RECENT DEVELOPMENTS
IN THE RESEARCH AND CONTROL
OF THEILERIA ANNULATA

PROCEEDINGS OF A WORKSHOP HELD AT ILRAD
NAIROBI, KENYA
17–19 SEPTEMBER 1990
The map on the front cover shows the distribution of the *Theileria annulata* parasite, the cause of tropical theileriosis, based on information given by workshop participants.
RECENT DEVELOPMENTS
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PROCEEDINGS OF A WORKSHOP HELD AT ILRAD
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Edited by
T.T. Dolan

THE INTERNATIONAL LABORATORY FOR RESEARCH ON ANIMAL DISEASES
BOX 30709 • NAIROBI • KENYA
The International Laboratory for Research on Animal Diseases (ILRAD) was established in 1973 with a global mandate to develop effective control measures for livestock diseases that seriously limit world food production. ILRAD's research program focuses on animal trypanosomiasis and tick-borne diseases, particularly theileriosis (East Coast fever).

ILRAD is one of 18 centres in a worldwide agricultural research network sponsored by the Consultative Group on International Agricultural Research. In 1992 ILRAD received funding from the African Development Bank, the Rockefeller Foundation, the United Nations Development Programme, the World Bank and the governments of Australia, Belgium, Canada, Denmark, Finland, France, Germany, India, Italy, Japan, the Netherlands, Norway, Sweden, Switzerland, the United Kingdom and the United States of America.
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Foreword

This workshop, Recent Developments in the Research and Control of *Theileria annulata*, is the first of two that the International Laboratory for Research on Animal Diseases (ILRAD), located in Nairobi, Kenya, has convened to assist it in research planning for the quinquennium 1993–1997. ILRAD has a global mandate for the improvement of livestock production in tropical and sub-tropical areas through developing better methods of disease control. The focus of its research since its inception in 1973 has been theileriosis caused by *Theileria parva* and tsetse-fly-transmitted animal trypanosomiasis. A method of immunization against *T. parva*, based on infecting animals with live parasites and simultaneous treatment with tetracyclines to control the severity of the infection, was already available when ILRAD was established. ILRAD has worked to improve the infection-and-treatment method of immunization by developing new methods of characterizing the antigenic diversity of *T. parva* stocks and strains in the field and by characterizing immunizing parasite stocks in several countries. To define the tick-borne disease (TBD) environments in which infection-and-treatment immunization is being used, ILRAD has also developed more sensitive and specific assays for the diagnosis of TBD.

A novel non-living vaccine would be a great improvement over the current live vaccine. Development of such a sub-unit vaccine must be based on a detailed understanding of the important protective immune responses of cattle to *T. parva* and of the antigens that provoke them; this has been the major focus of ILRAD's Theileriosis Research Program. In the longer term, the development of an antigen-based, vector-delivered vaccine for *T. parva* will have to be considered in parallel with other TBD pathogens as these parasites are frequently considered a single disease complex for control purposes. The progress made in research on *T. parva* at ILRAD, particularly in defining the nature of immune responses to this parasite, is of direct relevance to *T. annulata* research. ILRAD's establishment of a vaccine development project within its Theileriosis Program, for the purpose of investigating the induction of protective responses using different vector and adjuvant systems in cattle, provides another important opportunity for future ILRAD work on other TBDs in collaboration with other laboratories.

With these facts in mind, ILRAD hosted this workshop to discuss the magnitude of the *T. annulata* disease problem, the methods currently being used to control *T. annulata* infections, the problems encountered with these control methods, results of recent research on the epidemiology and molecular biology of *T. annulata* infections and developments towards new vaccines against this parasite. It is hoped that these discussions will help ILRAD to identify areas where its research program may contribute to research on *T. annulata*, and, should funding and mandate allow, to establish collaborations that will contribute towards the improvement in control of *T. annulata* and other TBDs using non-living, easily delivered vaccines.

I would like to thank Dr. Jim Lenahan and Mr. Kepher Nguli for their assistance in planning, organizing and running this workshop. Several ILRAD colleagues acted as rapporteurs and prepared the summaries around which the 'Concluding discussion' was written. The original summary of the concluding discussion was prepared by Ms.
FOREWARD

Susan MacMillan and Dr. Subhash Morzaria. Ms. Doris Churi and Ms. Susan Nduta retyped the manuscripts and Mr. William Umbima checked and corrected the references. The map showing the distribution of *T. annulata* was prepared by Mr. Russ Kruska and Mr. Joel Mwaura. Drs. Noel Murphy and Alan Young read and corrected the drafts. I am particularly grateful to Mr. Peter Werehire for proofreading and preparing the layout for these proceedings.

*Thomas T. Dolan*

*International Laboratory for Research on Animal Diseases*

*Nairobi, June 1992*
Opening address

A.R. Gray

Director General
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi, Kenya

It is my pleasant task to welcome you all to ILRAD as we convene this workshop on Recent Developments in Research and Control of *Theileria annulata*. A number of you have visited ILRAD previously and others are here for the first time. I hope you will take the opportunity during your visit to establish or renew friendships with ILRAD staff to learn what progress we are making, and perhaps also find time to see Nairobi and some of Kenya’s other attractions. For the moment, though, I would like to say a few words about why we have invited you here this week.

As most of you know, ILRAD’s task as an institution belonging to the Consultative Group on International Agricultural Research is to identify improved methods for alleviating the adverse effects of livestock diseases on the use of animals for food production and agricultural purposes in developing countries. For the past 15 years, the Laboratory has concentrated its research on African trypanosomiasis and East Coast fever caused by *Theileria parva*. Both disease complexes continue to cause substantial losses in livestock productivity over wide areas of the African continent.

In common with many other scientific organizations, ILRAD periodically revises its long-term strategic plan (on a ten-year horizon) and prepares program and budget proposals to prioritize and put the strategic plan into effect (on a five-year horizon). ILRAD’s next planning quinquennium is 1993–1997. It is quite possible that most of ILRAD’s proposals for the five-year period starting in January 1993 will be a continuation of current activities on tsetse-transmitted trypanosomiasis and East Coast fever, with associated socioeconomic studies and a cooperative research and training program. However, we have to remain alert to the possibility that other inadequately controlled disease complexes such as cowdriosis, anaplasmosis, babesiosis and streptothricosis, or parasites related to those we are already working on, such as *Trypanosoma evansi* and *Theileria annulata*, may qualify on various grounds for some of the Laboratory’s attention, through cooperative programs, sharing of materials, or even core programs and staffing changes.

In the next 18 months or so, ILRAD will be taking a look at progress being made in research on improved control of some of these disease conditions. We will do this through a series of literature reviews and workshops like this one, concerned with research on *Theileria annulata*. While ILRAD has been concentrating on *Theileria parva*, your organizations have been working on control of *Theileria annulata*, which is globally a more widespread parasite.

Our proposals for the next five years of work at ILRAD will also eventually be considered by the next major external review of the Laboratory scheduled for early 1992. Because all of you are distinguished scientists in the field of *Theileria annulata*,
we would like your input into this review and planning process, through your participation in this workshop.

The objectives of the workshop are to review the following:

- the present distribution and economic importance of tropical theileriosis
- the current state of control measures for this disease, including use of acaricides, immunization and chemotherapy
- the state of development of improved epidemiological, preventive and diagnostic tools
- how ILRAD might contribute either directly to research in these areas or indirectly by provision of reagents or transfer of technologies

I encourage your full participation in the meeting and urge you to strive for a useful outcome in the form of a high-quality position paper on recent developments in the research and control of *Theileria annulata*.

I wish you a successful workshop.
COUNTRY REPORTS
Theileria annulata and its control in China

Z.H. Zhang

Science and Technique Development Company of Ningxia
Animal Husbandry and Veterinary Institute of Ningxia,
Academy of Agricultural and Forestry Sciences
Yinchuan, Ningxia
People's Republic of China

Thelerosis is an epidemic disease recorded in ancient China. In this country, it kills cattle during the wheat harvesting season. The clinical signs are a continuous fever and an immediate loss of appetite and condition followed by death. Because of poor laboratory diagnostic techniques, the causative agent was not identified until the 1950s when a series of investigations showed that this disease was caused by Theileria annulata and transmitted by Hyalomma detritum detritum. In the 1960s, we began research into drug treatment and immunization methods. The 'Bovine Theileria annulata Vaccine' and 'Bovine TheilerioCide' were applied with great success in the 1970s. The application of these two specific measures and elimination of the ticks basically controlled theileriosis in China in the 1980s.

SYMPTOMS IN THE INFECTED CATTLE

The prepatent period from the attachment of infected ticks to the onset of fever is 10−13 days. For the first five days of the disease, the fever is high and continuous. A remittent fever follows for another five days, followed by a fall in body temperature before death. The precapeular and precrural lymph nodes are enlarged. The mucous membranes of the eyelid, anus and vagina develop hyperemia and pinpoint hemorrhages or petechiae appear. In some cases, jaundice and papular urticaria of skin lesions have been observed. The cattle show decreased rumination, reduced rumen peristaltic movement and occasionally a depraved appetite. There is constipation or diarrhoea with occult blood in the faeces, but no haemoglobin is detected in the urine. Lactation is reduced or ceases and abortion can occur. The animal becomes emaciated and often lies with the neck curved back to the shoulder. The animal becomes weak and is reluctant to move. The course of the disease from fever to death ranges from one to two weeks. The mortality rate is over 33% and survivors take a long time to recover.

PATHOLOGICAL CHANGES

On postmortem examination, numerous ulcers are detected in the mucous membrane of the abomasum of one to two millimetres diameter. Some ulcers are linked together to form wide, shallow lesions that may penetrate to the muscle layer. New ulcers are
red and older ones grey in colour. The spleen is enlarged with subcapsular haemorrhages. The cut surface bulges with a soft pulp. The liver is swollen with a brittle texture. The gall bladder is covered by serosal haemorrhages and distended with bile. Some of the lymph nodes in the thoracic, abdominal and pelvic cavities are haemorrhagic. The cut surface of the nodes is purple in colour and very moist. Ecchymotic haemorrhages are distributed in the serosal surface of the large and small intestines. Some ecchymotic haemorrhages appear on the surface of the heart and the blood is thin. The lungs are congested and there are small haemorrhages throughout the kidney.

HAEMATOLOGICAL CHANGES

The erythrocyte count is decreased to below 3 million per cubic millimetre and the haemoglobin count to less than 3 grams per 100 ml.

ETIOLOGY

The life cycle of *T. annulata* includes the following stages.

*Sporozoite stage:* When infected adult ticks attach to cattle, the sporozoites develop in the tick salivary gland and are injected with the tick saliva. The sporozoites invade the lymphoid cells and schizonts are detected in 10–13 days. This is the prepatent period of the disease.

*Schizont stage:* The schizont-parasitized lymphocytes proliferate and invade and damage the lymphoid system and produce lesions in the abomasum and skin, liver and spleen.

*Piroplasm stage:* The piroplasm parasitizes the erythrocytes and causes destruction of these cells with a decrease in the erythrocyte count and haemoglobin level. The parasitaemia pattern (infected erythrocytes per 1000) can be divided into two types causing different disease syndromes. The peracute form in which an initial rapid rise in parasitaemia to approximately 30% erythrocytes infected over three days is observed and the cattle die without a fall in parasitaemia. The cattle showing this syndrome are difficult to cure. The second is the acute form in which the parasitaemia does not rise as rapidly as in the peracute form and does not exceed 30%. The parasitaemia then falls slowly to below 1% over one to two weeks. A large number of these cattle could be cured by treatment, but usually only a few cases recover spontaneously. When ticks feed, they ingest piroplasms, which differentiate to gametocytes, fuse to form zygotes and then kinetes, which enter the tick gut cells and finally penetrate the salivary gland cells and develop to sporozoites. *Theileria annulata* completes its sexual generation in ticks and its asexual generation in cattle.

VECTOR TICKS

*Hyalomma* ticks carry and spread *T. annulata* in China. In Ningxia, the important species *H. detritum detritum*. The life cycle of this tick is divided into four
stages—adult, egg, larva and nymph. During the summer season in China, the adult ticks emerge from holes and cracks in the walls and floors of cattle sheds and attach, feed and mate on cattle. The females fall off and lay eggs in the holes and cracks. They die after oviposition. The males die after mating on the cattle. In autumn, the eggs hatch to larvae under suitable conditions of temperature and humidity. In early winter, the larva infest cattle to feed and keep warm through the winter. When spring comes, the larva moult to nymphs. Once engorged, the nymphs drop off, crawl into holes and moult to adults under suitable conditions. At each stage the tick plays a role in spreading *T. annulata*. The larvae and nymphs are infected by the blood in which the gametocytes are contained. The adult transmits the sporozoites, which cause disease in susceptible cattle.

**EPIDEMIOLOGY**

The disease is endemic in northern China, the region in which *Hyalomma* species exist. The disease is seasonal, being prevalent from June to September, with peaks in July and August when the adult ticks are active.

**TREATMENT**

Effective drugs for the treatment of theileriosis caused by *T. annulata* are available (Zhang, 1980, 1987). Primaquin phosphate is an effective drug which eliminates the causative agent. It was identified in our institute and has been used successfully for 15 years in China. The effective dose is as low as 0.75 mg/kg body weight given orally for three days. It is completely safe as the toxic dosage is 20 times greater than the effective dosage. The efficacy rate is 100%. The parasites in red blood cells begin to be destroyed within a few hours of administration and are eliminated totally at the end of the course of treatment. Primaquin phosphate not only eliminates the erythrocytic stage of *T. annulata* but also those of *T. sergenti* and of *T. hirci* in sheep and goats and *Babesia caballi* in horses.

Supportive treatment to arrest capillary bleeding is usually given in the form of calcium chloride (10%) solution at 50 to 100 ml intravenously in two doses at a 48-hour interval. The petechiae begin to discolor soon after injection. As treatment for anaemia, a chemical compound of iron and cobalt, together with liver extract and vitamin A, D, and B12, have been used, but satisfactory results have not been obtained. The most effective treatment is blood transfusion, but this is expensive and is usually only given to very valuable imported animals. Encouraging results have been obtained with a Chinese drug named 'millettia' which showed an effect on increasing the haemoglobin, but more research is required to evaluate its activity. To restore the rumen and intestinal motility, a mixture of 10% sodium chloride solution (50–100 ml), 10% vitamin C (10–50 ml) and 10% sodium benzoate caffeine solution (5–10 ml) is prepared and administered twice intravenously at a 48-hour interval.
TABLE 1. Number of cattle receiving *Theileria annulata* vaccine in 12 provinces of the People's Republic of China from 1975 to 1990.

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<td>286,127</td>
<td>344,150</td>
<td>250,000</td>
<td>352,473</td>
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<td>Cumulative total</td>
<td>75,647</td>
<td>200,447</td>
<td>486,574</td>
<td>830,724</td>
<td>1,080,724</td>
<td>1,433,197</td>
<td>1,624,317</td>
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<table>
<thead>
<tr>
<th>Epidemic area</th>
<th>Year</th>
<th>Vaccinated cattle (head)</th>
<th>Protected cattle (head)</th>
<th>Protection rate %</th>
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<td>MW</td>
<td>1976–1983</td>
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<tr>
<td>NH</td>
<td>1977</td>
<td>550</td>
<td>541</td>
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<tr>
<td>NHQ</td>
<td>1973–1983</td>
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<tr>
<td>MZ</td>
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<td>SR</td>
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<td>MA</td>
<td>1981–1983</td>
<td>570</td>
<td>570</td>
<td>100</td>
</tr>
<tr>
<td>GJ</td>
<td>1982</td>
<td>92</td>
<td>92</td>
<td>100</td>
</tr>
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<td>GCH</td>
<td>1982</td>
<td>976</td>
<td>976</td>
<td>100</td>
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<td>1,400</td>
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</tr>
<tr>
<td>SB</td>
<td>1985–1986</td>
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<td>100</td>
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<tr>
<td></td>
<td>1975–1988</td>
<td>428,848</td>
<td>427,070</td>
<td>99.59</td>
</tr>
</tbody>
</table>

VACCINATION

China has successfully produced a bovine *T. annulata* vaccine, which is highly effective in preventing disease. The safety coefficient is 100%, the prevention rate is over 99% and the duration of immunity is over one year. The Chinese Ministry of Agriculture has ratified the Animal Husbandry and Veterinary Research Institute of Ningxia Agriculture and Forestry Academy to set up a new laboratory to produce this vaccine and supply it to all the epidemic areas of China and to offer it for sale elsewhere in the world.

