Preliminary Report on Drug Research Involving *Acanthamoeba* and *Naegleria*

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The following is a preliminary report of work done with the assistance of Dr. Robert Neff, Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee. These are broad range dilution studies intended to show which drugs, and what dilutions, to investigate further. Most drugs were chosen based on reported anti-amoebic or anti-protozoal efficacy.

The following information was presented by Tony Chapdelaine, B.A., now M.D., at The Rheumatoid Disease Foundation's 1985 Seminar July 18, 1985

A series of drug tests was performed on two species of the genus *Acanthamoeba. Acanthamoeba castellanni* (Neff strain) and the pathogenic *Acanthamoeba culberrtsoni,* called A5, received from Ann Stevens who obtained it from Dr. Clyde Culbertson, the man who isolated it from contaminated tissue cultures in the late fifties.

The doubling time for a growing culture of this strain of *A. castellanni is* about 14 hours, while that of A. *culbertsoni is* about 25 hours.

Solvent dilution tests were first performed with both species. A final 0.8 molar (6.25%) solution was the highest concentration aad after twofold dilutions O.OO625 molar (8 one-thousandths of a percent) the lowest for both DMSO and DMF (dimethylformamide).

The tubes of amoebae were counted each 24 hours for 4 days and observations made of any morphological changes, such as clumping of cells or excystment. The A5 strain was not affected by a final concentration as high as 0.78% DMSO and 0.39% DMF whereas the maximum for the Neff strain was0.39% DMSO and 0.195% DMF.

A maximum of 0.195% DMSO and DMF was subsequently used for solubilizing drugs which were not soluble in water.

Counts of anoebae were made in counting chambers on microscope slides with ruled grid lines, each charriber being one-tenth mm deep and 0.4 microliters (one-tenth cubic mm). Each slide contains two of these chambers.

A concentration of one hundred thouiand cells per milliter was chosen since cells needed to be in the log phase of growth. This number represents a good sample for counting under the microscope and also avoids the problem of older cultures containing a mixture of dividing and non-dividing or encysting cells, which would make it difficult to know whether a drug was stopping growth.

A cell-counter or haemocytometer was not used because it is apt to count lysed cells and fragments of cells as well as viable whole cells, and does not allow one to observe important morphological changes in the amoebae as they oc**cur.**

The procedure was as follows:

Fifteen ml of grown; medium containing peptone-protease and glucose was placed into a 150 ml flask. From a tube containing one ml of amoebae sufficient cells were withdrawn and then inoculated into the 150 ml flask to provide approximately 100,000 cells per ml after a few days growth. Three to four drops of penicillin and streptomycin were added to discourage contamination by bacteria which tend to overgrow the medium and use up the oxygen. After carefully rotating the flask to remove the amoebae which cling to the bottom (by changing the surface tension), three quarter ml samples were pipetted into screw-cap tubes. Then one-quarter ml of the drug dilution, or in the case of controls either water or 0.78% DMSO or 0.78% DM F (which gives a final concentration of 0.195% to match that of the drugs) was added as appropriate. The tubes were gently shaken to mix the drug and growth medium and then placed into a horizontal position in a rack to provide sufficient oxygen diffusion.

The microscope used for counting is a phase-contrast type. Slight color changes tell several things about the state of the cell. Generally a magnification of 200 times was used for counting, although 400 times was used when the viability of the cell was in question or details and verification of cyst structure was desired. (Cysts contain cellulose and thus are birefringent. A microscope with birefringence filters was at times used to verify cells as encysted.)

After inoculating tlne tubes a count was made with a minimum of 3 separate samples taken from each tube, each sample representing a 0.4 microliter count. Often 4 or more samples were taken to provide a good statistical base. Samples were then counted approximately each 24 hours for 72 to 96 hours.

The concentration of cells per ml was calculated and a standard deviation made for each count. A graph showing the growth curve for both species and each drug dilution and control was made with the standard deviation shown. Separate sheets were prepared with comments on morphological changes, especially clumping and encystment.

The drugs tested which showed little or no effect on growth or morphology, and the highest drug concentration (which was sometimes limited by the requirement not to go beyond the initial 0.78% concentration of DMSO or DMF in preparing the dilution) are as follows:

Sulfamethoxazole 49.3 micrograms/ ml (David Casemore had only an inhibitory effect at 100 micrograms/ml when used with Trimethoprim)

Trimethoprim 113.1 micrograms/ ml.

