IFAR'S 2004 SMALL GRANT PROGRAM

Final report -first draft

A) TITLE:
FURTHER CHARECTERIZATION OF EFFICACY AND SAFETY OF MEDICINAL PLANT EXTRACTS USED TO TREAT EAST COAST FEVER (THEILERIA PARVA) INFECTIONS IN CATTLE

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BACKGROUND:
East coast fever (ECF) causes major limitation to livestock production due to deaths or loss of productions of infected cattle (Mukhebi et al 1991). Although there are standard drugs for the disease, these are costly and not always accessible due to high cost, yet, delayed treatment is often unsuccessful. The available vaccination option, the infection and treatment method, is not widely adopted due to limitations such as cold chain requirements during administration and the need for close monitoring of vaccinated animal (Morzaria and Nene 1990). Research on recombinant vaccine is in progress and when available its rate of adoption will depend on several factors such as cost and availability.

Alternative treatment methods mainly based on medicinal plants are often used in ECF endemic area in Africa, to alleviate the clinical symptoms and minimize the suffering. However, the efficacy and safety of these treatments is not determined for many plants due to limitations in research capacity in the regions, yet the search for new drugs from natural sources is increasing globally (WHO 2002), quite often using plant resources from tropical regions.

We have previously identified some plant extracts that show *in vitro* activity on *T. parva*. We propose to further characterize the efficacy and safety of these extracts, by testing their effects on viability of non infected host cells, at concentrations that are active on the parasite. We also propose to demonstrate the changes in *T. parva* genomic expression profile after exposure to active extract dilutions, in order to identify target genes.

The aim of this work was to enhance our capacity to undertake this research through training at ILRI on *in vitro* bioassay methods and on molecular techniques needed in the project.

Objectives

The grant was implemented with the following aims and objective:
To support the applicant train on *in vitro* drug assays and molecular techniques for further characterization of plant extracts already shown to have consistent activity against *T. parva in vitro*.

The training was based on laboratory procedures on *in vitro* cultures and drug assay methods and application of the acquired skills to test candidate plant extracts. Since this was a specialized training with specific targets, the initial work plan was set out such that training on *in vitro* assay methods preceded the molecular aspects, so that only those extracts that are non toxic to host cells were selected for genomic work.

The work plan was therefore divided in the following phases:

A. Acquire skills on *in vitro* propagation of *T. parva* infected cells (TPM) and non infected lymphocytes (F100 Con A blasts) and evaluate whether the concentrations of plant extracts with highest activity on the parasite were non- toxic to non infected lymphocytes.
B. Characterize the effects of non-toxic active extract on the genomic expression profile of *T. parva* in an attempt to identify target genes.
C. Data analysis and submission of final report.

METHODS

Samples of the plants earlier identified to have activity on *T. parva* were obtained from the field with the help of community groups already working in the project. Polar and non polar extracts were prepared and coded to ensure blind testing (the results on each extract were based on these codes). Training on *in vitro* methods of propagation of both infected and non infected lymphocytes was conducted from June 2004 to October 2004. The training was based on practical attachment in a busy tissue culture laboratory where hands on skills on *in vitro* culture methods and drug assay protocols were acquired. Procedures on the set up and practices of a standard tissue culture laboratory were learnt including preparation of reagents, media, serum, cryopreservation, resuscitation of stabilates, establishment and maintenance of cell cultures and other routine practices in a tissue culture laboratory. Protocols on cultivation of *T. parva* infected (TPM) and non infected lymphocytes (F100 ConA blasts) were learnt in the training and then applied on bioassay of selected plant extracts.