By 1990, 1.9 million cattle had been treated with the vaccine (Table 1) and disease losses have decreased to a very low level. The practical results are very noticeable. In 1985, a patent for the preparation technique of the vaccine was

<table>
<thead>
<tr>
<th>Epidemic area</th>
<th>Year</th>
<th>Non-vaccinated cattle</th>
<th>Diseased cattle</th>
<th>Incidence rate %</th>
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</tr>
<tr>
<td>NH</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MZ</td>
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<td>1981</td>
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<td>1981</td>
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<tr>
<td>GCH</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HS</td>
<td>1982</td>
<td>317</td>
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<td>MX</td>
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<td>1,996</td>
<td>114</td>
<td>5.71</td>
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|         | 1975-1988 | 8,507                  | 1,407           | 16.54           |

granted in the People's Republic of China. The technical regulations for the manufacture and examination of the vaccine have been approved by The National Control Institute of Veterinary Bio-Products and Pharmaceuticals of China. Field applications of the vaccine in the 1.9 million cattle in China for more than 16 years have shown that it is very safe and effective. A transient mild fever has been detected in only a few cattle in the second week following the injection of the vaccine. Vaccination generally does not produce any clinical signs. It does not cause abortion or a reduction in milk production. A most important factor is that the vaccinated cattle do not act as a source of infection to ticks because the erythrocytic parasites are not produced.

Clinical trials before the vaccine was marketed showed that all vaccinated calves were fully protected against lethal challenge with 50 infected ticks. The vaccinated cattle were widely distributed over the northern half of China and were of various
breeds, age and usage. Nevertheless, the protective rate was never below 98% and the average is 99.59% (Tables 2 and 3). The dose of vaccine is 1 ml intramuscularly for each animal irrespective of breed, sex, age or body weight. The vaccine can be stored in 4 °C in a refrigerator for two months or at room temperature for three days. For delivery, the vaccine can be kept in an ice bag in an insulated box for three days. Dry ice or liquid nitrogen containers are not needed. The immunity induced by the vaccine is maintained for over 13 months in cattle kept in isolation.

CONTROL MEASURES

- One injection of ‘Bovine Theileria annulata Vaccine’ should be given for all the susceptible herds in April or May, one to two months before the disease season each year.
- Ticks can be controlled by the use of organophosphorous or arsenical compounds in summer and autumn. The cattle should be examined frequently to ensure that there are no ticks on them.
- At the end of the epidemic season in September, cement or mud should be used to cover the crevices and holes in the walls of cattle sheds in order to kill the old adults, new eggs and larvae of ticks.
- The ‘TheilerioCide’ and other medicines described should be available for the treatment of sick animals such as unvaccinated cattle, newborn calves and recently imported cattle.

REFERENCES

Recent developments in research and control of *Theileria annulata* in India

D.K. Singh

Animal Disease Research Laboratory
National Dairy Development Board
Anand 338 001, Gujarat, India

During the first two decades of this century, theilerial organisms were regularly recorded in the blood of cattle maintained at Muktheswara, Uttar Pradesh. Since then, several reports describing the detection of theilerial piroplasms in the blood of cattle have appeared in the literature. Acute clinical cases of theileriosis were first recorded on 12 June, 1922, in hill bulls. From 1923 to 1924, the presence of theilerial piroplasms was reported in the blood smears of 1,368 out of 5,158 cattle examined. In 1930, outbreaks of clinical theileriosis were recorded in imported herds maintained at Lahore, Bangalore, Allahabad and Kirkee. Since then, occasional outbreaks of theileriosis have been recorded, mainly in cross-bred and exotic cattle. The disease signs recorded in these animals were high fever, lymph node enlargement, anaemia, petechiae of the mucous membranes and various degrees of jaundice.

Indigenous cattle and buffaloes (*Bubalus bubalis*) have an inherent resistance to disease and harbour theilerial piroplasms in their erythrocytes as symptomless carriers. Until the mid-1960s, theileriosis was enzootically stable. This stability was upset with the large-scale introduction of exotic (taurine) cattle and with the advent of extensive cross-breeding programs for the improvement of milk production. Today, India has approximately 200 million cattle, of which 10 million are either pure-bred or taurine crosses. With the establishment of many Bull Mother Farms of exotic breeds, there has been a rapid increase in the taurine blood in dairy cattle, which show a high degree of susceptibility to theileriosis. India has approximately 26,000 artificial insemination stations and several embryo transfer centres actively engaged in upgrading the existing 82% nondescript cattle. With the rapid increase in the number of cross-bred cattle, the previous enzootic stability of theileriosis has been upset and outbreaks of acute disease with 30 to 60% mortality have been recorded.

Because of the importance of tropical theileriosis, several research schemes were initiated in agricultural universities and other research institutes in India. Serological screening, organized under different programs sponsored by the Indian Council of Agricultural Research (ICAR), indicated that 30 to 60% of cattle maintained at organized farms had antibodies to *Theileria annulata*. Various species of *Hyalomma*, *H. anatolicum* anatolicum, *H. dromedarii*, *H. marginatum* isacii and *H. detritum* are potential vectors of *T. annulata* in India. Epidemiological studies conducted in Kheda District of Gujarat during 1989 indicated that 26.3% of males and 39.1% of females of *H. a. anatolicum* ticks feeding on cattle and buffaloes had *T. annulata* in their salivary glands. Screening of blood smears collected from apparently normal indigenous and cross-bred cattle and buffaloes in village conditions indicated that 45.95%,
Serological screening using the indirect immunofluorescent antibody (IFA) test showed that 6.18%, 72.68% and 21.13% of local and cross-bred cattle and buffaloes had mean titres of 172.50, 694.60 and 113.17, respectively.

Considerable immunological overlap was observed amongst the isolates of *T. annulata* from different geographical regions of the country and all were found to be immunogenically related. Most of these isolates were found to be highly pathogenic and their pathogenicity was dose dependent. In Kheda District alone, where the cross-bred and exotic cattle population is 40,000, a total of 3,800 acute cases of tropical theileriosis were recorded during the year 1988–1989. Considering the average cost of treatment at US$4.75 per visit and that each case involved an average of four visits, the total expenditure on treatment of these cases would be US$72,000. An additional attributed loss of US$173,780.50 would be incurred in the loss of milk by females at an average of four litres per day for an estimated three months. If there were a 5% mortality in the affected animals, an additional loss of US$57,926.80 would accrue assuming the value of an individual animal to be US$304.80. Using these figures as the base, the total losses due to theileriosis in India would be approximately US$77 million.

Drugs such as tetracyclines, halofuginone (Terit, Hoechst), parvaquone (Clexon, Coopers Animal Health) and buparvaquone (Butalex, Coopers Pitman-Moore) have been used for the treatment of clinical theileriosis. Buparvaquone is reported to be the most effective and safe drug when used at 2.5 mg/kg body weight on day 3 after detection of theilerial schizonts in biopsy smears. However, the drug is reported to be expensive and is presently not available commercially. Under these circumstances, in most cases, a combination therapy using tetracyclines, anti-malarials and antipyretics is practised. No organized program of any size is being operated in India for the control of ticks using acaricides. Only a few farmers maintaining valuable exotic cattle spray their animals with organochlorine or organophosphorous acaricidal compounds.

Many research centres established under an All India Coordinated Research Project are engaged in developing diagnostic, chemotherapeutic and prophylactic measures for control of theileriosis. A dot enzyme-linked immunosorbent assay (DOT-EIA) using piroplasm antigen has been developed by the Punjab Agricultural University, which awaits evaluation under field conditions. It is expected that the test will facilitate large-scale epidemiological studies.

Attempts have been made at different institutions to develop immunization as a control measure. Initial immunization using the infection-and-treatment method with tick stabilates and tetracyclines has been tried. However, this method has not been used outside the laboratories. Haryana Agricultural University, Hisar; the Indian Veterinary Research Institute, Izatnagar; the Punjab Agricultural University, Ludhiana; and the Madras Agricultural University, Madras, have all reported the development of tissue-culture attenuated schizont vaccines, which await testing under field conditions.

The Animal Disease Research Laboratory of the National Dairy Development Board, Anand, has developed a tissue-culture vaccine using the ODE (Anand) isolate of *T. annulata* at 150 passages. The vaccine has been extensively studied for safety and potency under laboratory and field conditions. Several experiments on the
standardization of dose, cross-protection against various isolates, storage, transportation, reconstitution and application of the vaccine to different categories of susceptible animals have been completed. The government of India has cleared the vaccine for large-scale production and Indian Immunologicals, a unit of the National Development Dairy Board, began commercial production and marketing in February 1989 under the trade name 'Rakshavac-T'. More than 100,000 pure-bred and cross-bred cattle over two months of age and at different stages of pregnancy and lactation have been vaccinated. These include immunization of several thousands of pure-bred Holstein-Friesian heifers in advanced stages of pregnancy. Serological screening and clinical observations of these vaccinated animals indicate that the vaccine provides protection against lethal tick challenge for a period of more than one year. The vaccine has been well received by veterinarians and farmers and the returns on investment for the mass vaccination program in a developing country are quite encouraging. Following vaccination of these susceptible animals in villages and on organized farms, a marked reduction in the incidence of theileriosis has been reported. In Kheda District alone, where 20% coverage has been achieved during the last three years, a marked reduction in the incidence of the disease has been recorded. Extensive use of this vaccine is likely to reduce the problem of theileriosis considerably in India.

It is essential to undertake extensive epidemiological surveys in countries where \emph{T. annulata} is prevalent. The presently available complement fixation and IFA tests are cumbersome and there is an urgent need for a cheap, simple and reliable diagnostic test, such as the DOT-EIA, and the transfer of the technology to affected countries. Attenuated schizont-infected cell-culture vaccines have shown promising results wherever they have been used. There is a need to evaluate the usefulness of these vaccines in countries where they are not available. The existing culture-derived vaccines have to be stored and transported in liquid nitrogen, which increases their costs considerably and there is always the risk of failure of the cold chain. Research should be directed at developing genetically engineered vaccines so that cheaper alternative vaccines are available that eliminate the need for a cold chain. Efforts should be made to understand the mechanism of attenuation/selection of virulent clones and the possible reversal of the virulence of culture-derived vaccines.

In tropical countries, cattle and buffalo are frequently heavily infested with many genera of ticks, which, apart from transmitting diseases such as theileriosis, babesiosis and anaplasmosis, also cause extensive damage to the health of these livestock. Research should be directed towards developing vaccines against ticks in view of the rapidly developing resistance to commonly used acaricides.
Theileriosis due to *Theileria annulata* in Iran

R. Hashemi-Fesharki

Department of Protozoology and Entomology
Razi State Serum and Vaccine Institute
P.O. Box 11365-1558, Tehran, Iran

Theileriosis caused by *Theileria annulata* is the most economically important cattle disease in Iran, causing major losses in livestock production. It is a serious constraint to the cattle breeding programs established to increase milk and meat production in the country. Research towards the control of this protozoan parasite has been in priority at the Razi Institute for a long time.

IDENTIFICATION, VECTORS AND DISTRIBUTION OF *THEILERIA PARVA*

Most of the early pure-bred cattle introduced into Iran became infected with *T. annulata* and many of them died. The first cases were recorded in 1935 when the government imported 16 pure-bred cattle from France, 12 of which died. Investigations into the deaths of these imported animals indicated that they had contracted theileriosis on arrival and that the species causing the mortality was *T. annulata*. The study also showed that the distribution of the protozoan in Iran broadly coincided with that of its ixodid tick vectors, the *Hyalomma excavatum* group and *H. detritum*. These ticks are the predominant species throughout the country. There are other species such as *H. savigny*, *H. rupipes-glubrum* and *H. dromedarii* that can transmit the disease, but their distribution is more limited. In the northern part of Iran, particularly the northwest and parts of the west, the winter is longer and the season of tick activity is short-lived. Thus the mortality and morbidity rate, in comparison to that of other parts, is lower.

SUSCEPTIBILITY OF CATTLE

Imported non-immune cattle are very susceptible to theileriosis, with a mortality rate of more than 70% in pure-breds, while cross-bred cattle have a mortality rate of less than 45%. In enzootic areas, indigenous calves become infected in early life and following recovery are immune. The mortality rate among these indigenous calves is approximately 15–20%. In 1988, the cattle population in Iran was eight million. Of the exotic cattle, 90% are Holstein-Friesian and the rest are Shitz, Red-Danish and Jersey. Among these cattle, the Holstein and Red-Danish are more susceptible than the others. A cattle improvement program is now under study to reduce reliance on imported meat and dry milk. Vaccination against theileriosis is the most important strategy to reduce the incidence of the disease.
STORAGE AND DELIVERY OF VACCINE

Studies of the pathogenicity and immunizing capacity of different strains of *T. annulata* maintained at -70 or -196 °C for periods lasting more than 10 years have shown that no changes occurred. Storing the vaccine at -70 °C and transporting it to the field in liquid nitrogen has proved more practical than other methods and has become routine. Although the transportation of the vaccine to distant parts of the country is possible, difficulties sometimes occur due to shortages of liquid nitrogen. Therefore, other delivery methods are being studied to facilitate transportation in case there are liquid nitrogen shortages.

CLINICAL SYNDROME

Clinical theileriosis due to *T. annulata* in Iran is similar to that in neighbouring Turkey, Iraq and Pakistan. During the past two decades, we have observed some unusual cases and clinical syndromes such as skin theileriosis presented as nodules spreading over the body. Biopsy smears from these nodules revealed more than 15–20 schizonts per microscopic field. In these cases, the pathological changes in internal organs were negligible. These nodules were observed only in calves less than two months old. Further studies are being undertaken on the pathogenesis of these lesions.

CELL-MEDIATED IMMUNITY

Delayed-type hypersensitivity and migration inhibition tests have been performed. These studies indicated that delayed-type hypersensitivity in cattle with febrile and parasitic reactions was positive but the degree of skin thickening did not correlate with the severity of the disease response. The study revealed that more than 60% of infected or vaccinated animals showed positive skin test reactions and the thickness of the skin reached two and a half times that of normal skin. Pathological findings revealed that a high number of eosinophils and a low number of lymphocytes infiltrated the site of inoculation. The positivity of skin tests showed that cell-mediated immunity could have an effect on theileriosis but the degree of protection afforded by this immunity is not clear. Whether cell-mediated immune responses alone are effective in protecting the animal against acute theileriosis or whether these responses, together with humoral immunity, are required is not known. Further study of cell-mediated immunity is required.

CHEMOTHERAPY

Beginning with the importation of susceptible cattle to Iran over 50 years ago, many different antiprotozoal drugs and antibiotics have been tested for the treatment of pure-bred cattle that were infected naturally or experimentally with stabilates of virulent or mild strains of *T. annulata*. No satisfactory results were obtained until recently when oxytetracycline and two naphthoquinones were used. Oxytetracycline
Hashemi-Fesharki, *THEILERIA ANNULATA IN IRAN*

dihydrochloride (Terramycin, Pfizer) at high doses in cattle experimentally infected with *T. annulata* was studied during the incubation period, at the onset of clinical signs and at the height of parasitaemia and fever. It was not effective as a therapeutic. Studies of treatment using naphthoquinones, parvaquone and buparvaquone showed that buparvaquone, at the recommended dose of 2.5 mg/kg, was more effective. The recovery rate of animals treated with parvaquone was 60.7% compared with 88.7% for those treated with buparvaquone.

