Brit. J. Pharmacol. (1958), 13, 458.

CHEMOTHERAPEUTIC SCREENING OF COMPOUNDS AGAINST *THEILERIA ANNULATA* IN TISSUE CULTURE

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(RECEIVED JULY 30, 1958)

Theileria annulata has been grown in tissue culture and these cultures have been used for screening compounds for chemotherapeutic activity against this parasite. The method described is simple and convenient for testing many compounds in a short time. Forty compounds, including antimalarials, trypanocides, and antibiotics, have been examined, but none has been found to be active.

A technique is described for screening of compounds for therapeutic activity against *Theileria annulata* growing in tissue culture. Preliminary results with compounds are reported; no activity was discovered in the present series.

METHODS

Tissue samples of lymph nodes, spleen, and liver containing *Theileria annulata* were kindly sent to me by air by Dr. I. Tsur of the Veterinary Institute, Tel Aviv, Israel. They were packed in tubes containing Hanks' or Earle's solution (both seemed equally good). When smeared, the tissues usually showed 1 to 10 Koch bodies/oil immersion microscope field. On arrival at this laboratory, they were washed in a solution containing penicillin (100 units/ml.) and streptomycin (100 units/ml.). The tissue was then cut into implants which were set up in Carrel flasks by the technique decribed by Brocklesby and Hawking (1958) which was adapted from that originally described by Tsur (Tchernomoretz), 1945. Briefly implants were fastened by a plasma clot to small coverslips (0.7 cm.^{2}) glued to the floor of a Carrel flask with plasma. The fluid medium consisted either of calf serum 40%, embryo extract 2.5%, and medium 199 57.5% (Morton, Morgan, and Parker, 1956); or of calf serum 40%, embryo extract 2.5%, tyrode 57%, phenol red to colour, and 0.5% of a solution containing glutamine 60 mg., pyridoxine 12 mg., pyridoxal 10 mg., riboflavin 2 mg., inositol 80 mg., water 100 ml. The latter medium is referred to here as Tsur's medium, but in fact differs from Tsur's original medium in some minor details and in that it contains embryo extract. Both media contained penicillin 50 units/ml. and streptomycin 50 units/ml.

Growth of cells and of parasites was somewhat irregular, but there was no constant difference between the two media. Spleen tissue grew more vigorously than lymph node, but lymph node, which usually contained more parasites initially, often gave a better growth of parasites; implants of liver did not yield significant growth. After 2 to 4 days, when it was established that growth of cells was taking place, the cultures were prepared for chemotherapeutic testing in two alternative ways. In one method, the coverslips carrying the cultures had been set up in large Carrel flasks (8 cm. diameter) each containing 20 slips. In this case the slips were removed and were placed individually in test tubes (15 ml.) each containing 2 ml. of fresh medium. In the other method, the slips were set up in small flasks (3 cm. diameter), each flask containing 3 slips; in this case the old medium was removed and 3 ml. of new medium was inserted. Drugs dissolved in Ringer solution were then added to the tubes or small flasks (0.1 ml. to 2 ml. or 0.15 ml. to 3 ml.). The concentrations of drugs were arranged in steps of $\times 3.2(\sqrt{10})$. The tubes were placed on a roller machine at 36° and the flasks were incubated at the same temperature. After 5 to 6 days slips were removed and prepared for examination. The exact times in the above scheme can be varied according to experience and to the acidity of the medium; if the flasks became yellow too soon, 1 to 4 drops of 1.3% sodium bicarbonate were added to neutralize the acidity. Probably the best results were obtained with a preliminary period of 2 days and exposure to drug for 5 days.

For examination, the slip was removed from the tube or flask. The centre of the implant was picked off with fine forceps and dabbed on a slide to make a series of small smears. This slide was later dried, fixed in alcohol, and stained with Giemsa. The slip with the outgrowth from the implant was dropped into methyl alcohol for fixation, stained with Giemsa, dehydrated by drying in the incubator, and mounted culture downwards with any of the customary mountants. The growth of the parasites, which varied considerably even in the control tubes without

drug, was patchy and unevenly distributed. Some smears showed five or more parasites (Koch bodies)/oil immersion field; others showed only one or two parasites during a 10 min. search. Examination of the smears was usually more rewarding than examination of the cultures fixed *in situ*; parasites were difficult to find in the cultures unless many were present. On the other hand, the cultures were better than the smears for showing the reaction of the cells to the drug. In view of this uneven growth, it is necessary to examine each concentration of drug several times or in several experiments. If well-preserved parasites were discovered, it was concluded that the drug had not destroyed them. If no parasites were discovered, although the cells seemed relatively healthy no conclusion was drawn. The finding of healthy parasites in two separate experiments was considered evidence that the drug failed to act. Demonstration of an active compound (which did not occur during the present work) would depend upon the consistent absence of parasites from cells which remained healthy.

TABLE I

COMPOUNDS TESTED

The values in this table give an approximate estimation of the maximum concentration tolerated by lymphocytes and macrophages.