Using *in vitro* methods (Malmquist, Nyindo and Brown 1970, Brown 1979, Spooner 1990), plant extracts were monitored daily for at least three days for effects on infectivity of the parasite (parasitosis) and the viability of the infected lymphoblastoid cells in culture. For each extract, 3 to 4 logarithmic dosage levels were mixed with culture medium and incubated at 37C in 5% CO_2. Controls for each extract included appropriately diluted solvent and solvent-free sets. The effects of the extracts on the parasite and the host cells were determined in cell lines from different animals and in animal-matched infected and non–infected cells. Infectivity of the parasite and viability of the host cells was determined daily by counting the number of the viable and the non-viable cells and determining the percentage of the viable cells containing the parasites

(percent parasitosis). Viability (cell growth) was expressed as per cent control growth and as percent survival rate. Prior to testing, each extract was adequately dissolved in a solvent that was compatible with the culture medium Dimethylsulfoxide (DMSO) or Dimethylformamide (DMFO) for organic extracts or sterile de-ionized-triple distilled water for aqueous extracts. Some extracts were tested at repeated times, either due to extreme toxicity of the initial trial dose ranges to the cultured cells or due to unexpected reactions such as precipitation of the dissolved extract in culture medium during incubation.

Extracts were ranked for activity on the parasite and toxicity to infected and non infected host cells based on comparison of the mean changes in parasitosis and viability of host cells from day 1 to day 3 in SPSS statistical package. The most suitable extract was the one combining a reduction in parasitosis and increased viability of the non infected host cells. The most active and least toxic extract (a non polar extract) was fractionated in a column, eluted with different solvent systems. Seven fractions were tested in order to locate the active fraction. Due the to the intensity of *in vitro* bioassay of plant extracts and some unexpected outcomes of the tests when cell lines from different animals were used and some unforeseen reactions after extracts were mixed with culture medium, some experiments had to be repeated more than once with commensurate use consumables. The *in vitro* bioassay component of the training took longer than was originally planned necessitating a readjustment of the work-plan by replacing the genomic component of the training with *in vitro* tests.

DATA ANALYSIS

The data was analyzed using SPSS statistical software. The summary statistics on the parasitosis and viability were carried out using summary of groups (Tabulation procedure) with Experiment, Extract and Concentration, Time (Day) and Cell Type as classification variables. Thereafter, ranking of differences in percent parasitosis and cell viability day 1 to day 3 was used to select the extracts with highest activity on the parasite and best viability of host cell.

RESULTS

Infectivity of *T. parva* (parasitosis) and the viability of infected cells varied between extracts. Based on the changes in cell line TPM H12 which was the prototype cell line used in the initial screening, extract 13SBC/N produced the highest reduction in parasite infectivity (negative change in parasitosis days 1 to 3) and good viability of infected host cells (positive change in viability days 1 to 3) (Table 1). A comparison of infectivity and viability changes, at different extract concentrations showed that extract 13SBC/N at a concentration of 10µg/ml, caused the best reduction in parasitosis and highest viability of the infected cells (Table 2).

Using cell lines from different animals including the animal-matched infected and non infected cells (cell linesTPMBW13 and CONBW13 were infected and non infected cells respectively from the same animal. The same was true for TPMBX215 and CONBX215), it was observed that those extracts that caused a reduction in parasite infectivity also produced varying changes in viability of infected cells and non infected cells. Infected cell lines had lower viability than the non infected cells at the conscripted extract

concentration. Extract 13SBC/N concentration10µg/ml, produced the highest reduction in parasitosis and the highest viability in matched infected and non infected cell lines respectively in two animals (Table 3).

Based on its capacity to reduce infectivity of the parasite and retain good viability of the non infected host cells in different animals, extract 13SBC/N was selected for more evaluation. Disregarding its effect on viability of different infected cell lines, this extract caused a time dependent reduction in infectivity of the parasite in cell lines from three different animals -TPMBW13, TPMBX215 and TPMH12 (Fig 1). However, the reduction in parasite infectivity at this extract dilution was accompanied by a corresponding decline in viability of infected cell lines, a feature that was best illustrated by responses in cell line TPMH12 (Fig 2). The decline in viability of infected cell lines caused by this extract dilution, varied between animals, the lowest viability occurring in infected cell line TPMBX215 (Table 4). Titration of activity of this extract at lower dilutions showed that concentrations of 5ug/ml supported cell growth and significantly reduced parasite infectivity in TPMBX215 (Table 3).