**IMMUNIZATION**

The early research on theileriosis at the Razi Institute attempted vaccination of cattle using blood collected from sick animals at the peak of the disease reaction. The cattle had been infected with mild strains of *T. annulata* that had been used by Sergent in Algeria (Sergent, E., *et al.*, *Theileriose a Theileria dispor, Etudes sur les Piroplasmoses Bovins*, Algeria: Institute Pasteur d’Algerie, 1945). A few cattle were also vaccinated using the Kuba strain, which had been sent to the Razi Institute by Sergent. Later it was observed that the blood used for vaccination could transmit other parasites, such as *Babesia, Anaplasma, Eperythrozoon* and other rickettsiae, and viruses, and this approach was abandoned. With progress in the culture of *Theileria*, the Institute started to grow schizont-infected lymphoid cells using the suspension culture method. Initially, fibroblasts were used as feeder cells for growing schizont-infected cells and then the method was changed and they were grown without feeder layers. There are different methods of vaccination against *T. annulata*, but the schizont-tissue-culture vaccine plays the main role in our current vaccination program, which began in 1973. To date more than 150,000 pure-bred and cross-bred cattle two months and older have been immunized in Iran. The vaccine being used in various provinces, representing different climatic conditions and livestock management practices, is prepared from local strains grown in cultured lymphoid cells and has proved to be very effective. No reports of adverse reactions have been received and the vaccinated cattle have shown very good resistance to challenge. We have found that animals may not be able to tolerate the *Theileria* vaccine if they are already suffering from another infection, especially a viral one. Similarly, the immune reaction may be compromised if a viral vaccine such as foot-and-mouth disease is administered during the reaction period of the *Theileria* vaccine. As a result, we do not recommend vaccination of sick animals nor do we suggest concurrent administration of other vaccines. It is recommended that other vaccines be given 25 days before or after the *Theileria* vaccine. A few cases of abortion in cows more than five months pregnant have also been observed after vaccination. The cause of abortion is not known, but the possibility exists that the vaccine may have been responsible, so it is recommended that it should not be administered to pregnant cows. Until 1988, two strains were used routinely but since then only one is used. More than 50,000 cattle have been vaccinated using this method and no adverse effects have been observed and no reports of animal deaths due to theileriosis have been received.

We have attempted to vaccinate calves with up to $300 \times 10^6$ inactivated schizonts either alone or with Freund's adjuvant, but in all cases the animals developed
theileriosis on challenge with virulent strains. Many of our livestock centres are not well maintained and since the whole country is infested with ticks transmitting *T. annulata*, the vaccinated cattle are automatically re-exposed to the parasite. This repeated challenge with sporozoites appears to reinforce the immunity induced by the schizont vaccine.

In heavily tick-infested areas, the use of acaricides is required; however, spraying cow sheds and dipping or spraying cattle with acaricides once a week or more frequently rarely results in effective control. The schizont tissue culture vaccine is, at present, the best means of minimizing the productivity and mortality losses in cattle due to theileriosis. During the last 16 years, over 2000 calves have been inoculated in an attempt to monitor the quality of the vaccine under laboratory conditions, and over 150,000 cattle, representing eight breeds of *Bos taurus* cattle, have been vaccinated and monitored in the field. Indigenous cattle (*Bos indicus*) are much more resistant to *T. annulata* than imported cattle; nonetheless, they suffer up to 15–20% mortality in some parts of the country and we plan to commence vaccination of these cattle.
Epidemiology and control of theileriosis in Morocco

H. Ouhelli and E. Flach

Institut Agronomique et Veterinaire Hassan II
B.P. 6202
Rabat-Instituts, Morocco

Theileriosis caused by *Theileria annulata* is one of the most pathogenic and economically important diseases in Morocco. The mortality rate is 70% in imported breeds and 40% in local breeds. Sera and blood were collected from 714 cattle from 130 farms in the Doukkala area after the theileriosis peak (May–August) in 1989. Seventy-eight of the sera were positive in the indirect immunofluorescent antibody test (at a dilution of 1/160 or more) and *Theileria* piroplasms were found in 55% of blood smears. Similarly, more than 50% of animals in the Gharb region (400 km from Doukkala) had *Theileria* piroplasms in their blood smears.

Twelve farms in Doukkala were chosen for a longitudinal study. A representative number (varying from 50 to 70% of total) of mainly cross-bred cattle were examined monthly or twice a month in the summer for ticks and symptoms of theileriosis and were sampled for serum and blood smear examination. Prior to the disease season in May–August, 50% of cattle had *Theileria* piroplasms at parasitaemias of <1% and usually <0.1%. None of the calves born after the theileriosis season (category 1) had piroplasms detected, whereas 39% of calves born before then (category 2) and 18% of adult cattle showed *Theileria* piroplasms in their blood.

All *Hyalomma* species infesting cattle in Morocco (*H. detritum, H. marginatum, H. a. excavatum, H. dromedarii* and *H. lusitanicum*) can transmit *T. annulata* experimentally. However, only *H. detritum* is recognized as a natural vector. This species is widely distributed in Tunisia, Algeria and Morocco. Its adult activity begins in May, peaks in June and ends in August, corresponding to the curve of theileriosis. Ticks infest adult cattle more heavily (X̄=32.5) than young animals (X̄=3). *Hyalomma detritum* infection with *T. annulata* was assessed in the salivary glands of unfed ticks (in winter) or those attached to cattle for less than 12 hours. The infection rate was 48% with 1 to 34 *Theileria* sporoblasts.

The control of theileriosis in Morocco is by control of *H. detritum* (taking into account its biology and distribution), the use of parvaquone (Clexon, Coopers Animal Health) as a specific treatment for *T. annulata* and vaccination using schizont-infected lymphoblastoid cells. It was shown that 10^2 infected cells could protect cattle and a dose of 10^4 cells was selected for use in vaccination. Experimental application and large-scale field vaccination on a farm of 1,500 cattle over four years showed its ability to protect against artificial challenge one month later, and against natural infection. However, the protection does not last for more than six months and revaccination or natural challenge is needed. The vaccine is safe. No morbidity or mortality has been recorded in vaccinated animals and ticks do not appear to become
infected when fed on vaccinated animals. A program of meetings with cattle owners to inform them of the efficiency and limitations of the different tick and disease control measures has been running for several years.
Theileria annulata in Sudan

O.M. Osman

Veterinary Research Administration
P.O. Box 8067
Khartoum, Sudan

Sudan extends across 2.5 million square kilometres in north and central Africa and is rich in agropastoral potential. There are about 81 million hectares of arable land together with rich pastoral land in the south and central regions of the country. Sixteen million cattle together with sheep, goats and camels are distributed in different agroecological zones with marked areas of overlap. Livestock are under fairly continuous challenge with contagious and vector-borne diseases. Theileriosis due to Theileria annulata constitutes a major obstacle to the development of the milk and meat industry in northern Sudan.

Bovine theileriosis was first reported in Sudan in 1908 (Annual Report of Sudan Veterinary Services) and by 1923 it was reported regularly from all over the country. Theileria annulata piroplasms are frequently found in the blood of indigenous Zebu cattle kept by pastoralists but the parasite is not considered to cause a major disease problem. The piroplasms are detected in the blood of cattle especially after rinderpest vaccination using goat-adapted virus vaccine. However, more importance is attached to contagious killing diseases among adult cattle. There is an inherent resistance to theileriosis among indigenous pastoral cattle. Animals that survive disease challenge at an early age become immune and this immunity is reinforced by repeated exposure to infected ticks. The mortality among indigenous calves is not known, although field veterinarians attributed a high calf mortality to T. annulata infection.

The growing urban communities around the cities and towns and within large agricultural schemes have created an increasing demand for milk. When dairy farming was integrated with crops, exotic breeds were introduced and many local breeds were upgraded. With the exception of government research farms in Khartoum and Gezira, dairy producers keep either pure Friesian or their crosses with indigenous breeds. About half a million of these animals are distributed in highly endemic T. annulata areas where the conditions are conducive for vector multiplication.

Hyalomma anatolicum anatolicum is the major vector of T. annulata in central Sudan. The disease vector in other parts of the country, outside the distribution of H. a. anatolicum, are H. rufipes, H. impeltatum and H. dromedarii and H. truncatum in the south and south central areas.

MORTALITY AND INCIDENCE RATE OF THEILERIA ANNULATA

Calf mortality due to T. annulata has been estimated at 17% in cross-bred calves. Antibody surveys showed a prevalence rate of 46% in indigenous herds in the White Nile area, 67% on farms north of Khartoum and 84% on dairy farms south of
Khartoum. It was generally found that antibodies appear in the blood of calves two to seven months after birth.

There is a general awareness that *T. annulata* is of considerable significance in the economy of dairy farming. The economic effects vary with the standard of hygiene, the status of nutrition and the degree of tick challenge. Tick control using insecticides is expensive and therefore are used at irregular intervals, in many cases after the disease has appeared in the herds. Chemotherapy using buparvaquone (Butalex, Coopers Pitman-More) is very expensive. Infection-and-treatment immunization has also been tried on a very limited scale and proved effective.

*Theileria annulata* is a constant threat to the development and expansion of dairy farming. Recent clinical records show a 30% calf mortality. Newly imported Friesian cattle suffer up to 70% mortality. Ecological factors and host availability are highly conducive for the establishment of vector tick populations in farm establishments and vegetation cover in irrigated areas where naturally resistant indigenous cattle which are carriers of *T. annulata* and infested with *Hyalomma* species vectors graze.

The diverse ecological environments of Sudan make it a good model in which to study *T. annulata*, but such studies are not possible for national institutions because of a lack of funds.
Studies on tropical theileriosis in Turkey


T.C. Ankara Universitet
Vetiner Fakultesi Protozoologi Entomologi
Bilim Dali Backanligi
Irfan Bastug
Caddesi Difkapi
0o226 Ankara, Turkey

Theileria annulata has been reported from every part of Turkey (Lestoquard, 1931; Samuel and Raif, 1930; Tüzül, 1936, 1953; Urtipinar, 1955; Göksu, 1959, 1970; Özcan, 1961; Mimioglu et al., 1971; Ünsüren, 1976; Tasci, 1984) and is a major threat, particularly to European and cross-bred cattle. A blood smear survey carried out in Central Anatolia showed that 178 out of 996 cattle were infected with T. annulata. Sixty-nine of the infected cattle showed clinical signs of tropical theileriosis and 29 died. Tropical theileriosis was diagnosed in 216 out of 1,225 sick cattle brought to the clinic of the Ankara Veterinary Faculty for treatment between 1982 and 1986 (Ünsüren, 1986). Of the 216 infected cattle, 191 were European breeds. In a serological survey conducted in Beytepe Village, Ankara, high titre antibodies against T. annulata were detected in the sera of 12 out of 185 cattle examined using the indirect fluorescent antibody (IFA) test (Cakmak, 1987). During the summer seasons in 1989 and 1990, nearly 11% of the sick cattle brought to the Ankara Veterinary Faculty for treatment suffered from tropical theileriosis. All the cattle with tropical theileriosis were of European breeds.

Several species of ixodid ticks have been reported from Turkey and they were found all over the country (Mimioglu, 1954; Merdivenci, 1969; Hoffman et al., 1971; Karaer, 1983; Sayin and Dumanlı, 1982). In the last few years, 7,029 cattle and sheep were examined for ixodid ticks at 42 collection points in Central Anatolia. A total of 11,586 ticks, composed of 15 species, were collected. Potential vectors of T. annulata such as H. anatolicum anatolicum and H. detritum were commonly present in the collections. Hyalomma a. excavatum and H. detritum were also observed in the shelters of livestock. The mean infestation percentages of cattle and sheep with ticks were 46 and 50, respectively (Sayin and Karaer, unpublished).

The Parasitology Section of the Pendik State Veterinary Institute is responsible for the production of 70,000 doses of live, attenuated T. annulata cell-culture vaccine a year. The vaccination program is aimed at protecting the large number of highly susceptible cattle being imported into Turkey from Europe and North America (with World Bank funds). However, despite these efforts to control the disease, the importance of tropical theileriosis in Turkey is increasing. The inability to control the disease by conventional methods is probably related to the lack of epizootiological knowledge and the lack of regular acaricide application to control the vector ticks.

Currently a joint project is being carried out by the Faculty of Veterinary Medicine (Ankara), the Centre for Tropical Veterinary Medicine (Edinburgh) and Pendik State
Veterinary Institute (Istanbul) to investigate several aspects of tropical theileriosis in Turkey. These are to define the epidemiology of tropical theileriosis using seroepidemiological methods to study tick transmission patterns, to characterize stocks of *T. annulata* using immunological, biochemical and molecular biological methods and to establish the value of the Pendik tissue-culture vaccine in laboratory and field trials.

Ankara had already initiated cell cultures of *T. annulata*, established colonies of *H. detritum* and *H. a. excavatum*, built large and small animal isolation facilities, isolated local stocks of *T. annulata* in ticks and cell culture, established a liquid nitrogen cryobank to store the above stocks as stabilates of infected blood, cell cultures and sporozoites and set up the IFAT for serodiagnosis of *T. annulata*. During this project we have collected infected blood from cattle with tropical theileriosis in the field and inoculated 15 susceptible calves of two to three months old with the infected blood in the laboratory. Eight calves developed severe tropical thilceriosis. Blood stabilates were obtained from these calves and cell cultures were isolated to study the characteristics of *T. annulata*. Nine stocks were isolated from Ankara County as tissue culture stabilates, of which blood stabilates have been prepared from eight and tick stabilates from three.

Uninfected nymphs of either *H. a. anatolicum* or *H. detritum* or both were fed on infected calves. After moult ing, the adult ticks were fed on rabbits for four days and dissected to assess the infection rate. In Table 1, the tick species, their infection rate and mean number of infected acini are shown. The ticks fed on calves with high parasitaemia had a higher percentage infection and an increased number of infected acini.

Sera were collected from six calves 17, 28, 35 and 42 days after inoculation and tested with piroplasm and schizont antigens using the IFAT (Cakmak, 1987). Positive and negative sera were obtained from the Parasitology institute, Hannover Veterinary School, Germany. Rabbit antibovine IgG FITC (Sigma) was used in the test. Schizont and piroplasm antigens were produced in our laboratory from cell cultures and infected blood obtained from experimental calves.

Antibodies were detected in the sera from infected calves 17 days after inoculation and increased by 28, 35 and 42 days after inoculation. Antibody titres against

<table>
<thead>
<tr>
<th>Stocks</th>
<th>Engorged ticks</th>
<th>Infection rate %</th>
<th>Mean number of infected acini</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. annulata</em> Hidirsih</td>
<td><em>H. a. anatolicum</em></td>
<td>100</td>
<td>410</td>
</tr>
<tr>
<td><em>T. annulata</em> Eryaman-i</td>
<td><em>H. a. anatolicum</em></td>
<td>17</td>
<td>2.1</td>
</tr>
<tr>
<td><em>T. annulata</em> Balıçıkhisar</td>
<td><em>H. a. anatolicum</em></td>
<td>75</td>
<td>7.5</td>
</tr>
<tr>
<td><em>T. annulata</em> Gülselen</td>
<td><em>H. a. anatolicum</em></td>
<td>78.5</td>
<td>11</td>
</tr>
<tr>
<td><em>T. annulata</em> Balıçıkhisar</td>
<td><em>H. detritum</em></td>
<td>75</td>
<td>8.8</td>
</tr>
<tr>
<td><em>T. annulata</em> Eryaman-2</td>
<td><em>H. detritum</em></td>
<td>60</td>
<td>23.3</td>
</tr>
<tr>
<td><em>T. annulata</em> Sarioba-1</td>
<td><em>H. detritum</em></td>
<td>100</td>
<td>160</td>
</tr>
<tr>
<td><em>T. annulata</em> Akdere</td>
<td><em>H. detritum</em></td>
<td>100</td>
<td>159.2</td>
</tr>
<tr>
<td><em>T. annulata</em> Mamak</td>
<td><em>H. detritum</em></td>
<td>100</td>
<td>114.5</td>
</tr>
</tbody>
</table>
piroplasm antigen were high when compared with titres against schizont antigens. Serological cross-reactivity among *T. annulata* isolates obtained from different villages of Ankara was studied using the IFAT. All the isolates cross-reacted with each other.