Copper Aspirinate 41 micrograms/ ml,

Sulfadiazine 680 micrograms/ml. Ve:y slight inhibition of growth for A5. Casemore had a slight inhibition effect with it at 100 micrograms/ ml and mentions that Dr. Culberston used it with good protective results in mice experimentally infected with the pathogenic strain of A. *culbertsoni.* Ann Stevens showed it has no effect)n various species of *Acanthcmoeba* at 100 micrograms/ml.

Chloroquine diphosphate 1290 micrograms/ ml had a very slight inhibitory effect. 12,900 micrograms/ ml killed them within 24 hours but this represents a 25 rnillimolar concentration and osmotic effects are the likely reason. Casemore had slight unspecified morphological changes at 100 micrograms/ml.

Metronidazole 428 micrograms/ml. Casemore shawed variable results at 100 micrograms/ml. Prasad showed no effect at 1000 micrograms/ mil. The mechanism with other protozoa involves interference with electron transfer in the pyruvate phosphoroclastic reaction involving reduced ferredoxin. In effect its nitro group is reduced and it acts as an electron sink. Since *Acanthamoeba* have an alternate pathway available in their electron transport system, metronidazole will have a very limited effect on this genus.

Allopurinol 250 micrograms/ml. There was an inhibitory effect on A5 only.

Chenodeoxycholic acid 306.5 micrograms/ ml had a slightly inhibitory effect.

Dimetridazole 275 micrograms/rnl had no effect.

Niridazole 83.5 micrograms/ml had no effect. Also, it is a potent, long-lasting suppressor of cell-mediated immunity.

Idoquinol or Diiodohydroxyquin 38.8 micrograms/ml had no effect. I was unable to obtain a higher concentration and do not know if an effect would be seen.

Diphenylhydantoin 6,850 micrograms/ml showed an initial slight inhibition which disappeared after thirty hours. This is a 25 millimolar concentration.

Tlie drugs which nave a pronounced effect are:

Copper Sulfate 400 micrograms/ml was amoebicidal for both species and at 40 micrograms/ml inhibitory for A5 for 48 hours but not afterwards.

Ornidazole 1,372 micrograms/mil was inhibitory for both species but caused 17% encystment, for the Neff strain. Cells were generally small indicating blocked protein synthesis. The highest concentration (50 millimolar or 2,745 micrograms/ rnl) was amoebicidal, probably an osmotic effect. At 549 micrograms/ml no effect was seen.

Paromomycin sulfate is an antibiotic aminoglycoside of the neomycin group which acts directly on amobae and is also antibacterial to normal and pathogenic microorganisms in the gut. Little of the drug is absorbed into systemic circulation. It is ineffective against E. *histolytica* outside the gut. Hound that at 90 micrograms/ml it was inhibitory for the first 48 hours and amoebicidal afterwards for both species. Concentrations lower than 90 have not been tried yet, but Casemore showed A. *castellanni* inhibited at 100 micrograms/ml and .Jones showed it to be amoebicidal at 12.5 micrograms/ml and inhibitory at 5 micrograms/ml for A. *polyphaga.* There is probably some blockage of protein synthesis.

Rifampin or rifamycin is a marcrocyclic antibiotic. After 96 hours at 321 micrograms/ml, slight inhibition was seen with 3 to 5% encystment and at 80 micrograms/ml no inhibition occured but 19% encystment was induced for the Neff strain, inhibition for the A5 occured at 321 micrograms/ml. Peak plasma concentrations of 7 micrograms/ml and a half-life of 1.5 to 5 hours occur for this drug. Some immunosuppression has been shown in animal models (related to its inhibition of protein synthesis by cells in the immune process). When used alone for tuberculosis it induces rapid resistance. There are many minor side effects possible but they are supposedly infrequent.

