in sterile vials. The vials were labeled, protected with paper covers, and stored in a dry place until the day of the test. Depending upon the solubility of the chemicals, 10 ml amounts of distilled water or of N/100 sodium hydroxide were added, and the vials shaken, gently heated if necessary, and stoppered. All of the sulfonamides dissolved readily in N/100 sodium hydroxide. Highly insoluble chemicals usually formed suspensions suitable for injection. Doses of 0.5 ml contained 0.2 mg. Each chemical was tested by injecting a group of ten mice at four hour intervals beginning 1 hour after the culture dose. Retests using larger numbers of mice (20–30) were made whenever chemicals showed activity. In spite of the rapid mortality of animals infected with *Vibrio cholera*, we were able to make 4 or 5 injections of inactive chemicals before the mice died. A total of a hundred untreated mice, inspected at four hour intervals, were found dead in an average of 22.9 hours, and none in less than 17 hours. On each initial test, we gave eight injections of chemicals showing activity; six injections were used on retests.

EXPERIMENTAL RESULTS

Mouse-protective tests were carried out to determine the inhibitory effect of fifty-seven different chemicals on *Vibrio cholera*. The chemicals tested were those that showed the greatest activity in *in vitro* tests of 650 different chemicals listed in Table 3 of the report by McKenzie and co-workers (1948). The chemicals which were found active in mouse-protective tests are shown in table 1. The most active compounds were 2-sulfanilamidopyrimidine and its heterocyclic substituted chloro-, bromo-, and methyl forms.

Some activity was demonstrated by 2-sulfanilamidothiazole, by 2-sulfanilamidopyridine and its 5-chloro derivative, and by 2-sulfanilamidopyrazine. The slight activity of the xanthene dye, pyronin *Y*, and the thiazine dye, new methylene blue N, are of interest because of the fact that discovery of the activity of the azo dye, chrysoidin, led to the development of sulfanilamides.

Sulfanilamide, itself, and all of the following *sulfanilamide* derivatives were found inactive: sodium *N*-acetylsulfanilamide, N^1-*n*-valerylsulfanilamide, N^1-(2,5-dimethylbenzoyl)-sulfanilamide, N^1-(3,4-dimethylbenzoyl)-sulfanilamide, N^1-furoylsulfanilamide, sulfanilamidothymol, 2-sulfanilamido-4-methyl-5-carboxythiazole, and sulfaguanidine.

As shown in table 2, the test dose of *Vibrio cholera* (1 or 10 million organisms in 5 per cent mucin) produced active immunization of the animals which were protected by treatment with chemicals. The mice which were protected by 2-sulfanilamidopyrimidine, 2-sulfanilamido-5-bromopyrimidine, 2-sulfanilamido-5-chloropyrimidine, and 2-sulfanilamido-4-methylpyrimidine were re-inoculated after two weeks with a dose of 0.5 ml of 10^{-2} culture (10 million organisms). Ninety-two per cent of sixty-five mice survived re-inoculation and all control mice died.

The following chemicals of the aromatic series were found inactive: benzophenone, mercaptobenzothiazole, *B*-benzoylacrylic acid, *N*,*N'*-4,4'-bisdimethylaminoazobenzene butyramidine; *p*-aminophenol, diaminodiphenyl disulfide, *p*,*p'*-dihydroxydiphenyl sulfone, 4,4'-diamino-3,3'-dimethyldiphenyl methane, 2,2'-dihydroxy-3,3'-5,5'-6,6'-hexychlorodiphenylmethane, methylene-bis-4-chlorophenol, 1-phenyl-2(4-pyridyl) acetylene, *N*,*N'*-bis(*p*-aminophenyl)-caproamidine, 1-dodecyl-5-phenylbiguanidine hydrochloride.

The following chemicals containing guanidine nucleus were found inactive: octadecylguanidine acetate, diguanidinefuchsin carbonate. The following dyes were found inactive: acridine, acridine orange, amethyst violet, aniline green, anthracene (benzyl) violet, brilliant cresyl blue, brilliant violet, crystal violet,

TABLE 1

Chemicals Found Active Against Vibrio cholera *in Mouse-Protective Tests Employing a Series of Eight Injections of 0.2 mg Doses At Intervals of Four Hours*

CHEMICAL NAME	NUMBER OF TESTS MADE	TOTAL NUMBER OF MICE USED	PERCENTAGE OF MICE PROTECTED
2-Sulfanilamidopyrimidine	7	140	53
2-Sulfanilamido-5-bromopyrimidine	3	55	60
2-Sulfanilamido-5-chloropyrimidine	3	55	41
2-Sulfanilamido-4-methylpyrimidine	3	45	51
2-Sulfanilamidopyrazine	2	20	15
2-Sulfanilamidopyridine	2	20	30
2-Sulfanilamido-5-chloropyridine	2	20	30
2-Sulfanilamidothiazole	2	25	24
New Methylene Blue *N*	3	45	11
Pyronin *Y*	3	60	18

Culture dose:
1 million organisms in initial test.
10 million organisms in re-tests.

TABLE 2

Immunity of Mice After Having Been Protected By Treatment With Chemicals

TEST	CHEMICALLY PROTECTED MICE*			UNTREATED CONTROL MICE		
	Re-inoculated with *Vibrio cholera* (0.5 ml 10-2 dilution)			Inoculated with *Vibrio cholera* (0.5 ml 10-2 dilution)		
	Total Mice	Number Lived	Number Died	Total Mice	Number Lived	Number Died
1	15	14	1	15	0	15
2	25	24	1	25	0	25
3	25	22	3	10	0	10

* Mice were protected by use of 0.2 mg doses of 2-sulfanilamidopyrimidine, 2-sulfanilamido-5-bromopyrimidine, 2-sulfanilamido-5-chloropyrimidine, 2-sulfanilamido-4-methylpyrimidine.

Janus black II, Janus green B, malachite green HCl, naphthazarin, victoria green.

The following *miscellaneous* chemicals were found inactive: pinacyanole and 2-amino-6-hydroxy-8-carboxypteridin.

SUMMARY AND CONCLUSIONS

Mouse-protective tests were carried out against *Vibrio cholera* using fifty-seven different chemicals. The chemicals were selected because of activity in *in vitro* tests against *Vibrio cholera* as reported by McKenzie and co-workers. The only chemicals showing superior activity were 2-sulfanilamidopyrimidine and its 5-bromo, 5-chloro, and 4-methyl derivatives. Animals having been protected by chemicals were immune when re-inoculated with *Vibrio cholera*. The xanthene dye, pyronin *Y*, and the thiazine dye, new methylene blue *N*, showed slight activity.

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