Serum samples were collected from a calf infected with the *T. annulata* Gülseren stock at different times over a year and tested with homologous schizont and piroplasm antigens in the IFAT (Table 2). Antibody titres against piroplasm antigen and schizont antigen appeared 17 days after inoculation of the calf and persisted for 347 and 270 days, respectively. The peak antibody titres against schizont (1:10240) and piroplasm (1:10240) antigens were detected 50 days after inoculation.

<table>
<thead>
<tr>
<th>Days</th>
<th>Schizont</th>
<th>Piroplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>17</td>
<td>1:1280</td>
<td>1:2560</td>
</tr>
<tr>
<td>28</td>
<td>1:5120</td>
<td>1:5120</td>
</tr>
<tr>
<td>35</td>
<td>1:5120</td>
<td>1:5120</td>
</tr>
<tr>
<td>42</td>
<td>1:5120</td>
<td>1:5120</td>
</tr>
<tr>
<td>50</td>
<td>1:10240</td>
<td>1:10240</td>
</tr>
<tr>
<td>68</td>
<td>1:10240</td>
<td>1:5120</td>
</tr>
<tr>
<td>115</td>
<td>1:5120</td>
<td>1:2560</td>
</tr>
<tr>
<td>176</td>
<td>1:640</td>
<td>1:2560</td>
</tr>
<tr>
<td>216</td>
<td>1:320</td>
<td>1:2560</td>
</tr>
<tr>
<td>236</td>
<td>1:160</td>
<td>1:2560</td>
</tr>
<tr>
<td>270</td>
<td>1:160</td>
<td>1:1280</td>
</tr>
<tr>
<td>311</td>
<td>1:20</td>
<td>1:320</td>
</tr>
<tr>
<td>325</td>
<td>1:10</td>
<td>1:320</td>
</tr>
<tr>
<td>347</td>
<td>1:10</td>
<td>1:320</td>
</tr>
</tbody>
</table>

TABLE 2. The IFAT titres in serum collected from a calf experimentally infected with *Theileria annulata* Gülseren at different times throughout the year and tested with homologous schizont and piroplasm antigens.

Three stocks of *T. annulata* identified as Mamak, Akdere and Sarioba were characterized using glucose phosphate isomerase (GPI) isoenzyme electrophorosis (Melrose and Brown, 1979; Melrose *et al.*, 1980, 1981). The stocks were isolated from the Ankara areas and grown as bovine lymphoblastoid cell lines. Lysates of the parasitized cells were examined using thin-layer starch-gel electrophoresis for GPI patterns. The isoenzyme patterns associated with each stock were different. Lysates prepared from the salivary glands of *H. detritum* ticks engorged on the three stocks of *T. annulata* were also examined for GPI patterns and different bands of enzyme activity were detected in each stock. The isoenzyme patterns of different life cycle stages of *T. annulata* also differed in the same stock. Piroplasms from three cattle infected naturally in the field were isolated and characterized and the isoenzyme pattern of each isolate was also different.

Two years earlier, three farms (Ortaköy, Saray and Igmir) in Ankara Province were selected as trial sites. A total of 38 imported European breed cattle were introduced
TABLE 3. The results of examination of cattle for *Theileria annulata* in Ortaköy, Saray and Igmir farms, Ankara.

<table>
<thead>
<tr>
<th>Date</th>
<th>Farm</th>
<th>Number of cattle examined</th>
<th>Number of cattle infected with <em>T. annulata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ortaköy</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>March 88</td>
<td>Saray</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>April 89</td>
<td>Igmir</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>May 88</td>
<td>Ortaköy</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>June 88</td>
<td>Saray</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>July 88</td>
<td>Igmir</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>August 88</td>
<td>Ortaköy</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>September 88</td>
<td>Saray</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>October 88</td>
<td>Igmir</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>November 88</td>
<td>Ortaköy</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>December 88</td>
<td>Saray</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>January 89</td>
<td>Igmir</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>February 89</td>
<td>Ortaköy</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>March 89</td>
<td>Saray</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>April 89</td>
<td>Igmir</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>May 89</td>
<td>Ortaköy</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>June 89</td>
<td>Saray</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>July 89</td>
<td>Igmir</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>August 89</td>
<td>Ortaköy</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>September 89</td>
<td>Saray</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>October 89</td>
<td>Igmir</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>November 89</td>
<td>Ortaköy</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>January 90</td>
<td>Igmir</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

TABLE 4. The results of examination of cattle for ticks in Ortaköy, Saray and Igmir farms.

<table>
<thead>
<tr>
<th>Tick species</th>
<th>Number of cows infested with ticks</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hyaloma detritum</em></td>
<td>Ortaköy: 0/10, Saray: 0/15, Igmir: 3/13</td>
</tr>
<tr>
<td><em>Hyaloma anatolicum anatolicum</em></td>
<td>Ortaköy: 0/10, Saray: 1/15, Igmir: 1/13</td>
</tr>
<tr>
<td><em>Hyaloma anatolicum excavatum</em></td>
<td>Ortaköy: 0/10, Saray: 1/15, Igmir: 1/13</td>
</tr>
<tr>
<td><em>Dermacentor marginatus</em></td>
<td>Ortaköy: 0/10, Saray: 1/15, Igmir: 1/13</td>
</tr>
<tr>
<td><em>Rhipicephalus bursa</em></td>
<td>Ortaköy: 0/10, Saray: 2/15, Igmir: 0/13</td>
</tr>
<tr>
<td><em>Rhipicephalus turanicus</em></td>
<td>Ortaköy: 0/10, Saray: 1/15, Igmir: 0/13</td>
</tr>
<tr>
<td><em>Haemaphysalis otophila</em></td>
<td>Ortaköy: 0/10, Saray: 0/15, Igmir: 2/13</td>
</tr>
<tr>
<td><em>Haemaphysalis punctata</em></td>
<td>Ortaköy: 0/10, Saray: 0/15, Igmir: 1/13</td>
</tr>
</tbody>
</table>

to the farms (10, 15 and 13 cattle in each farm, respectively) and examined for the development of antibodies to *T. annulata* at three-month intervals. Thick and thin blood smears were prepared at each visit and the animals were examined for ticks. The results of examination of cattle for *T. annulata* infection in Ortaköy, Saray and Igmir farms are shown in Table 3. *Theileria annulata* was observed microscopically...
in few animals, although many of them developed high antibody titres during the two years of observation. No clinical cases of theileriosis were recorded on the farms during this investigation.

Table 4 shows the tick species detected and the number of cattle infested with these ticks on the farms. The number of the cattle infested with ticks was low but severe tick infestations with *Rhipicephalus bursa*, *R. turanicus*, *H. a. anatolicum*, *H. a. excavatum*, *Haemaphysalis otophila* and *Haemaphysalis punctata* were observed on sheep and goats on the farms or on cattle in nearby areas.

REFERENCES


Specific prevention of bovine theileriosis in the USSR

V.T. Zablotsky

The All-Union Institute of Experimental Veterinary Medicine
Viev, Kuzminki 109472
Moscow, USSR

Tropical theileriosis is one of the most important protozoan diseases of cattle. It causes considerable economic losses in herds located in areas where the tick vectors *Hyalomma detritum* and *H. anatolicum* are present. The disease is reported from many countries of Africa, Central and Southeast Asia and southern Europe. Losses due to theileriosis include high mortality (from 30 to 90%), abortion, infertility, reduction in milk yield, loss of body weight, deterioration of meat quality of slaughter cattle and the cost of keeping and treating affected cattle. The upgrading of local cattle through cross-breeding with high-producing imported stock is greatly hindered because of susceptibility of the imported stock to theileriosis. Regular acaricide treatment of cattle no longer gives the expected results because of the occurrence of acaricide-resistant tick populations. Following many years of research conducted in the Protozoology Laboratory of the All-Union Institute of Experimental Veterinary Medicine, in Moscow, on the protective properties of *Theileria annulata* schizonts grown in bovine lymphoid cell cultures, a cell-culture vaccine against theileriosis was developed and evaluated in the field.

An important point in the specific prevention of theileriosis was to evaluate the efficiency of the vaccine in different climatic regions of the country where parasites with different biological properties occur. Opinions vary as to the immunogenic identity of different strains of *T. annulata* isolated in different regions. Some investigators believe that animals that recover from theileriosis acquire immunity only to homologous strains and they have partial or no immunity to heterologous strains. According to another opinion, *Theileria* strains originating from different geographic regions are immunogenically identical and induce cross-immunity in vaccinated cattle. Our studies showed that strains isolated in various infected regions of the country were immunogenically related and were cross-protective. In addition, a single inoculation of susceptible calves with attenuated cell-culture vaccine protected them from infection with highly virulent heterologous field strains. Because of these findings, a vaccine prepared from a single attenuated *T. annulata* strain is used to immunize cattle in all *Theileria*-affected regions.

It has been shown by many investigators that the great majority of European cattle breeds are highly susceptible to theileriosis, suffer severe disease and usually die (up to 90% or more). Zebu and Zebu cross-bred cattle, which are bred mostly in tropical and subtropical countries and in southern USSR, are generally resistant to piroplasmoses, including theileriosis. For these reasons, we studied the susceptibility to infection and the safety and immunogenic properties of the theileriosis vaccine in
naive calves of different breeds. Calves of Black-and-White, Red Latvian, Red Estonian and Boushouyev (mixed Zebu-like and Dutch cattle) breeds were used. There were no significant differences in the response to vaccination between calves of these breeds. Experimentally immunized and control non-vaccinated calves were kept on tick-infested pastures in a theileriosis-infected region to evaluate their immunity. All control calves became ill with theileriosis and 33% of them died. The disease was as severe, and caused losses just as high, in Boushouyev calves and in calves of other local breeds. None of the vaccinated calves became ill with theileriosis. Thus all experimental calves acquired a strong immunity to theileriosis.

Host age is an important factor on which parasite development and manifestation of its pathogenicity depend. Because of contradictory evidence about the effect of host age on the severity of the disease, we studied the post-vaccinal responses of calves of various ages from one month to two years. These age groups were chosen because vaccination was being planned for theileriosis-affected regions where the adult cattle populations were already immune. Therefore, only young animals that had not yet had contact with tick vectors were to be vaccinated together with newly introduced animals usually no more than two years of age. It was demonstrated that post-vaccinal responses were more pronounced in animals two years old than in calves from two to seven months old. The main difference was in the duration of temperature response, which was one or two days longer in the former group. Insignificant differences in the response to vaccine application subsequently allowed us to vaccinate young cattle two months to two years old using the same vaccine dose.

Our studies showed that humoral and cellular immune responses in calves of various ages differed. One-month-old calves had the lowest responses. Two-month-old calves reacted to inoculation of the vaccine with an increase in T lymphocytes in blood of up to 12% and of B lymphocytes up to 19%; whereas one-month-old calves had considerably fewer T and B lymphocytes than their older counterparts. The calves vaccinated at one month of age showed a peak complement fixing antibody response on day 65 after vaccination that was never higher than 1:40–1:80; whereas older calves had the maximum titre of 1:80–1:320 35 days after vaccination. All calves vaccinated at one month of age fell ill with theileriosis following tick challenge.

The prophylactic efficacy of the cell-culture vaccine was evaluated from 1976 to 1989. Altogether over 2.5 million animals were vaccinated in the Caucusus and central Asian areas of the USSR. Breeding females usually introduced as calves from disease-free parts of the country late in autumn or in winter were the largest group of vaccinated cattle, together with young stock born to introduced cattle that had not yet had any contact with tick vectors. Vaccinated and non-vaccinated animals were kept on tick-infested pastures in summer and autumn and were not subjected to treatment with acaricides. The studies revealed that all control non-vaccinated animals became ill with theileriosis at different times after returning to pasture, with a mortality rate of 27 to 40% or more. One hundred and ten cases of theileriosis were recorded among vaccinated calves. During long periods of observation it was established that immunity in once vaccinated calves persisted for their lifetime if animals were attacked by Theileria-infected ticks each year. Field evaluation of the theileriosis vaccine showed that it possessed high prophylactic efficacy and gave animals full protection against the disease.
The vaccine is manufactured on a commercial scale at a plant using a Soviet nutrient media. The delivery of the product by aircraft and vehicles has been organized to the zones of its application where storage and distribution centres have been established. Scientifically substantiated principles for the product application in the field were developed, including the route of inoculation, time and regimen of vaccination, dose of the product and the duration of immunity. Annual vaccination has led to the establishment of *Theileria*-immune cattle populations in infected zones of the country. It has also allowed the introduction of high-producing animals and a reduction to a minimum in the frequency of treatments with acaricides.
MODELLING
Modelling

G. Gettinby

Department of Mathematics
University of Strathclyde
Livingstone Tower
26 Richmond Street, Glasgow G1 1XH, UK

INTRODUCTION

This paper presents an overview of modelling and will be based around a description of the development and uses of ECFXPERT, a computer model for tick populations and East Coast fever (*Theileria parva* infection) at sites throughout Africa. It will also discuss modelling concepts and future trends.

ECFXPERT

ECFXPERT is a model of the interaction of *Rhipicephalus appendiculatus* ticks, cattle and *T. parva* developed at Strathclyde University, UK. It has been developed to run on an IBM microcomputer or compatible with VGA graphics. Incorporated into the model are help and dictionary facilities which aid the user both in using the model and understanding the disease complex it models. The model is based largely on empirical data taken from over 80 years of published literature and on observations of experts in the field. Using these data, mathematical algorithms were formulated and rules based on the expert knowledge were built into the model. Since climatic factors are important to the survival of the tick, mean monthly temperatures and rainfall inputs were of primary importance in ECFXPERT.

The model actually comprises four models: a tick, disease, dipping and chemotherapy model. These are interconnected to provide a model for the disease complex. Output is graphically displayed and includes numbers of larvae, nymphae and adult ticks, numbers of cattle infected and uninfected and other data. As ECFXPERT is stochastic, it gives different outputs for each run, thus providing confidence limits.

It was found that rules for superinfection need to be better researched to describe the course of the disease. In addition, it would appear that at most sites, carrier cattle with a persistent transmissible infection are necessary for disease maintenance.

ECFXPERT was tested on several published data sets, and broad agreement on many was found although further investigation is needed. An important use of ECFXPERT is that it provides a tool for undertaking computer experimentation. This allows 'what if' questions to be investigated. For example, if temperature
were to either increase or decrease, what impact would this have on tick development?

MODEL BUILDING

To build a model, say for *T. annulata*, one should consider the following:

<table>
<thead>
<tr>
<th>Taxonomy</th>
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<th>Scale</th>
<th>Level</th>
<th>Goal</th>
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<td>global</td>
<td>DNA</td>
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In a model taxonomy, index models are the simplest and rely on statistical techniques to establish cause and effect relationships. More recently, artificial intelligence (AI)/expert systems use the expert opinions of people. Other considerations are whether the model should be deterministic or stochastic, the scale of the model—ranging from global to site—and the level, which may be at the DNA or the herd. The goal of the model is usually predictive, although it can be a tool to understand relationships. The end user of models has often been the researcher. However, as models become easier to use and change, they can be aimed at planners and managers.

Once the above points have been considered, the typical model building cycle is:

**formulate-calibrate-validate-implement-maintain**

The final step, maintenance, is often overlooked and the model should be flexible enough to be changed easily. As new developments/discoveries are made, these need to be implemented in the model.

FUTURE TRENDS

As computing technology delivers more easily usable power, modelling will be taken to new horizons. One of these will be merging geographical information systems (GIS), which are databases containing spatial referenced data such as climate and vegetation, with classical modelling techniques. This will lead to integrated geographical information systems. Within the next two decades, models could attain a predictive power close to 90%.

Another development is the discovery that biological systems are not always linear and that non-linear phenomena can lead to chaos. Chaos highlights the effects of small changes in starting values which then ripple through and can cause very different results to be obtained. An example of this is in the epidemiology of disease (W. Byrom, unpublished data), where a given number of infected ticks can produce very different outcomes for the disease. For one year there may be many occurrences of the disease and a few the following year. This could be because of the chaotic nature of the disease under observation.
DISCUSSION POINTS

Carrier status

It was noted that the lack of a carrier state disrupted the course of the disease. In the model, ticks did not become infected if the carrier state was absent. Also, buffalo provide a major reservoir for the disease, which would be impossible to control. Wildlife is an accepted part of the epidemiology of disease caused by *T. parva*.

