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SULPHADIMIDINE, PYRIMETHAMINE AND DAPSONE IN THE TREATMENT OF TOXOPLASMOSIS IN MICE

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Sulphadimidine, dapsone, and pyrimethamine have been tested alone and in various combinations for their therapeutic effect against toxoplasma infection in mice. In the treatment of active infection, sulphadimidine by itself was effective, but relapses were common. Pyrimethamine gave complete cures and prevented the carrier state when used in doses near to the toxic level. Dapsone alone was not as good as either of the other two drugs tested. The best combination was found to be sulphadimidine and pyrimethamine, which were synergic. In doses well below the toxic level, this combination not only controlled the acute infection but also prevented relapses and the development of the carrier state. Dapsone and pyrimethamine were also synergic, but were not as effective as the previous combination. No synergy was found between dapsone and sulphadimidine. The mechanism of relapse and the development of the carrier state and the modes of action of the drugs alone and in combination are discussed.

Warren and Sabin (1942) reported discrepancies between the *in vitro* and *in vivo* effects of drugs against *Toxoplasma gondii*. Most subsequent experiments on the action of drugs against this organism have therefore been done *in vivo*, chiefly with mice and rabbits. Antibiotics are almost without effect and the little action they have probably results from their influence on secondary infections. Fungicides and compounds containing arsenic and antimony are likewise ineffective; so are the majority of the antimalarial drugs, the exceptions being the sulphonamides and the 2:4-diaminopyrimidines. Sulphonamides have been shown to be toxoplasmicidal by many workers. Eyles and Coleman (1953a) showed that there was little to choose between the three pyrimidine sulphonamide derivatives, namely sulphadiazine, sulphamerazine and sulphadimidine, and that these compounds were superior to sulphathiazole, sulphapyridine and sulphanilamide. The diphenylsulphones were shown to be active by Summers (1949) and by Cross (1951). Eyles and Coleman (1952) showed that pyrimethamine (Daraprim) was the most active of a group of 2:4-diaminopyrimidines, and that the activity was enhanced by the concurrent administration of sulphadiazine (Eyles and Coleman, 1953b).

With mice as the test animals and using sulphadimidine, dapsone and pyrimethamine as representatives of the three groups of active drugs, we have attempted to assess (1) their relative value as short-term therapeutic agents and as substances which may prevent or eliminate the carrier state, (2) the ratio of effective dose to toxic dose, and (3) whether a synergy exists between pairs of the drugs. A preliminary account of our results has been given elsewhere (Beverley and Fry, 1956).

METHODS

Test Animals.—Mice were caged in groups of 6 and were allowed as much food and water as they wished. They were fed on a diet compressed into cubes. (Diet No. 86, North-Eastern Agricultural Co-operative Society Ltd., Aberdeen).

Inoculation.—The *RH* strain of toxoplasma (Sabin, 1941) was used. It had been maintained in our laboratory by twice-weekly mouse passage for the past six years. The inoculum consisted of peritoneal fluid from infected mice, diluted with saline so as to contain 15,000 to 20,000 extracellular toxoplasms in 0.013 ml. The number of extracellular toxoplasms was determined by counting in a Neubauer chamber. In order to simulate a natural infection, the mice were anaesthetized lightly with ether and one drop (0.013 ml.) of inoculum was allowed to fall from the tip of a 27 S.W.G. needle held 0.25 in. above the nostrils. Tests for potency showed such an inoculum to contain from 750 to 1,250 LD50 doses, determined by giving tenfold saline dilutions of the test dose to groups of 6 mice and calculating the titre by the methods of Reed and Muench (1938). In some of the experiments large numbers of mice, up to 300, were used. Even

though it was possible to inoculate all of them within 1 hr. it was thought that the virulence of the inoculum might lessen within that period. To determine if this was so, one set of control mice was inoculated before any of those to have treatment, a second set in the middle of those to be treated, and a third set at the end. No appreciable difference in survival time was found in these three groups in any of the experiments.

Treatment.—The drugs were given in the diet because it is the most practical way of treating large numbers of mice for a long period, and because mice eat at frequent intervals and a more uniform blood concentration can be maintained by this method than by oesophageal intubation. Drugs were added in the required amounts to 200 g. of powdered diet. After mixing, 100 ml. of water was added and the mixture worked into a doughy mass. This was recast into cubes by means of an apparatus designed to force the mixture through jets of suitable size. The wet cubes were dried in a well-ventilated oven at 56° C. overnight. Treatment started immediately after inoculation (unless otherwise stated) and was continued for 1, 2, 3, or 4 weeks.