A comparison of the viability of infected and non infected cells from the same animal showed that extract 13SBC/N concentration 10ug/ml supported a higher viability of non infected cell lines than for infected cells from the same animals; the viability of non infected cells was more than 60 percent that of the controls by day 3 when that of infected cell lines from the same animal (BX215) was lower than 10 per cent by day 1(Fig 3, Table 4), cell lines TPMBX215 versus CONBX215).
Preliminary tests on fractions of 13SBC/N using cell line TPMH12 (Tables 1, 2 & 3) showed two fractions (F2 and F3) that caused a moderate reduction in parasite infectivity. This effect will be examined in other infected and non infected cell lines.

Discussion
These results have confirmed the activity of different plant extract concentrations on *T. parva* in different cell lines. This activity was ranked against the viability of infected and non infected cell lines (Table 3). Although many extract dilutions that caused a reduction in parasite infectivity also caused a reduction in viability of the infected host cells, their corresponding effect on viability of non infected cells was much higher. The work underscores the fact that evaluation of toxicity of host cells at effective extract concentrations should be based on viability changes of non infected cells; low viability in infected cell lines probably being an outcome of combined factors in the extract and the cell infection. Extract concentrations causing the highest reduction in parasite infectivity and highest viability of non infected cells are the most desirable.

Extract 13SBC/N concentration 10ug/ml was therefore selected for further work on this basis and the fact it repeated these changes in cell lines from three different animals (Fig 1), two of which were animal-matched infected and non-infected lines (Fig 3 and Table 4). Fractions of this extracts are therefore being tested for activity in prototype cell line, TPM H12 and preliminary data shows that the activity is located within fractions F2 and F3 tables 1, 2&3).

It is expected that fractionation of extract 13SBC/N will yield pure compounds that can be further developed to ethno-therapeutic products. Even if the active compounds might

proof difficult to isolate, the refined fractions that will replicate the activity in experimentally infected animals can be formulated for clinical use, as long as they will be non toxic to laboratory animals. This will form the basis of future work if the project will attract more funds

Selective toxicity of effective extract concentrations observed in infected than in non infected cells from the same animal suggests that, if the *in vitro* activity will be replicated in experimentally infected animals, then a more enhanced resolution of clinical infection will occur, as the parasite and the infected host cells are eliminated in a sick animal. Selective toxicity of effective extracts to infected cells is an interesting observation that can be extended to anticancer research, since *T. parva* infected cells are cancer-like transformed cells.

OUTPUTS

Skills acquired in this training on *in vitro* bioassay methods will enhance the research capacity in evaluation of ethno-therapeutic value of plant extracts used against ECF. The applied component of the training has established a criterion for raking the efficacy and safety of plant extracts on the basis of parasite infectivity drop in infected cells and increased viability of non infected cells. Extract 13SBC/N has been identified for further work on this basis in cell lines from different animals and preliminary work on its fraction bioassays in progress.

PROBLEMS

Unexpected reaction of the extracts and culture media for non infected cell occurred at the initial phase of the training, necessitating a repeat of some bioassay tests and refinement of the methodology. Responses of infected cell lines from different animals to extract dilutions earlier selected in prototype cell were initially unpredictable, calling for titration of extracts at lower dilutions. Thus, more time than was anticipated in the work plan, in the in vitro bioassay training.

CHANGES

The unexpected reactions of the extracts in the *in vitro* bioassay procedures at the early phase of the training resulted in use of more material than was budgeted for. This caused a modification of the previous work plan to fit with resources and time available. The training was therefore based on in vitro bioassay methods only.

LESSONS

Training on molecular aspects should have been left out from the original proposal and applied for as a separate study. The initial assumptions were that *in vitro* work would be short, quickly yielding results to be used in genomic training. The reactions of plant extracts in vitro bioassay methods at the initial phase were unforeseen

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Acknowledgement
I sincerely thank the following: IFAR for financial support, Luka Juma, S. Kemei, Edward Okoth and John Nyanjui of ILRI for technical support *in vitro* work; J. Mwarukumbi of University of Nairobi for plant extraction; Nicholas Ndiwa of ILRI, for statistical analysis; ILRI and University of Nairobi for approving and supporting this training.

Signed----------

Date-------31-Dec.2004----------

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