Chaos

The difficulties in determining whether a system is chaotic or not were discussed. One way to determine this would be to vary inputs and see how these affect the outputs.

Models

Is a model only as good as the weakest part? It may be necessary to adjust some parameters to obtain better fit to certain data. In building a complex model, all the pieces have to be validated and tested. One of the major problems of ECFXPERT is in making radical changes, which would involve altering the computer code. It is hoped that the newer generation of expert systems/AI and simulation models will enable such changes to be made much more easily.

Parasitaemias

The parasitaemia level in relation to tick infection needs better understanding for incorporation within infection models.
CURRENT METHODS OF DIAGNOSIS AND CONTROL
Research developments in diagnosis and control of bovine tropical theileriosis in India

A.S. Grewal

Tick-Borne Diseases Research Laboratory
Department of Immunology
College of Veterinary Science
Punjab Agricultural University
Ludhiana 141 004, India

In developing countries such as India, which have introduced mass cross-breeding between native low-milk-yielding cattle (Bos indicus) and imported high-milk-yielding cattle (Bos taurus), bovine tropical theileriosis has emerged as a clinical entity of economic significance for the dairy industry. India is importing both live animals and frozen semen for mass cross-breeding schemes.

IMMUNODIAGNOSIS

The development of a Theileria annulata dot enzyme-linked immunosorbent assay (DOT-EIA) for detection of antibodies in bovine species is a major achievement for control of bovine tropical theileriosis. The test has been developed by the Punjab Agricultural University (PAU), Ludhiana. The antigen used in the test is derived from erythrocytic piroplasms. Preparation of good-quality antigen material free of host cell contaminants (leukocytes and erythrocytes) is critical for the specificity of the test. In contrast to the tube method of DOT-EIA, we designed comb-type multiple-spot dipsticks suitable for conducting the test in 96-well microtitre plates. The advantages of this system are conservation of reagents and fast processing for mass screening of samples.

Sera from cattle vaccinated with T. annulata schizont parasitized lymphocyte cell culture become antibody positive after three to four weeks using the DOT-EIA, with titres of up to 1:256,000, but always higher than 1:3,200. Pre-vaccination sera from these calves were negative even when tested undiluted. Sera from Anaplasma- or Babesia-infected calves did not cross-react in the T. annulata assay. It will be of interest to do cross-reaction studies with sera from cattle infected with other Theileria species. The assay was found useful even for screening of hybridoma culture supernates with traces of antibodies to T. annulata piroplasm antigens. In February 1990, a workshop co-sponsored by the Indian Council of Agricultural Research (ICAR) and the PAU was organized by our department to transfer the T. annulata DOT-EIA to some 35 participants representing research centres and universities from all over India. The ICAR has recommended PAU to produce standardized antigen material for use by the participating stations. This is being done to collect information, over the next few years, on the following aspects of bovine tropical theileriosis: to determine the incidence/prevalence of theileriosis in cross-bred cattle, to assess
the role of water buffalo and indigenous cattle as reservoirs of infection, to monitor field vaccination trials and to estimate the effect of infection on the productivity of animals.

EPIDEMIOLOGY

In India in recent years, the occurrence of clinical theileriosis in new-born calves of one to six weeks of age has been recorded as an emerging epidemiological feature in endemic herds. The disease occurrence in young calves is in significant numbers of not less than 40%, leading to a mortality of approximately 33%. The dams of these calves do not always suffer from theileriosis. On farms with large numbers of cattle, groups of new-born calves have been affected. Random serum samples from cows whose calves become infected were consistently DOT-EIA antibody positive. Previously unexposed adults are an equally susceptible group. Recurrence of clinical theileriosis in carrier adults under stress conditions needs to be studied. An obvious explanation for these observations is that in endemic herds new-born calves, especially those born in the peak tick season, become infested with ticks from birth. Passive immunity through colostrum is not beneficial as cytotoxic T lymphocyte immunity is of prime importance in protection against theileriosis. Also, endemic stability alone may not be effective in preventing clinical theileriosis in highly susceptible breeds of cattle.

An important issue in India is the successful immunization of young calves. Will field vaccination of new-born calves be feasible and, if so, will the vaccine be effective in preventing disease in the face of simultaneous natural exposure to ticks, as cell-culture vaccine requires two to three weeks to induce immunity? On top of these difficulties, the commercially available vaccine recently introduced by India Immunologicals, Hyderabad, is not recommended for use in calves under four months of age (see vaccines and vaccination approaches discussed below). The majority (up to 80%) of cattle are likely to be already exposed to the parasite and to be carriers at the time of adult vaccination.

A few sera tested in the DOT-EIA showed that some buffalo sera contain *T. annulata* antibodies. A large sampling survey is under way to assess the incidence/prevalence of infection in different types of animals (discussed above under DOT-EIA immunodiagnosis).

Immunization with *T. annulata* cell-culture vaccine leads to a carrier state. It is of great importance to understand the nature, of the carrier state with regard to the epidemiology of the disease. Important questions to be addressed are the transmission potential and virulence, as well as the antigenic nature, of carrier state parasites. We have examined vaccinated calves for the carrier state by feeding clean *Hyalomma anatolicum* nymphs. The moulting adult ticks were activated to examine their salivary glands for infected acinii, and by the inoculation of ground-up tick stabilates (GUTS) into susceptible calves. The tick pick-up infection, however, was too low to cause clinical disease on sub-inoculation of GUTS. In a separate experiment, we observed a linear relationship between the degree of piroplasm parasitaemia in calves and the infectivity of fed ticks. The ticks fed on calves with higher parasitaemia (10-40%) were more potent than those fed on lower (1-2%) parasitaemia. The carrier state
parasitaemia in vaccinated calves was rare, varying from negligible to 0.01%. The inability of ticks fed on vaccinated calves to transmit clinical theileriosis cannot be interpreted as a qualitative change in virulence; it was more likely due to a low parasite challenge. The carrier state parasite given at a higher infection dose could be pathogenic. The vaccine-induced carrier state could be seen as beneficial for maintaining immunity both in the vaccinated animals and by tick transmission to the rest of the cattle population.

IMMUNIZATION WITH THEILERIA ANNUlATA-PARASITIZED LYMPHOCYTE CELL-CULTURE VACCINE

Two strategies for immunization are suggested. Immunization with cell-culture vaccine alone is recommended during the winter months when the tick vector is inactive. A number of cell-culture vaccines are now available, developed by different centres to meet the regional needs of their respective State Animal Husbandry Departments. In the past, cross-protection trial studies reported full cross-protection between isolates from distant regions. However, a report from the Indian Veterinary Research Institute (IVRI), Izatnagar centre, describes an isolate (Parbhani) against which the standard vaccine stock does not protect. Cross-protection between different isolates (cultures) is being studied further this year. Secondly, the ICAR is aiming at further evaluation of the efficacy of vaccines in new-born calves (as discussed earlier under the Epidemiology Section). Two such effective vaccines (PAU, Ludhiana, and IVRI, Izatnagar) are at different stages of testing in the field. In laboratory testing it was observed that with high passage cultures (more than 300 days), the post-vaccination reaction consisted of mild fever for a day in some calves and no parasitaemia, but challenge produced 1–7% parasitaemia with fever lasting for two to four days. On the other hand, with the lower passage cultures (100 days), mild post-vaccination reactions were produced with transient fever of one to three days, very low and irregular parasitaemia, varying between negligible to <0.05%, but the calves were fully protected against challenge with no fever and no change in the parasitaemia profile from that of the vaccination phase. All calves survived in both the vaccinated groups and became DOT-EIA antibody positive three to four weeks after vaccination. The control calves suffered clinical theileriosis with parasitaemias of 40–85% and 100% mortality, respectively, within two to four weeks. These high challenge infections are unlikely to be encountered under field conditions. Even so, it would be safer not to use the higher passage cultures and accept a mild reaction after vaccination with the lower passage cultures that ensure solid immunity to heavy challenge. It was also demonstrated that vaccination (300 days culture) of Anaplasma-infected calves showed higher Theileria parasitaemias of 5–15% on subsequent challenge.

Immunization with cell-culture vaccine in conjunction with buparvaquone (Butalex, Coopers Pitman-More) is recommended during the peak tick vector season (summer months), since some of the animals may be incubating the disease at the time of vaccination. Depending on the time of exposure to infected ticks, prior to vaccination, it is likely that such animals could succumb to clinical theileriosis because the vaccine takes two to three weeks to induce protective immunity. Our recent findings confirm that the use of Butalex, which has a prolonged antitheilerial
activity in cattle, in conjunction with cell-culture vaccine ensures safety against any likely tick exposure while the vaccine induces immunity. Butalex, although reported to be an anti-schizontal drug, did not interfere with the efficacy of schizont parasitized lymphocyte cell-culture vaccine. The mechanism of the drug’s action in this application is not fully understood. Neither attenuated parasitized lymphocytes nor unattenuated parasitized lymphocytes, in conjunction with Butalex, produced clinical disease, but they did induce immunity to sporozoite challenge infection. The control calves treated with drug alone were fully susceptible to challenge infection four weeks after treatment. The drug was effective in reducing the virulence of unattenuated parasitized lymphocytes but it did not reduce the immunogenicity of the attenuated parasitized lymphocytes. The parasitized schizonts from allogeneic lymphocytes in the vaccine transfer to the lymphocytes of the vaccinated animals in order to induce recipient host MHC compatible cytotoxic T lymphocytes. Since Butalex in conjunction with the parasitized lymphocyte cells could induce solid immunity, as do the sporozoites when used with the drug, the transfer of infection from the allogeneic cells to the lymphocytes of vaccinated animal was not inhibited.

The modified approach to infection-and-treatment immunization using cultured parasitized lymphocytes in place of tick sporozoites has other advantages. The reproducibility of infective dose can be more easily obtained than with sporozoites; infected lymphocyte culture can be scaled up more easily and economically than bulk sporozoites for large-scale field immunization. In situations where new strains appear, against which the existing vaccine may fail to protect, it is even safer to use the unattenuated parasitized lymphocyte cultured in vitro. This offers the obvious advantage of avoiding the long time required to achieve optimal attenuation of the culture for use as vaccine. In India, as discussed above, our aim is to vaccinate cattle when young; the amount of drug required would be small, which minimizes the cost of vaccination with Butalex plus cell-culture vaccine. Young calves are also easier to handle.

**INFECTION AND IMMUNITY STUDIES WITH THEILERIA ANNULATA PIROPSAMS**

Preparation of erythrocytes by centrifugation and removal of the buffy coat from infected calf blood still leaves mononuclear cells as contaminants. These erythrocyte preparations induce clinical *Theileria* infections. Parasitized erythrocytes, freed of mononuclear cells by repeated centrifugation on Lymphoprep (Nygaard) gradients, did not set up active infections, even with 2 ml of packed red cells from blood with 70–80% parasitaemia. Pre- and post-inoculation (four weeks) sera from these calves were negative for antibodies to *T. annulata* using the DOT-EIA. Blood smear examinations revealed extremely low parasitaemia that may be of inoculum origin. Inoculation of purified freshly released piroplasm preparations also did not establish infections.

Immunization by injecting piroplasms emulsified with adjuvants into calves and subsequent boosting at four weeks induced high DOT-EIA antibody titres of up to 6,400–512,000. However, on sporozoite challenge, these calves developed typical clinical theileriosis with high erythrocytic parasitaemias and died at the same time.
as control calves. These results suggest that the merozoites from lymphocytic schizogony were not blocked by antibodies to the intra-erythrocytic piroplasms. Secondly, transfer of infection from one erythrocyte to another is an uncommon event or is too limited to be a suitable vaccine target. These suggestions, of course, do not exclude that intra-erythrocytic multiplication of the parasite can take place, as reported convincingly by other workers. The parasitaemia is most likely to be a direct reflection of merozoites produced by lymphocytic merogony. Research on merozoite invasion of erythrocytes will be of great interest as an alternative vaccine target. In our experience with experimental infections in more than 200 calves (accumulated data), development of erythrocytic piroplasms was associated with the severity of illness, and death occurred after piroplasm development.

CONFLUENT MONOLAYER CULTURES OF ADHERENT MONONUCLEAR CELLS FROM PERIPHERAL BLOOD OF *THEILERIA ANNULATA*-INFECTED CATTLE

Mononuclear cells from venous blood of infected animals were isolated on Lymphoprep gradients and cultured. The non-adherent cells were removed, initially at three hours, then daily for four days, until no non-adherent cells were seen. At five to seven weeks a few small foci of dividing adherent cells, forming epithelial-like cell monolayers, were noticed, which by 8–12 weeks became confluent monolayers. At this time, small numbers of floating refractile dividing cells, seen mostly in pairs or small clumps, also began to appear. The number of these non-adherent dividing cells increased rapidly. Stained cytospin preparations revealed that all the non-adherent cells were parasitized with *Theileria* schizonts and had a lymphocytic morphology. Transfer of the non-adherent cells using selective trypsination allowed the establishment of pure adherent cell monolayer cultures. The adherent cell monolayers could be subcultured at 1:3 split ratio and became confluent again in one to two weeks. Cytospin-stained smears of these adherent cells revealed no parasite forms and they were macrophage-like in morphology. The adherent cells injected into calves did not produce *Theileria* infection and the calves were fully susceptible to sporozoite challenge. The susceptibility of bovine monocyte/macrophages as targets for *T. annulata*-infection needs to be studied further because it had been reported that these cells are susceptible to infection and transformation. The non-adherent, actively dividing *Theileria* parasitized cells in the primary adherent cell cultures most likely represented a few passively attached lymphocytes that had not been removed initially, rather than monocyte/macrophage cells that had been infected and transformed by *T. annulata*. 

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Vaccination of cattle against *Theileria annulata* using culture-derived schizonts

E. Pipano

The Kimron Veterinary Institute
Beit-Dagan, 50 250, Israel

*Theileria annulata* (Dschunkowsky and Luhs, 1904) has a complex life cycle involving *Hyalomma* spp. ticks and cattle (Mehlhorn and Schein, 1984). Three of the developmental stages—sporozoites, schizonts and erythrocytic merozoites—are infective for cattle. The clinical manifestations and pathological alterations are mainly associated with the presence of microscopically detectable schizonts in the infected animals. *Theileria annulata* has been diagnosed in North Africa, southern Europe, the Near and Middle East, southern USSR, India and southern China (Dolan, 1989). The geographical distribution of *T. annulata* in the Far East is not yet completely known.

HISTORICAL REVIEW OF VACCINATION AGAINST *THEILERIA ANNULATA*

During the 1920s and 1930s an intensive study of theileriosis caused by *T. annulata* in the Mediterranean Basin was carried out in Algeria by a team of French investigators headed by Edmond Sergent. The results of their work were summarized in a classic monograph (Sergent *et al.*, 1945), which provided the fundamental characterization of *T. annulata* that has been the basis for all subsequent studies. Based on the observation that blood from cattle acutely infected with *T. annulata* was invariably infective for susceptible cattle, the French investigators proceeded to select field isolates of natural low virulence. The low-virulence isolates were maintained in the laboratory by passage through susceptible calves, and this eventually resulted in loss of the capacity of the schizonts to yield erythrocytic parasites (Sergent *et al.*, 1945). Such a strain was used for vaccination of field cattle in North Africa for a period of 10 years (1929–1938).