Toxicity.—Drugs were fed in different concentrations in the diet of six-week-old mice for a fortnight. The toxic dose (TD) was taken to be that concentration which just prevented the mice from gaining weight.

Determination of Optimum Ratios of Drug Mixtures.—Various combinations of four different geometric fractions of the toxic dose of sulphadimidine and pyrimethamine were fed to 24 groups of mice. The survival times, relapse times, carrier states, and complete cures for each group were recorded. A similar experiment was made with mixtures of dapsone and pyrimethamine. The effective dose was taken as the lowest concentration of drug or drugs in the diet which, when given for 28 days, ensured that all mice so treated survived for 28 days ; these concentrations were expressed as fractions of the TD.

Assessment of Results.—An autopsy was done on each animal which died during the period of treatment or in the subsequent observation period of 4 weeks. Smears were made from the liver, lung, spleen, brain, and exudate (if present), and stained with methylene blue. In order to demonstrate the presence or absence of the carrier state, all animals which survived the observation period were killed by dislocation of the neck. The whole of the brain, half the spleen, and a portion of the liver about 0.75 in. square from the anterior edge were removed and ground with 2.5 ml. of saline containing 1,000 i.u. of penicillin and 100 i.u. of streptomycin/ml. After standing for ½ hr., the supernatant fluid and as many of the finer pieces of tissue as possible were aspirated into a syringe through a 20 S.W.G. needle. The fluid thus collected usually amounted to 2.0 ml., and one-half of it was inoculated intraperitoneally into each of 2 mice. If toxoplasms were present the inoculated mice died in 9 to 11 days. In early experiments a second passage was made from the mice which survived the first ; this procedure failed to reveal any further carriers.

Concentrations of Drugs.—Groups of 6 mice were fed for 3 days on a diet containing 1/2 TD of each of the drugs, and then bled under ether anaesthesia. The blood levels of sulphonamide and sulphone were estimated by the method of Bratton and Marshall (1939), as modified by Francis and Spinks (1950).

RESULTS

Toxicity.—The toxic doses were for sulphadimidine, 1.6 g./100 g. diet ; for dapsone, 200 mg./100 g. diet ; and for pyrimethamine, 50 mg./100 g. diet.

Duration of Treatment.—Four groups of 6 mice were treated with 1/2 TD of sulphadimidine for 1, 2, 3, and 4 weeks respectively. All of the mice in the first two groups, 3 of those in the third and only 1 of the fourth group relapsed after treatment was stopped. A comparable experiment with pyrimethamine showed that, if treatment lasted 1 or 2 weeks, all the animals relapsed ; whereas, if treated for 4 weeks, very few relapsed. As a result of these preliminary trials, it was decided that in further experiments the drugs would be given for 4 weeks. Mice which relapsed did so most frequently in the second and third weeks after cessation of treatment.

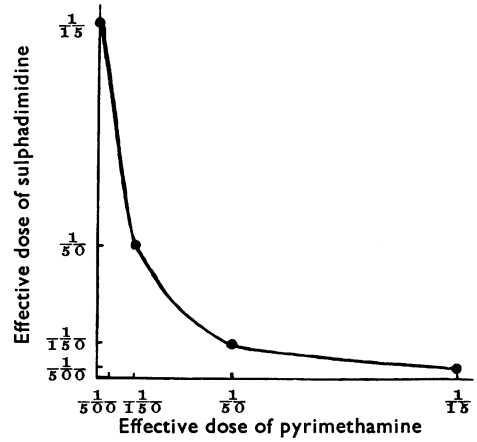

FIG. 1.—Synergic effect of sulphadimidine and pyrimethamine in causing survival of mice infected with *Toxoplasma gondii* for 28 days. The effective dose is expressed in terms of a fraction of the toxic dose. Definition of these terms may be found in the methods.