Compound	mg./100 ml.
Antimalarials	
Quinine bisulphate (1 expt.)	? 1
Pamaquin	0·01–0·03
Pentaquin diphosphate	0·05
Rhodoquin dihydrochloride	0·03
Chloroquine phosphate	0·03
Sontoquin sulphate (1 expt.)	0·03–0·1
Mepacrine hydrochloride	0·2
Pyrimethamine	3–10
Diamidines	
Stilbamidine nitrate	0·3–1
Berenil	1
Sulphonamides, etc.	
Sulphadiazine	5
Dapsone	3
Maphenide (Marfanil)	3
Metachloridine, 2-(*m*-aminobenzenesulphonamido)-5-chloropyrimidine	3
Arsenicals	
Melarsoprol (Mel B; Arsobal)	0·1
Arsenamide sodium, p-NH_2.$CO.C_6H_4$.$As(S.CH_2.CO_2Na)_2$ (Otto and Maren, 1947; 1950)	0·1
Antimonials	
Stibophen	0·1
Antimony potassium tartrate (tartar emetic)	0·1
Trypanocides	
Suramin	5
Quinapyramine (Antrycide)	1
Prothidium (R.D. 2801) (Watkins and Woolfe, 1956)	0·1–0·3
Styryl 314, 2-(*p*-acetamidostyryl)-6-dimethylamino-quinoline methochloride (1 expt.)	?0·3
Bayer 7602 (Fulton, 1943)	1
1-Methyl-1-phenyldithiobiuret (RD 1660) (Woolfe, 1953)	f
Miscellaneous	
Lucanthone hydrochloride	0·1–0·3
Cyanine 863, 1′-ethyl-3 : 6-dimethyl-2-phenyl-4-pyrimidinyl-2′-cyanine (Welch, Peters, Bueding, Valk and Higash, 1947)	0·1–0·3
Methylene violet, 3-amino-7-dimethylaminophenosafranine (Hawking, Ormerod, Thurston and Webber, 1952)	0·1–0·3
Isoniazid	10–30
BIQ 20, eicosamethylenebis(*iso*quinolinium iodide) (Austin, Lunts, Potter and Taylor, 1959) (1 expt.)	0·1–0·3
BIQ 12, dodecamethylenebis(*iso*quinolinium iodide)	0·1
BAC 20, eicosamethylenebis(4-aminocinnolinium iodide)	0·1
3 : 4-Dimethyl-ω-piperidinoacetanilide hydrochloride (W 2)	1–3
1-(3 : 4-Xylylcarbamoylmethyl)pyridinium chloride (W 6)	3–10
N-β-Piperidinoethyl-3 : 4-xylidine dihydrochloride (W 55) (1 expt.)	3
Antibiotics	
Chlortetracycline (aureomycin)	10
Chloramphenicol	10
Neomycin	3
Viomycin sulphate	10
Bacitracin	10
Erythromycin (insoluble)	?10
Vulcamycin (insoluble)	?10

RESULTS

To test the screening technique and to make a preliminary survey of the chemotherapeutic reactions of *T. annulata*, a series of compounds was tested (Table I). A range of concentrations of each drug was employed, the highest concentration being sufficient to kill or severely injure the host cells. The values given in Table I give an estimate of the maximum concentration (mg./100 ml.) tolerated by the cells (lymphocytes and macrophages) without great degeneration. None of the 40 compounds showed activity in preventing the survival of Koch bodies for 5 to 6 days. In view of reports suggesting possible activity in cattle, particular attention was paid to aureomycin, to the 8-aminoquinoline compounds (pamaquin, pentaquine and Rhodoquin) and to mepacrine, but these compounds were not effective in our cultures. Penicillin and streptomycin (which were present in all the culture media) did not prevent growth of parasites.

DISCUSSION

The above technique provides a method for laboratory screening of compounds for chemotherapeutic activity upon *Theileria annulata*. Provided a source of infected tissue is available, it is simple and convenient; over a hundred drug concentrations or duplicates can be easily examined in a single experiment. Like all *in vitro* tests, it might fail to indicate compounds the activity of which depended upon co-operation of the tissues of the host; but, since some host cells are present in the cultures, these might be sufficient for many of such compounds. Theoretically, the activity of some compounds might depend upon an antimetabolite effect; accordingly the sensitivity of the test would probably be improved if a medium less rich in growth stimulants than medium 199 was used; for example, Tsur's medium or simple Tyrode solution. The method examines for activity against *T. annulata*, the Middle East species of theileria. It is not known

whether *T. parva,* the East African parasite, differs from *T. annulata* in its chemotherapeutic reactions since no highly effective drug is yet known for either of them. Growth of *T. parva* in tissue culture is still unsatisfactory (Tsur, Neitz and Pols, 1957 ; Brocklesby and Hawking, 1958) ; until this position has been remedied, *T. annulata* would appear to be the best organism for laboratory tests. *T. mutans* (English strain) has been examined in our laboratory, but no Koch bodies could ever be found by gland puncture.

The experimental results described above failed to reveal therapeutic activity in any of the 40 compounds studied. This agrees broadly with experience in the field ; although many compounds have been claimed to be curative for *T. annulata,* none of these claims has received general acceptance. The number of compounds tested so far on this infection is still, however, small, and further systematic screening might be expected to reveal activity in due course.

Grateful acknowledgments are due to Dr. I. Tsur for the supply of infected tissue, and to Mr. K. Gammage for technical assistance.

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