This method of vaccination was investigated by the Israeli scientists Adler and Ellenbogen (1934), with the aim of protecting Friesian-type cattle imported from Europe to improve local breeds. Since they could not isolate an indigenous *T. annulata* strain with low virulence for mass vaccination of cattle, they acquired a mild strain sent by Sergent and his collaborators. However, this exotic strain provided poor protection against the local virulent strains (Adler and Ellenbogen, 1934, 1935, 1936). To circumvent this obstacle, the Israeli investigators adopted a two-step vaccination procedure: first inoculating the mild exotic strain, and two months later inoculating a local strain to reinforce the partial immunity induced by the exotic strain. After 420 passages, the latter failed to infect susceptible calves and, by that time, no other low virulence strains were available (Pipano, 1966).
CURRENT METHODS OF DIAGNOSIS AND CONTROL

The successful cultivation of *T. annulata* schizonts in plasma clot cultures by Tsur (1945) was another major step in the progress of research in theileriosis. This line of investigation was pursued in the search for an immunizing agent against this disease. The achievement of mass propagation of schizonts in monolayer cultures (Tsur *et al.*, 1964) and the fact that the pathogenicity of the parasite decreased after prolonged cultivation *in vitro* (Pipano and Tsur, 1966) provided the basis for development of a safe cell-culture-derived vaccine.

SCHIZONT VACCINE

The process of producing an antitheilerial schizont vaccine begins with the isolation of *T. annulata* parasites to initiate cultures. Blood from acutely ill cattle drawn during the period when schizonts are detected in smears from lymph node or liver biopsy usually induces theileriosis when inoculated into susceptible animals. A few ml of blood drawn into a non-toxic anticoagulant are enough to cause infection, even after storage for several days at 4 °C (Sergent *et al.*, 1945).

*Hyalomma* ticks from theileriosis enzootic areas are also a reliable source of parasites since field studies have shown that a considerable percentage of such ticks carry *T. annulata* infections (Walker *et al.*, 1983). Adult ticks feeding on cattle or water buffalo can be carefully detached and allowed to complete their blood meal on susceptible calves in the laboratory. Most of the ticks will re-attach and transmit theileriosis to susceptible calves. Infected adult ticks can also be obtained in enzootic areas by collecting engorged nymphs from cattle and allowing them to moult, or by collecting mouling nymphs or unfed adults from cracks and crevices of barns. The adults can then be fed on cattle with a high probability of producing infections, or they can be macerated after stimulating the development of sporozoites and the suspension inoculated into calves (see sporozoite vaccine).

Establishment of schizont cultures

The techniques for establishing cultures of *T. annulata* schizonts have been described by Brown in three comprehensive reviews (Brown, 1979a, 1980, 1983). Cultures are initiated by incubating schizont-infected cells collected from cattle suffering from theileriosis. Although schizonts are scarce in blood smears of such animals, incubation of peripheral blood leucocytes results in a high percentage of schizont-infected cultures (Pipano *et al.*, 1989). Similarly, schizont-infected cells obtained by trypsinization of aseptically removed pieces of lymph node, liver or spleen can be used for initiating cultures. An elegant technique for initiating cultures was developed by Brown (1979b) and collaborators. Peripheral blood leucocytes of healthy cattle were infected *in vitro* with sporozoites obtained from infected ticks. This technique opened important experimental possibilities to the *in vitro* study of *T. annulata*.

Attenuation of schizonts

*In vitro* cultivation of *T. annulata*-infected lymphoid cell lines for months or years results in an attenuation of their pathogenicity for cattle. Schizont-infected cells are
subcultured by transfer every three to four days in small culture vessels (Pipano, 1989a). A portion of every tenth passage is stored frozen. The degree of attenuation is assessed by periodic inoculation of $2 \times 10^4$ to $3 \times 10^4$ infected cells into susceptible calves. Pathogenicity is evaluated according to the response of the inoculated calves. During the early period of cultivation, the schizonts produce clinical theileriosis in most calves and kill some of them. Later, milder clinical reactions, accompanied by rare schizonts in lymph nodes and liver, are observed (Tsur et al., 1964; Zablotsky, 1967; Gill et al., 1976). Attenuation is complete when inoculated calves show no clinical symptoms, schizonts or erythrocytic merozoites (Pipano and Israel, 1971; Pipano et al., 1973).

We know too little about the phenomenon of attenuation. It may be that attenuated schizonts derived from culture undergo only a few replication cycles when inoculated into cattle (Pipano, 1977). Consequently, they may never reach the minimum number of parasites needed to provoke a rise in temperature, nor the lower limit of infected lymphocytes that can be detected by microscopic examination of tissue smears. There are no indications that the time needed for attenuation is proportional to the initial virulence of the schizonts (Ozkoc and Pipano, 1981; Pipano, 1971). Moreover, the same theilerial isolate may become attenuated after different periods of cultivation (E. Pipano, unpublished data).

Production of schizont vaccine

Production of a batch of schizont vaccine begins with the retrieval of an aliquot of frozen, infected seed cells. For initiating the culture, 25 cm$^2$ plastic flasks are usually used. The cells are further passaged, transferred and expanded into larger vessels until the desired number of cells has been obtained (Pipano, 1989a). *Theileria annulata*-infected cells have been grown as monolayers (Zablotsky, 1967; Tsur and Adler, 1962) or as suspension cultures (Hooshmand Rad and Hashemi-Fesharki, 1968; Van den Ende and Edlinger, 1971; Mutuzkina, 1975). For monolayer cultivation, large vessels such as Roux or Blake bottles are used. These yield about $10^8$ cells per bottle. Monolayers can also be grown in roller bottles that may generate 30 to 40% more cells than the static vessels with the same amount of medium (Pipano, 1989a). The schizont-infected cells are harvested and concentrated by centrifugation. The vaccine can be used directly as a fresh suspension of infected cells but in most cases doses of vaccine are cryopreserved in liquid nitrogen (Pipano, 1989a; Wathanga et al., 1986).

Protection engendered by the schizont vaccine

Completely attenuated schizonts do not induce any clinical response and no parasitaemia is detected (Pipano et al., 1973). When attenuated schizonts retain the ability to produce erythrocytic merozoites, the latter can be detected on peripheral blood smears from a portion of the vaccinated cattle. In these cases, a few schizonts probably also occur in the lymph nodes or liver, but mass needle biopsy of vaccinated animals cannot be considered a practical means for evaluating the
response to the vaccine. On the other hand, even when schizonts are completely attenuated, specific antibody can be detected (Pipano et al., 1973, 1969; Frank et al., 1971; Mutuzkina, 1983). The presence of antibody during the post-vaccination period implies that some multiplication of schizonts in the vaccinated animals has occurred. Challenge of vaccinated animals with virulent blood-derived schizonts is not of great practical value, but may be useful as a preliminary indication of the protective capacity of the attenuated schizonts. There is good evidence that the main targets of the immune response to *T. annulata* are the schizont-infected cells (Hall, 1988). However, cattle recovered from schizont infection possess a stronger immunity to reinfection with schizonts than to infection with sporozoites (Sergent et al., 1945; Pipano, 1974).

Infection of schizont-immune cattle with *T. annulata* sporozoites has yielded variable results. In India, 14 out of 15 schizont-vaccinated calves were totally protected against challenge with infected ticks and only one showed mild parasitaemia (Gill et al., 1976). In the USSR, seven vaccinated calves were challenged by ticks; five exhibited transient fever and all of them showed a mild rise of parasitaemia above that resulting from the vaccine (Zablotsky, 1967). In Israel, 7 out of 22 calves vaccinated with completely attenuated schizonts remained asymptomatic and 15 exhibited low parasitaemia and mild fever after sporozoite challenge (Pipano, 1981). In all these trials, control susceptible cattle challenged together with the vaccinated cattle showed severe clinical theileriosis and a considerable portion of the animals succumbed to the challenge.

In the past, vaccination-challenge trials using blood-derived parasites from various isolates have shown that different immunogenic properties may exist among the isolates (Adler and Ellenbogen, 1936; Pipano et al., 1974; Sergent et al., 1935; Gill et al., 1980). Cattle vaccinated with culture-derived schizonts and then infected with sporozoites from remote geographical areas were well protected in some cases (Preston and Brown, 1988) but poorly-protected in others (Ozkoc and Pipano, 1981). To reinforce the immunogenic property of the schizont vaccine, a combination of schizonts from isolates obtained in different geographical regions was proposed (Stepanova and Zablotsky, 1989).

Field observations have confirmed the efficacy of the schizont vaccine in inducing immunity against natural infection. In a large field trial in the USSR, 410 vaccinated and 66 susceptible calves were introduced into theileriosis enzootic pastures. None of the vaccinated animals showed clinical theileriosis, while all control calves contracted the disease and 33% of them died (Stepanova et al., 1977). A summary of field trials in Uzbekistan showed that 98 to 100% of schizont-vaccinated cattle were protected against clinical theileriosis when grazing in infected pastures (Stepanova et al., 1986). Similar results were obtained in Turkmeenia where 397 calves were protected by the schizont vaccine when kept in barns infested by *T. annulata*-infected ticks. Thirteen out of 20 non-vaccinated calves contracted theileriosis (Divanov and Khudainazarova, 1988). In Israel, about 3,000 bulls imported from theileriosis-free, western European areas survived in theileriosis-enzootic pastures when protected by vaccination with schizonts. Non-vaccinated local or imported cattle exposed accidentally on these pastures have invariably contracted theileriosis (Pipano, 1989b). In Iran, observations of about 100,000 cattle showed that mortality caused by field-acquired theileriosis was around 0.04% in schizont-vaccinated cattle versus up
to 80% in non-vaccinated ones (Hashemi-Fesharki, 1988). Under experimental conditions, the immunity conferred by the vaccine lasted for at least three and a half years (Zablotsky, 1983).

Although the schizont vaccine decreases considerably the economic loss from theileriosis, there is no evidence, for the time being, that it has an influence on the epizootiology of the disease. Cattle vaccinated with completely attenuated schizonts do not provide a source of infection for ticks (Samish et al., 1984). On the other hand, even when complete protection is conferred, the immunity does not prevent the appearance of erythrocytic merozoites resulting from the bites of infected ticks. Although it has not yet been proven experimentally, it is likely that ticks engorging on such cattle will become infected. Consequently, the use of schizont vaccine does not result in the eradication of theileriosis in enzootic areas.

Testing the schizont vaccine

The nomenclature used below follows that employed in the United States Code of Federal Regulations (1989). The use of schizont vaccine has already reached the stage of commercial production in several countries. However, little has been done to establish standard requirements for testing and use of this vaccine. It would be desirable for an ad hoc committee of representatives from vaccine-producing centres to elaborate a standard protocol covering production, testing and application of the vaccine, rather than having each centre follow its own procedures. The basic tests that a veterinary vaccine produced in cell culture should undergo are for purity, safety, sterility, potency and efficacy. At present, perhaps with a few exceptions, a master seed (a) is kept in the production centres and most use a working seed (b) as a stock material. Since the parasite is incorporated in the cells used for its propagation, several consecutive batches of vaccines are produced from cells obtained from the previous production cycle. This means that the production seed (c) is used as working seed.

The following is the testing procedure for schizont vaccine used in the Kimron Veterinary Institute. It should not be considered a final recommendation for other centres but rather a provisional protocol that should be completed and improved according to the experience gathered in other production centres.

(a) Master Seed. An organism at a specific passage level that has been selected and permanently stored by the producer from which all other seed passages are derived.

(b) Working Seed. An organism at a passage level between Master Seed and Production Seed.

(c) Production Seed. An organism at a specified passage level that is used without further propagation for initiating preparation of a fraction.

Testing for sterility

A sample from each batch of frozen vaccine is thawed and 0.2 ml of concentrated vaccine is introduced into 120 ml of Soybean Casein Digest Medium and incubated
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for 14 days at 35 °C to test for bacterial contamination. To test for fungi, a separate vaccine sample is incubated with the same medium at 25 °C for 14 days.

Testing for specific infections

The vaccine seed is free from common viral infections such as bovine virus diarrhoea, infectious bovine rhinotracheitis, Blue tongue virus, bovine leucosis virus, *Mycoplasma* and *Clamydia*, having been tested for these agents before storage. On the other hand, another biological product is used in the production of the vaccine, namely bovine serum, all other medium components being of synthetic origin. Therefore, the use of serum certified to be free of potential contaminants of bovine origin is an acceptable, practical measure in producing the vaccine. However, whether this type of procedure is entirely adequate is a subject for further elaboration.

*Testing for purity* (freedom of extragenous organic or inorganic material) is not performed with this type of vaccine. It is not clear how the presence of the large volume of bovine lymphoid cell material should be regarded.

*Testing for safety* as a practical matter is simply the testing for pathogenicity to cattle. It appears from presently accumulated experience that no reversion to virulence occurs during further subcultivation of attenuated schizonts. Furthermore, the frozen vaccine is prepared in relatively small batches, so that testing samples from each batch of vaccine in cattle is economically unpractical. However, for legal purposes, two susceptible calves are inoculated once a year from a batch of vaccine chosen at random.

*Testing for potency* is in effect a determination of the number of viable schizonts (infected cells) included in a dose of vaccine necessary to induce a serological response in cattle. If fresh vaccine is used, a constant decrease of viable schizonts occurs, proportional to the time of storage and temperature. Because such vaccine is subjected to variable ambient conditions, it is difficult to standardize.

With frozen vaccine, the time of storage in the frozen state does not play a major role in the survival of the schizonts, but the freezing-thawing process does. With this in mind, and considering the possible environmental conditions in the field after thawing and diluting the vaccine, the number of infected cells per dose is fixed at 10 million.

The plating efficiency of the infected cells after thawing may represent a potential test for potency (Wathanga *et al.*, 1986). However, there is no evidence that multiplication of schizont-infected cells in culture is equivalent to infectivity for cattle. The serological response is assessed by measuring the antibody level in vaccinated cattle using the indirect fluorescent antibody test (Pipano *et al.*, 1969).

*Testing for efficacy* is performed by challenging vaccinated and susceptible cattle with sporozoites. Challenge is performed about six weeks after vaccination using a suspension of macerated *T. annulata*-infected adult *H. excavatum* ticks that has been stored as a stabitate in liquid nitrogen. A dose containing the equivalent of five ticks is inoculated subcutaneously. Usually, the two calves used earlier for safety tests, and
a susceptible calf, are inoculated with the challenge stabilate. A considerable percentage of challenge calves show a few parasites and a rise in body temperature (see protection engendered by the schizont vaccine). However, no criteria for a standardized objective evaluation of the severity of response to challenge with sporozoites have yet been elaborated.

SPOROZOITE VACCINE

A technique for vaccinating against *T. parva* theileriosis using sporozoites to infect the cattle and chemotherapy to mitigate the clinical response was developed during the late 1960s and early 1970s (Cunningham, 1977; Purnell, 1977; Radley, 1981). This technique was tried also with *T. annulata* and research in this field was recently summarized in a comprehensive review (Pipano, 1989a).

The main stages in preparation and use of a sporozoite vaccine are as follows.

**Breeding and infecting ticks.** To maintain a tick colony, non-infected *Hyalomma* ticks are reared on rodents. To prepare the vaccine, preimaginal stages are allowed to engorge on cattle infected with erythrocytic merozoites of *T. annulata* (Samish *et al.*, 1983). A high parasitaemia in the donor calf is more likely to ensure a high yield of sporozoites in the engorged ticks. Adult ticks developing from infected preimaginal stages are fed on cattle, goats, rabbits or other mammals for a period of three to five days to stimulate maturation of sporozoites in their salivary glands (Samish and Pipano, 1976; Singh *et al.*, 1979). Incubation at 37 °C at high humidity (Samish, 1977) may also stimulate maturation of parasites without a blood meal, but fed ticks provide a higher number of sporozoites (Walker and McKellar, 1983; Reid and Bell, 1984).

**Preparation, storage and testing of sporozoite suspension.** The adult infected ticks are removed from the host, washed free of host tissue exudate, then triturated manually (Brown, 1983) or by means of a homogenizer (Samish *et al.*, 1983; Food and Agriculture Organization, 1984) in the liquid phase of cell culture medium complemented with bovine plasma albumin. The supernatant, containing infective sporozoites, is decanted and cryoprotectant is added (Samish *et al.*, 1983). The suspension is distributed in aliquots of 1.0 to 1.5 ml and preserved in liquid nitrogen. Infectivity of the vaccine is tested by inoculating susceptible cattle with serial dilutions of thawed suspension (Pipano *et al.*, 1982).