Optimum Ratios of Drug Mixtures.—Fig. 1 was constructed from the results of an experiment using various combinations of doses of sulphadimidine and pyrimethamine. It shows that the combination which produced an effect on toxoplasmosis with the least summation of toxicity was

1/100 TD of each compound. Of the 24 groups of mice, there were 4 in which all animals survived the 4-week period of treatment, but nearly all relapsed or were proved to be carriers. In a similar experiment using combinations of dapsone and pyrimethamine, 1/16 TD of each compound was found to be the optimum ratio for an effect comparable with that of the former experiment (Fig. 2). These experiments show that there is

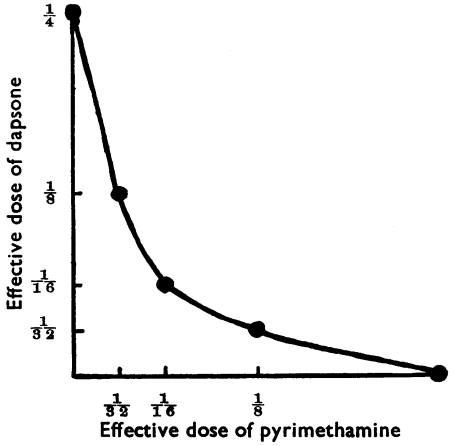

FIG. 2.—Synergic effect of dapsone and pyrimethamine in causing survival of mice infected with *Toxoplasma gondii* for 28 days. Doses, see Fig. 1.

synergy between pyrimethamine and either sulphadimidine or dapsone and that the optimum ratios of the drugs are equal fractions of their toxic doses. A third experiment showed that there was no synergy, but an additive effect between dapsone and sulphadimidine. From these preliminary results it was decided that in further experiments combinations of drugs would be used only in equal fractions of their toxic doses.

Effect of Drugs When Given Singly.—The results of giving the drugs alone are shown in Table I. Of 18 untreated control mice used in these experiments, all died within 6 to 9 days (average 7½).

Effect of Combinations of Drugs.—The results of giving the drugs in combinations are also shown in Table I. These experiments were performed at the same time and using the same controls as those in which the drugs were given singly.

At the end of the treatment period all the surviving mice were weighed. The gains in weight were more than those of normal uninoculated mice in all groups except for those receiving (*a*) 1/2 TD of each of sulphadimidine and pyrimethamine, (*b*) 1/2 TD of each of dapsone and pyrimethamine (these two groups gained less than normally), and (*c*) 1/4 TD or more of either sulphadimidine or pyrimethamine, (*d*) 1/4 TD of each of dapsone and pyrimethamine, (*e*) 1/2 TD each of dapsone and sulphadimidine (these three groups gained normally). There did not appear to be any synergy of toxicity.

TABLE I

RESULTS OF TREATMENT OF TOXOPLASMOSIS IN MICE WITH DRUGS GIVEN SINGLY AND IN PAIRS

See Methods for the definition of Toxic Dose (TD). The column headed Response gives the number of mice out of the groups of 6 which were completely cured (A), which survived 4 weeks (B) and which survived 1 week longer than the controls (C). The numerals in brackets give the number of survivors in each group which were subsequently found to be carriers.

Drug	Dose (Fraction of TD)	Response		
		A	B	C
Sulphadimidine ..	1/2	5	6	6
	1/4	2	6	6
	1/8	4	6	6
	1/16	1	6	6
	1/32	4	6	6
	1/64	0 (2)	2	5
	1/128	0	0	0
Pyrimethamine ..	1/2	6	6	6
	1/4	0 (2)	6	6
	1/8	0	0	3
	1/16	0	0	0
Dapsone	1/2	0	6	6
	1/4	1 (3)	6	6
	1/8	0 (1)	1	4
	1/16	0	0	0
Sulphadimidine + pyrimethamine ..	1/2	6	6	6
	1/4	5	6	6
	1/8	6	6	6
	1/16	6	6	6
	1/32	6	6	6
	1/64	3	6	6
	1/128	3 (1)	4	5
	1/256	0	0	0
Dapsone + pyrimethamine ..	1/2	4	6	6
	1/4	4	6	6
	1/8	6	6	6
	1/16	2 (1)	6	6
	1/32	2 (2)	5	6
Sulphadimidine + dapsone ..	1/2	3	6	6
	1/4	4	6	6
	1/8	3	6	6
	1/16	4	6	6
	1/32	1	6	6
	1/64	0 (5)	5	5
	1/128	0	0	2
	1/256	0	0	0

TABLE II

RESULTS OF DELAYED TREATMENT OF TOXOPLASMOSIS IN MICE WITH 1/4 TOXIC DOSE OF EACH OF SULPHADIMIDINE AND PYRIMETHAMINE

See Methods for definition of Toxic Dose. Response recorded as in Table I.