5-flurocytosine or flucytosine at 3 micrograms/ml up to 30 micrograms/ml showed inhibition for the Neff strain. Most cells were shrunken, but encystment occured at 30 and 300 micrograms/ml concentrations, the 300 micrograms/ml being amoebicidal after 48 hours. Tnhibition for the A5 required a minimum of 15 micrograms/ml and 300 micrograms/ml was inhibitory but not amoebicidal. Clumping showed evidence for pre-encystment for A5. Casemore showed flucytosine to be inhibitory at 12.5 micrograms/ml and amoebicidal at 100 micrograms/ml. Stevens showed the Neff strain to be killed at 40 micrograms/ml and the *A. culbertsoni* at 10 micrograms/ml.

Flucytosine is a nucleotide analogue and is antifungal. Peak plasma levels reach 70 to 80 micrograms/ml with a half-life of 3 to 6 hours. Bone marrow functions may be depressed with anemia, leukopenia and thrombocytopenia, usually in patients with underlying hematological disorder or undergoing radiation treatment or drugs that injure bone marrow. Five percent of patients have heptomegaly or elevation of hepatic enzymes in the plasma which is reversible when therapy is stopped. All these complications are more frequent in patients with azotemia or when plasma levels of the drug reach 100 to 125 micrograms/ml. Some caution that this may not be a desireable drug due to its potential toxicity.

Clotrimazole is chemically similar to another synthetic imidazole, miconazole, being antifungal and antibacterial. According to Sawyer et al, like miconazole and Amphotericin B it is preferentially bound to the cell membrane where it probably interacts with the phospholipid bilayer altering membrane permeability, resulting in a loss of various precursors, metabolites and ions and thus inhibits macromolecular synthesis. Very limited resistance to Clotrimazole has been observed. Serum levels after oral adminisrtation reach a mean peak of 1.29 micrograms/ml, with about 0.3 micrograms/ml mean serum level after continued 100 milligram oral doses.

Low Toxicity, with gastro-intestinal disturbances are the most frequent complaint after oral administration. 67.2 micrograms/ml was amoebicidal after 24 hours for both species. Some shrinkage occured and encystment for the Neff strain was about 33% after 48 hours. At 6.7 and 0.67 micrograms/ml the amoebae were inhibited with shrinkage and some encystment. For the A5, 6.7 micrograms/ml showed inhibition, with the 0.67 micrograms/ml showing slight inhibition after 24 hours. There is a rounding which indicates possible preencystment at the 67 micrograms/ml dose. Duma showed no effect for Acanthamoeba at 100 micrograms/ml, but Jones showed inhibition at 12.5 micrograms/ml and an amoebicidal effect at 25 micrograms/ml, while Stevens showed that 0.5 to 5.0 micrograms/ml was amoebicidal for *A. castellanni, A. culbertsoni* and two other Acanthamoeba species. The cells remaining were cysts which were viable on subculture to fresh medium.

Naefleria according to Duma are inhibited or killed at 0.39 to 39 micrograms/ml depending on the strain, while Jamieson showed Clotrimazole was amoebicidal to *N. fowleri* at 0.12 to 1.0 micrograms/ml.

Discussion

Different researchers unfortunately use differing testing techniques and differing definitions of "inhibition" and "amoebicidal" so it is not always practical or wise to use the current literature data, other than to get a "ballpark" idea of a drug's potency. However, it is clear that Acanthamoebae are much more resistant to the drugs that have been tested than Naegleria. Also, in vitro tests with amoebae do not always project what happens in vivo, Culbertson's sulfadiazine/mouse challenge being a good example. Even the two similar Acanthamoebae used in this study show widely varying responses to the same drugs. On the other hand, drugs which have been shown to be effective in vitro against amoebae thus far have been effective also in vivo.

Clotrimazole shows the best results at low dosages against both *Acanthamoebae* and *Naegleria.* Amphotericin B shows some promise if the dosage can be lowered through synergism with one of the protein blockers, otherwise its use is limited to cases of primary amoebic meningoencephalitis.

Further testing needs to be done on these two drugs to see if the other drugs showing promise (Paromomycin sulfate, rifampin, flucytosine and perhaps some others) will work synergistically, one drug altering membrane permeability, allowing the other to kill the amoebae. This would also allow a lower dosage and thus less toxicity for the host. One major difficulty which must be overcome is encystment. We hope to find an effective drug or combination of drugs which do not also induce differentiation or encystmcnt.

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