**Controlled infection and treatment of cattle.** Vaccination is performed by subcutaneous inoculation of a thawed suspension of sporozoites followed by treatment with tetracycline or naphthoquinone derivatives. Tetracyclines should be administered during the early prepatent period to have the required effect. To reduce the number of treatments, one or two inoculations with long-acting oxytetracyclines have been applied (Gill *et al.*, 1977, 1978; Pipano *et al.*, 1981; Khanna *et al.*, 1980). Naphthoquinones are administered simultaneously with the tick suspension (Dhar *et al.*, 1987) or at a later stage of infection (Bansal and Sharma, 1989).

As far as *T. annulata* is concerned, this technique of vaccination has not passed the stage of laboratory trials. A procedure that involves infecting cattle with highly
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virulent parasites and then preventing severe disease by chemotherapy cannot be considered safe. It appears, also, that the cost of preparing the sporozoite vaccine plus the cost of the drugs needed for treatment is considerably higher than that required for producing cultured schizont vaccine. It follows then that, aside from experimental purposes, the sporozoite vaccine has no practical application in T. annulata theileriosis.

FUTURE PROSPECTS

At present there are two effective tools available for control of T. annulata theileriosis, a completely safe and relatively efficient vaccine for preventing the disease, and an efficient chemotherapeutic agent (Bansal and Sharma, 1989) to decrease losses in sick animals. Further studies should be directed towards improvement of the existing vaccine and development of techniques for assessing the epizootiological situation and hence evaluation of the need of vaccination.

Little has been done in defining the antigenic complexity of T. annulata, or concerning the immunological responses of cattle to the different developmental stages of this parasite. More information may lead to improvement in the protective capacity of the vaccine.

A knowledge of the mechanism of attenuation of the schizonts in culture may shorten the period required for obtaining avirulent schizonts. If a gene for virulence was identified in the schizonts, the determination of virulence or avirulence using a DNA hybridization probe might be feasible. This would replace the need for inoculation of cattle in assessing the degree of attenuation.

It is not yet clear whether prolonged cultivation in vitro reduces the immunogenic capacity of schizonts, and therefore decreases the efficacy of the vaccine. This information would be indispensable for determining the optimal time for cultivation in vitro in order to obtain maximal safety with maximum immunogenicity of the schizonts.

The response of cattle to revaccination needs further clarification. To induce immunity, the schizonts should become established when inoculated into cattle. If the basic immunity conferred by an initial vaccination prevents the establishment of schizonts on a subsequent vaccination, no reinforcement of immunity will occur. It is likely that the use of vaccine prepared from antigenically different strains might help circumvent the elimination of the schizonts used for revaccination.

A specific serological test that would allow rapid screening of large numbers of samples is required to assess the need for vaccination of exotic cattle to be introduced in regions where local breeds enjoy enzootic stability. In addition to the indirect fluorescent antibody technique (Pipano et al., 1977), the enzyme immunoassay (Gray et al., 1980) should be adopted and expanded for use for this purpose.

Restricted preliminary trials have shown that immunization with non-viable antigens of T. annulata engenders weak or no protection against natural infection (Hashemi-Fesharki, 1988). It follows, therefore, that, for the present at least, the schizont vaccine remains the method of preference for preventing losses from T. annulata-theileriosis.
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Butalex (buparvaquone): A new therapeutic for theileriosis

N. McHardy
Coopers Pitman-Moore,
Berkhamsted Hill
Berkhamsted, Hertfordshire, HP4 2QE, UK

INTRODUCTION

Buparvaquone is a second-generation hydroxynaphthoquinone with outstanding activity against *Theileria* spp. and some small *Babesia* spp. Formulated as a 5% solution for injection (Butalex, Coopers Pitman-Moore), it achieved an overall cure rate of 92% against 1,800 field cases of theileriosis, caused by *Theileria annulata* and *T. parva*, in independent trials. High cure rates were achieved with a single intramuscular injection at a dose rate of 2.5 mg/kg buparvaquone, particularly against *T. annulata* infections, though supportive therapy to control anaemia and pulmonary oedema was found beneficial in advanced cases. Butalex is an extremely safe product, and has a recommended milk-withhold period of only two days. Work is progressing on several possible uses for Butalex in addition to the treatment of clinical cases of theileriosis. These include its use as a ‘blocking’ drug in immunization procedures against both *T. annulata* and *T. parva*, using, respectively, cultured schizonts and sporozoites as the infective material, and as a strategic therapy in the face of severe challenge among young calves in *T. annulata*-endemic areas. Several studies have examined the effect of Butalex treatment on the milk productivity of cattle with latent *T. annulata* infections, with encouraging results, and studies are in progress to confirm the ability of Butalex to eliminate latent infections of *B. equi* in horses and to treat the clinical disease. Its highly specific action against *Theileria*, allied to its very low mammalian toxicity, could make it an excellent tool in investigations on the nature of the immune response to *Theileria* spp. Cost efficacy analyses indicate that Butalex will play an important role in increasing productivity and profitability, particularly of ‘improved’ dairy cattle, in *Theileria*-endemic areas.

THERAPY OF CLINICAL THEILERIOSIS

Independent trials in at least 12 countries have demonstrated the efficacy of Butalex in curing field cases of theileriosis caused by both *T. annulata* and *T. parva*. A total of more than 1,800 cases have been treated with an overall cure-rate of 92%. Cure was achieved in around 80% of cases of *T. annulata* infection with only one intramuscular injection of 1 ml Butalex per 20 kg (2.5 mg buparvaquone/kg) (Table 1). Cure rates were particularly impressive when early or moderately severe cases
TABLE 1. Results of representative trials with Butalex in the treatment of field cases of theileriosis caused by *Theileria annulata* infection.

<table>
<thead>
<tr>
<th>Country</th>
<th>Trial Name</th>
<th>No. of cases treated</th>
<th>No. cured</th>
<th>No. excluded*</th>
<th>% Cured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>1 Dose</td>
<td>2 Doses</td>
<td></td>
</tr>
<tr>
<td>India</td>
<td>S. Dhar</td>
<td>19</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>India</td>
<td>B.B. Verma</td>
<td>41</td>
<td>36</td>
<td>5</td>
<td>97.3</td>
</tr>
<tr>
<td>India</td>
<td>R.D. Sharma <em>et al.</em></td>
<td>95</td>
<td>95</td>
<td></td>
<td>93.7</td>
</tr>
<tr>
<td>India</td>
<td>P.K. Banerjee <em>et al.</em></td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>100.0</td>
</tr>
<tr>
<td>India</td>
<td>U.V. Shastri</td>
<td>17</td>
<td>17</td>
<td>0</td>
<td>71.4</td>
</tr>
<tr>
<td>Iran</td>
<td>R. Hashemi-Fesharki</td>
<td>66</td>
<td>54</td>
<td>12</td>
<td>87.9</td>
</tr>
<tr>
<td>Iraq</td>
<td>N. Hawa <em>et al.</em></td>
<td>23</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pakistan</td>
<td>M.A. Choudhry <em>et al.</em></td>
<td>596</td>
<td>540</td>
<td>56</td>
<td>98.3</td>
</tr>
<tr>
<td>Turkey</td>
<td>H. Unsuren <em>et al.</em></td>
<td>35</td>
<td>32</td>
<td>3</td>
<td>84.4</td>
</tr>
<tr>
<td>Soviet Union</td>
<td>V. Zablotsky <em>et al.</em></td>
<td>402</td>
<td>310</td>
<td>92</td>
<td>66.1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1,309</td>
<td>1,136</td>
<td>173</td>
<td>1,208</td>
</tr>
</tbody>
</table>

A standard dose of 1 ml Butalex per 20 kg body weight was used throughout.

*Cattle that died of causes other than theileriosis or that died within 12 hours of treatment are excluded from the results.

were treated, but some advanced cases required a second, similar treatment 40 hours after the first to effect a cure. Dhar found that supportive treatment with B vitamins and iron were beneficial in cases of *T. annulata* infection in which the haemoglobin concentration was below 68% (Table 1). When it was below 38%, the prognosis was poor, even with supportive therapy.

It was generally observed that, following treatment of *T. annulata* infections with Butalex, clinical signs of disease were resolved in 24–48 hours and productivity was generally restored within days or weeks, depending on the severity of the disease at the time of treatment. ‘The completeness of cure’ was commented upon by many trialists, in marked contrast to their results when using tetracyclines together with supportive therapy. No signs of drug toxicity were reported from any of the trials.

Field trials against *T. parva* infections have been fewer and more mixed in their design. However, high cure rates with a single dose were reported from Zambia (Musisi *et al.*, see Table 2) and Uganda (Otim, see Table 2). In Tanzania, Mbwambo *et al.*, see Table 2) reported excellent results, though they routinely used two doses. They reported that use of a diuretic in any case showing respiratory distress was very beneficial. In Kenya (Dolan *et al.*, see Table 2), around 40% of cases required a second injection of Butalex, and several ‘cured’ cases developed into chronic theileriosis. The reasons for this relatively disappointing result are not known.

The overall cure rate of field cases of theileriosis of 92% with Butalex combined with the frequent need for only one injection and the milk withhold time of only two days indicates that its performance as a treatment for theileriosis is clearly superior to that of either Clexon (parvaquone) or Terit (halofuginone).

LABORATORY STUDIES WITH BUPARVAQUONE

In an *in vitro* drug screen, buparvaquone was shown to have an EC$_{50}$ of 0.0004 mg/L (ca. 10$^{-9}$M) against *T. parva*, (Muguga E174) but its specific activity against *T.*
TABLE 2. Results of representative trials with Butalex in the treatment of field cases of theileriosis caused by *Theileria parva* infection.

<table>
<thead>
<tr>
<th>Country</th>
<th>Trial</th>
<th>No. of cases treated</th>
<th>No. of cases</th>
<th>% Cured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kenya</td>
<td>T.T. Dolan <em>et al.</em></td>
<td>167</td>
<td>136†</td>
<td>81.4</td>
</tr>
<tr>
<td>Tanzania</td>
<td>H.A. Mbwambo <em>et al.</em></td>
<td>68</td>
<td>67</td>
<td>98.5</td>
</tr>
<tr>
<td>Uganda</td>
<td>C. Otim <em>et al.</em></td>
<td>51</td>
<td>46</td>
<td>90.2</td>
</tr>
<tr>
<td>Zambia</td>
<td>F.L. Musisi <em>et al.</em></td>
<td>68</td>
<td>62</td>
<td>92.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>354</strong></td>
<td><strong>311</strong></td>
<td><strong>88.1</strong></td>
</tr>
</tbody>
</table>

A standard dose of 1 ml Butalex per 20 kg bodyweight was used throughout.

*Cattle which died of causes other than theileriosis or which died within 12 hours of treatment are excluded from the results.

†Includes 12 ‘chronic’ cases.

‡Two doses were given routinely except to nine early cases.

*annulata* was rather less (around 10⁻⁸ M). This compares with an EC₅₀ of 0.006 mg/L against *T. parva* (Muguga E174) for both parvaquone and halofuginone (McHardy *et al.*, 1985).

Electron microscopic studies on the effect of buparvaquone on cultured *T. parva* showed a rapid and specific effect on schizonts, but no adverse effects on host cells, or host cell mitochondria. This conforms with the suggested mode of action of buparvaquone as a specific inhibitor of parasite mitochondrial electron transport (Fry *et al.*, 1984). The principal schizont lesion was progressive cytoplasmic vacuolation, while membranes and nuclear structure remained unaffected during 48 hours exposure. Similar lesions were seen in buparvaquone-treated piroplasms of *T. parva*.

Bioassay of the plasma concentration of buparvaquone following intramuscular injection into cattle of Butalex at the standard dose of 2.5 mg buparvaquone/kg showed that concentrations peaked at around 0.2 mg/L (300 × EC₅₀) three hours after injection, then fell steadily to low concentrations by around day 7. This observation is in close agreement with HPLC assay by Kinabo and Bogan (1988), which suggests that plasma binding of buparvaquone is relatively low.

**STUDIES IN ARTIFICIALLY INFECTED CATTLE**

Studies in calves infected with sporozoite stablates of either *T. annulata* (Ankara) or *T. parva* (Muguga) indicated an optimum dose of 2.5 mg/kg of buparvaquone, as Butalex, injected intramuscularly for both infections. As in field studies, clinical signs were generally controlled within 24–48 hours, while schizonts were clearly damaged within 24 hours. Their numbers then fell to undetectable levels, usually within two to seven days. Damage to schizonts took two forms. Either they became more densely staining, and progressively smaller, or they became more diffuse in appearance and progressively more difficult to identify. It is suggested that the former changes were due to the direct effects of buparvaquone, while the latter was due to an immunological effect. A low-level recrudescent schizont parasitosis was...
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sometimes observed following treatment of either *T. annulata* or *T. parva* infection. This invariably resolved without the need for further buparvaquone treatment. It emphasizes, however, that treatment with buparvaquone is unlikely to eliminate *Theileria* infections totally.

Piroplasms became densely staining and 'anaplasmoid' in form within 24 hours of buparvaquone treatment. They then showed a progressive decline in numbers over the subsequent seven days or so, but then small numbers of piroplasms of normal appearance were sometimes seen. In cattle treated before patent piroplasm parasitaemia was observed, no piroplasms were subsequently seen in blood smears. Similarly, when advanced cases with large numbers of schizonts detected were treated, microschizonts were seldom observed, suggesting that buparvaquone is particularly effective against this stage of the life cycle.

In calves infected with *T. annulata* and showing a declining haematocrit at the time of treatment, the fall was generally halted within 24 hours, before showing a slow but progressive increase. These observations (McHardy *et al.*, 1985) generally parallel those seen when field cases of theileriosis are treated with Butalex. This indicates that buparvaquone is effective against all theilerial life cycle stages in cattle, permitting rapid resolution of clinical symptoms and return to normal productivity, particularly when cattle are treated early in clinical disease.

In a small trial, McHardy and Wekesa (1984) showed that buparvaquone may be a good candidate drug for use in the infection-and-treatment method of immunization against *Theileria*. In ten cattle injected with 2.5 mg/kg buparvaquone as Butalex at the same time that they were infected with a sporozoite stabilate of *T. parva* (Muguga), a very mild episode of theileriosis ensued, and no piroplasms were seen in blood smears. Ten untreated cattle developed typical fatal theileriosis, characterized by large numbers of both schizonts and piroplasms (Table 3). This encouraging lead has been pursued by various workers with both *T. parva* and *T. annulata* with some success.

| TABLE 3. Effect of simultaneous administration of buparvaquone at 2.5 mg/kg body weight intramuscularly and infection of calves with *Theileria parva* (Muguga) stabilate. |
|---|---|---|
| **10 calves/group** | **Untreated controls** | **Treated** |
| | mean ± s.e. | mean ± s.e. |
| **Survival** | 0 | 10 |
| **Days to first schizonts** | 8.2 ± 0.9 | 9.7 ± 1.6 |
| **Days of ‘healthy’ schizonts** | 12.8 ± 1.3 | 0 |
| **Days of damaged schizonts** | 0 | 6.5 ± 1.9 |
| **Maximum% piroplasms** | 57.7 ± 10.9 | 0 |

In a second trial, Butalex was administered at the same dosage to groups of five calves, either seven days before infection, at the same time as infection, or ten days afterwards. All treated calves survived to day 28 after infection, but two of those treated seven days before infection had died by day 35. All five untreated calves had died by day 22. The weight changes up to day 28 are shown in Table 4. Clearly, treatment seven days before infection afforded only partial protection against severe challenge, but demonstrates that Butalex does exert a significant prophylactic effect against theileriosis, which could be beneficial in the endemic
TABLE 4. Prophylactic effect of Butalex at 2.5 mg buparvaquone/kg body weight in groups of five calves infected with *Theileria parva* (Muguga).