Interval between Inoculation and Start of Treatment (Days)	Response		
	A	B	C
0	6	6	6
1	6	6	6
2	2	4	6
3	3 (1)	4	5
4	0	0	1

Delayed Treatment with Sulphadimidine and Pyrimethamine Combined.—The results of treatment with 1/4 TD of each of sulphadimidine and pyrimethamine commencing at different times after inoculation are shown in Table II. Six untreated control mice died within 6 to 9 days (average 6½). Most of those mice whose treatment was delayed until 96 hr. were already sick and ate little of the medicated diet.

Autopsy Findings.—In mice which died within 6 to 9 days, organisms were usually most abundant in the lungs; in those which died within 10 to 12 days, in the liver; and those which died within 13 to 14 days, in the spleen. In mice which died still later, toxoplasms were found most easily in the brain. In mice which relapsed after treatment had ceased, there was a similar distribution of parasites in their organs according to the time of their death.

Blood Concentrations of Drugs.—After 3 days of treatment with diet containing 1/2 TD of drug, the concentrations of sulphadimidine were 214, 166, 208, 220, 192, and 164 μg./ml. of blood (average, 194 μg./ml.) and those of dapsone were 4.1, 6.4, 3.4, 3.2, 4.0, and 4.6 μg./ml. of blood (average, 4.3 μg./ml.). Attempts were made to assay pyrimethamine in the blood by the technique of Schmidt, Hughes and Schmidt (1953), but the method was not sufficiently sensitive.

Discussion

From the results it would appear that sulphadimidine alone is more active than either pyrimethamine alone or dapsone alone against the acute stages of toxoplasmosis in mice.

For the prevention of death during the acute stage of the infection there does not appear to be any advantage in combining pyrimethamine either with dapsone or sulphadimidine, but for the prevention of chronic infection a combination of sulphadimidine with pyrimethamine is much superior to the other combinations tried, or to any drug alone. This combination is active if withheld until infection is well established.

There is definite synergy between sulphadimidine and pyrimethamine and this is most marked if the criterion of effectiveness is a complete elimination of infection. There is also synergy between dapsone and pyrimethamine, less marked than with the former pair of drugs when considering complete cures, but more marked when considering treatment of the acute stages.

Nearly all the carriers (Tables I and II) were found in groups of mice which had been given only just sufficient drug to prevent death during the treatment period. It is possible that in these animals the infection was only partially controlled and that sufficient multiplication of organisms took place to stimulate active immunity which prevented relapse when treatment ceased.

Mice which had received larger doses of drugs and which subsequently relapsed did not become carriers, but died. This might be because during the treatment period the toxoplasms were present either in numbers too small to act as an antigenic stimulus, or in a form such as a terminal colony or a cyst which may be incapable of acting as an antigen. On stopping treatment proliferation would occur. Synergy between pyrimethamine and either sulphadimidine or dapsone may be connected with the fact that, whilst sulphadimidine and dapsone are competitors of *p*-aminobenzoic acid, pyrimethamine is a competitor of folic acid (Hitchings, 1952). If toxoplasms normally synthesize their own folic acid, one would expect drugs of the sulphonamide type to act against them. These drugs may not, however, be effective either in preventing the formation of cysts or in eliminating organisms already in cysts. In this phase there is no active growth and the organisms presumably contain sufficient folic acid for their reduced metabolism. Consequently, in an animal given dapsone or sulphadimidine alone, not all of the parasites would be eradicated and, if immunity had not been acquired in the meantime, relapses would occur on cessation of treatment. However, since it is possible that pyrimethamine can interfere with the metabolic effects of folic acid whether synthesized by the parasite or absorbed from the tissue fluids of the host, the combination of dapsone or sulphadimidine with pyrimethamine could lead to the complete eradication of the parasite. The reason why such a large dose of pyrimethamine is required when given alone may be that toxoplasma synthesize so much folic acid that an effective concentration of the drug is one which is near the level toxic to the host. If the rate of synthesis of folic acid by the parasite is reduced by a sulphonamide drug, less pyrimethamine is required.

We suggest that sulphadimidine interferes with the synthesis of folic acid in toxoplasma and that it may be especially effective against the proliferative forms found in acute infections. It may not, however, be able to prevent the development of cysts or affect the relatively inactive parasites in terminal colonies or cysts. It is suggested that the latter may rely on the slow metabolism of their stores of folic acid and that pyrimethamine may

interfere with such metabolism and thus prevent subsequent relapse or development of the carrier state. Pyrimethamine may even prevent the development of terminal colonies and cysts.

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