<table>
<thead>
<tr>
<th>Treatment, relative to time of infection</th>
<th>Mean % weight change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0–14</td>
</tr>
<tr>
<td>Day −7</td>
<td>−9.2</td>
</tr>
<tr>
<td>Day 0</td>
<td>−2.0</td>
</tr>
<tr>
<td>Day +10</td>
<td>−3.9</td>
</tr>
<tr>
<td>Infected, untreated</td>
<td>−9.2</td>
</tr>
<tr>
<td>Not infected, untreated</td>
<td>−1.2</td>
</tr>
</tbody>
</table>

situation. This observation is in close agreement with the pharmacokinetic findings reported above, which showed that buparvaquone persists at schizont-inhibitory concentrations in plasma for at least seven days after injection.

SAFETY OF BUTALEX

Extensive studies of the safety, metabolism and residues of buparvaquone, conducted in support of the registration dossier of Butalex, show that the production is remarkably safe. They support a two-day withhold period for milk, but 42 days for meat. The relatively long recommended meat withhold period is due solely to persistent residues at the intramuscular injection site in some cattle. Residues in other tissues reach low levels long before this.

In overdose studies, a single intramuscular injection of five times the recommended therapeutic dose (12.5 mg/kg buparvaquone) or of the recommended dose (2.5 mg/kg) on six occasions at two-day intervals caused no adverse effects beyond transient local painless swelling at the injection site in some cases. Studies in laboratory animals and using various *in vitro* tests show that buparvaquone is not carcinogenic, mutagenic or teratogenic.

FURTHER STUDIES WITH BUTALEX

Infection-and-treatment immunization

Studies have been conducted with both *T. parva* and *T. annulata*. Mutugi *et al.* (1988) obtained initially very encouraging results against cattle-derived *T. parva* type infections, but more equivocal effects against buffalo-derived *T. parva*. They also highlighted the difficulty of obtaining a satisfactory balance between dose of sporozoite stabilate and blocking dose of Butalex. Musisi *et al.* (1990) also obtained equivocal results, possibly because injection of Butalex shortly before the stabilate may have interfered with establishment of the infection.

Studies with *T. annulata* have been conducted in India. Dhar *et al.* (1987) obtained excellent results, using either sporozoite stabilate or schizont cultures as the infective material, even in very young calves, in which a cultured schizont ‘vaccine’ alone was of low efficacy. Grewal and co-workers (personal communication) have conducted
large trials in very young calves in which Butalex was used simultaneously with the schizont ‘vaccine’. They showed excellent protection with this system, where ‘vaccine’ alone was ineffective, and they are working towards a system which would be effective in endemic areas where mortality among young calves from theileriosis is a severe problem. Shastri (1989) has successfully used Butalex strategically in young calves facing heavy challenge with *T. annulata*, indicating that, under these circumstances, no artificial infection may be required.

Enhancement of milk productivity in latent carriers of *Theileria annulata* infection

Initial study by Michael et al. (1989) in a herd of Egyptian Friesians with poor milk productivity in the presence of a low-grade *T. annulata* infection showed a highly significant improvement in milk productivity following a single injection of Butalex (2.5 mg/kg buparvaquone). It was suggested that the effect was due to a restoration of immune responses as a result of suppression of the *Theileria* infection, such that resistance to various intercurrent infections was increased. It appeared that suppression of the very low grade *Theileria* infection was not likely, alone, to account for the observed increase in milk yields. Similar studies have been conducted by D.K. Singh and M.S. Kwatra, in India. It is speculated that, should this role for Butalex be confirmed, then it could represent a valuable additional use for the product.

Effect of Butalex on *Babesia equi* infection

In a study by Zaugg and Lane (1989), it was shown that Butalex is highly effective in curing clinical cases of *B. equi* infections in horses, and that multiple doses may be capable of reliably eliminating latent infections. This role could be very useful in facilitating the free movement of horses from *B. equi*-endemic areas into infection-free areas, such as USA.

COST EFFICACY OF BUTALEX TREATMENT

The efficacy of Butalex in curing *Theileria* infections of cattle has been demonstrated in independent trials in many countries in both *T. parva* and *T. annulata*-endemic areas. While the cost of Butalex treatment will vary between countries, because of differences in the distributor chain from manufacturer to end-user, its cost-efficacy is likely to be excellent for several key reasons.

- High cure rates can be expected, particularly if the disease is diagnosed and treated relatively early in the syndrome. Overall cure rates of 92% are considerably better than with any other therapy.
- High cure rates have been achieved with a single injection. The cost of multiple treatments is greatly reduced, and frequently only a single attendance by the veterinarian is necessary. The need for supportive therapy for oedema and anaemia is also less. Thus, both the cost and the difficulty of veterinary care are significantly
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reduced in comparison to alternative treatments, all of which routinely require repeated veterinary attendance.

- Rapid responses and restoration of productivity. Field trialists, particularly in *T. annulata* areas, have been impressed by the rapid cure of disease following Butalex therapy. They have commented that, except with the most advanced cases, normal production of milk, meat and of recovery in calves are vastly superior to that following any other therapy.

- The milk withhold period of only two days is remarkably short, and permits a minimum of production loss. This feature would be of particular significance if the treatment of apparently healthy *Theileria* carrier cattle was found to improve their milk yields reliably.

CONCLUSIONS

The efficacy of Butalex in curing clinical cases of both *T. parva* and *T. annulata* infections has been established in extensive, independent field trials. Further studies in progress may indicate additional uses for Butalex in the infection-and-treatment method of immunization against both *T. parva* and *T. annulata* and its ability to protect very young calves could be particularly beneficial, since very small quantities of Butalex are required. Development of these possible new uses for the drug, together with its use as a safe and specific anti-theilerial tool in basic studies on the pathology and immunology of theileriosis, make Butalex a significant advance in the control and study of theileriosis.

REFERENCES


CURRENT METHODS OF DIAGNOSIS AND CONTROL


IMMUNOLOGY
Stage-specific immune responses in *Theileria annulata* infection and their relevance to the design of novel vaccines

E.A. Innes

International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi, Kenya

*Present address*
Moredun Research Institute
408 Gilmerton Road
Edinburgh EH17 7JH, UK

The main factors to be considered in designing effective vaccines are the identification of relevant antigens, the identification of relevant immune responses, the methods necessary to induce and maintain these responses and the identification of individuals likely to respond poorly to the vaccine. In host-parasite relationships it is unlikely that any one anti-parasitic mechanism operates alone to effect host resistance. The net effect of any one component of the spectrum of immune responses induced by the various parasite life cycle stages will either be host protective, parasite protective, irrelevant or harmful to the host. The immune responses induced by a vaccine need not necessarily cover the whole spectrum of immune responses; the effects of a few 'key' responses may tip the balance in favour of the host.

Animals that recover from primary infection are immune to subsequent homologous and often heterologous challenge with various stocks of *Theileria annulata* parasites and vaccination has already proved to be an effective method of control. Current vaccination measures rely essentially on live immunization of cattle using attenuated *T. annulata*-infected cell lines. However, if novel vaccines are to be designed, characterization of relevant immune responses is essential for the identification of appropriate antigens. The following sections review the current state of knowledge concerning immune responses involved in *T. annulata* infection at each stage of the parasite life cycle. The pathogenic stages in *T. annulata* infection are the schizont-infected cell and the piroplasm-infected erythrocyte.

**IMMUNE RESPONSE TO THE TICK**

The host response to tick attachment and feeding involves a local inflammatory reaction, the extent and complexity of which depends on the species of tick and on whether or not the host has been sensitized (Allen, 1973). A study of the cellular responses in rabbits induced by *Hyalomma anatolicum anatolicum*, a natural tick vector for *T. annulata*, was recently reported by Gill and Walker (1985). Neutrophils,
IMMUNOLOGY

Mononuclear cells, eosinophils and basophils were present, the main effectors of resistance to the tick being mediators released by mast cells, basophils and eosinophils. However, in the case of *T. annulata* infection, it is not known whether or not this inflammatory response is beneficial or detrimental to the host in that it may either facilitate or hinder establishment of the parasite in its target host cell. A novel strategy of immunizing animals against tick gut antigens to elicit antibodies that would damage feeding ticks has provided some very promising results against the one-host tick, *Boophilus microplus* (Willadsen and Kemp, 1988). The application of such a strategy to other tick species is presumably possible although, as discussed by Morrison (1989), in the case of two- and three-host ticks, the immunity would have to be effective against different tick stages and over a shorter time period.

IMMUNE RESPONSE TO THE SPOROZOITE

Despite the fact that the sporozoite stage of the parasite, which is inoculated by ticks and infects the host cell, is only exposed to the host’s immune system for a very short time (Brown *et al.*, 1978), it is possible to generate immune serum, *in vivo*, that will inhibit the penetration of sporozoites into uninfected host leucocytes *in vitro* (Gray and Brown, 1981; Preston and Brown, 1985; Ahmed *et al.*, 1988). This sporozoite neutralizing activity was found in recovery sera or sera from animals which had experienced multiple infections and it was found to be equally effective against different geographical isolates of *T. annulata* (Gray and Brown, 1981). However, antisera raised in *T. parva*-infected animals did not neutralize the infectivity of *T. annulata* sporozoites *in vitro*, implying that the neutralizing activity is parasite-species specific. The antiparasitic activity of immune serum was investigated further by Preston and Brown (1985). Two effects were observed; serum could inhibit invasion of the target cell by the sporozoite and/or suppress the intracellular development to schizont of the trophozoite stage of the parasite, depending on whether the serum was derived from animals recovered from primary or multiple challenge infections. The serum from the multiple challenge animals was more likely to inhibit sporozoite invasion. In field challenge situations, where animals are presumably exposed to repeated infection, immunity against the sporozoite may play a protective role by reducing the number of host cells that become infected.

Monoclonal antibodies (MAbs) have been raised against the surface of the sporozoite and two were found to neutralize sporozoite infectivity significantly for normal peripheral blood lymphocytes (PBL) *in vitro* (Williamson *et al.*, 1989). These MAbs were used to isolate the genes coding for the antigens recognized. Antisera raised in rabbits to a recombinant antigen encoded by a 300-base-pair (bp) fragment of the gene was found to neutralize sporozoite infectivity *in vitro*. Therefore, this antigen would seem to be a strong candidate for a sporozoite vaccine. However, any immune response generated against the sporozoite stage would have to be extremely efficient to completely prevent infection of host cells. There is no evidence to date that the schizont-infected cell is susceptible to attack by anti-sporozoite antibody. A more likely role for the anti-sporozoite response is to reduce the infectivity of the initial sporozoite dose sufficiently to allow the host immune system to control the infection.
IMMUNE RESPONSE TO THE SCHIZONT-INFECTED CELL

The parasite, by choosing an intracellular location, resembles a biological Trojan horse and is effectively protected from the humoral arm of the immune response. Cell-mediated immune mechanisms are most likely to be effective against this stage, as is the case with other intracellular pathogens. During intracellular replication of the parasite, various antigens will appear on the surface of the parasite-infected cell in association with major histocompatibility (MHC) antigens that can be recognized by T lymphocytes. The action of the T lymphocytes may be categorized into 'direct' and 'indirect' effector mechanisms. Direct effector mechanisms involve cytolysis of the infected cell as occurs in many viral infections (Townsend and McMichael, 1985). Indirect effector mechanisms involve the activation of T cells to produce various cytokines, which in turn activate other cells of the immune system.

An early study by Preston and Brown (1981) reported that irradiated *T. annulata* infected cells could induce proliferative responses in autologous peripheral blood mononuclear cells (PBM) irrespective of the immune status of the responder animal. It was unclear how relevant this autologous *Theileria*-mixed lymphocyte response was in the induction of a parasite-specific immune response in vivo. Following the more extensive work on the closely related parasite, *T. parva*, studies were carried out to examine the in vivo generation of cytotoxic cells capable of lysing parasite-infected target cells. The first study on cytotoxic responses in animals infected with *T. annulata* sporozoites was reported by Preston et al. (1983). During recovery from primary infection, two peaks of cytotoxic activity were observed. The first appeared to be genetically restricted, the second was not. After challenge, the majority of the cytotoxic cells were directed against the autologous *T. annulata*-infected cell line. A further study examined the development and specificity of cytotoxic cells in cattle immunized with autologous or allogeneic *T. annulata*-infected lymphoblastoid cell lines (Innes et al., 1989). After primary inoculation of the infected cell lines, the two groups showed distinct differences in both their clinical responses and the target specificity of the cytotoxic cells detected. The allogeneic *T. annulata* cell line recipients showed a mild clinical response, and on day 9 after inoculation a strong cytotoxic response was detected, which appeared to be directed against the allogeneic MHC antigens of the inoculated cell line in some form of graft rejection response. By day 23 the predominant cytotoxic response was directed against the autologous infected cell line. In contrast, the animals that received autologous *T. annulata*-infected cells showed very severe clinical symptoms and the cytotoxic cells, although specific for parasite-infected target cells, were not MHC restricted until day 20. Both groups were found to be immune to a heterologous sporozoite challenge and in both groups a peak of cytotoxicity was detected on day 10 after challenge and was directed against the autologous infected target cell. This would suggest that the cytotoxic response to challenge was MHC restricted and cross-reactive between the heterologous stocks of *T. annulata* parasites used.

A cytotoxic T-cell line was generated by repeated stimulation of immune PBM with irradiated autologous infected cells supplemented with a 20% T-cell growth factor obtained as a supernatant from a transformed gibbon lymphoid cell line, MLA-144. The functional specificity and phenotypic analysis of the CTL line provided further evidence that the cytotoxic response to *T. annulata* was MHC class
I restricted. Firstly, the CTL line recognized and killed only infected target cells that were autologous to or shared BoLA class I specificities with itself. Secondly, the effector cells appeared to reside in the BoT4- subpopulation. Thirdly, the effector function of the CTL line was effectively blocked by the addition of anti-MHC class I sera (Innes, Miller, Brown and Spooner, in preparation).

Several investigations have examined the possible role of cytokines produced by sensitized leucocytes. Studies reported for *T. annulata* (Singh *et al.*, 1977; Rehbein *et al.*, 1981; Ahmed *et al.*, 1981) emphasize the presence of leucocyte migration inhibition factor (MIF) when using immune PBM incubated *in vitro* with either schizont or piroplasm antigen. The cytostatic effect on schizont-infected cells by immune adherent cells has been reported by Preston (1981) and Preston and Brown (1988). Cytostasis usually reached a sustained peak around three to four weeks after primary immunization and would peak again after challenge with similar magnitude and kinetics as in primary immunization. The pattern of adherent cell-mediated cytostasis was consistent despite the differing immunizing material, either sporozoites or infected cell lines, and the different geographical origin of the stocks of *T. annulata* used in the study. Cytostasis was apparent when using both autologous and allogeneic *T. annulata*-infected target cells, although the mechanism by which the adherent cells mediate this effect is unknown. It seems likely that adherent cells, presumably macrophages, will play some form of immunoregulatory role during *Theileria* infection, either immunopotentiating or immunosuppressive. Further studies are needed to elucidate the induction of these responses *in vivo*, and their potential host protective or host damaging roles.

Circulating antibody has been detected against schizont antigen in cattle infected with *T. annulata* using complement fixation, haemagglutination and immunofluorescence techniques (Pipano, 1974). The protective role of this antibody has not been resolved and it has not been possible to correlate serological titres to the degree of protective immunity (Pipano, 1981). The antibody produced in infected animals appears to recognize the schizont antigen consistently within the cell and not an antigen on the surface of the infected cell. In a recent study (Ahmed *et al.*, 1988), it was demonstrated that serum from immune cattle was not opsonic for infected cells and would not lyse these cells in the presence of complement.

**IMMUNE RESPONSE TO THE MEROZOITE AND PIROPLASM**

Antibody responses to piroplasm antigen detected in cattle recovering from infection with *T. annulata* (Pipano, 1974). Ahmed *et al.* (1988), using a chemiluminescence technique, showed that immune serum would react with free merozoites (produced by lysis of infected erythrocytes) but not with the surface of infected erythrocytes. Monoclonal antibodies have been raised using piroplasm antigen (Glascodine *et al.*, 1990) which will react with *in vitro*-derived merozoites but not schizonts. In summary, it would appear that the humoral immune response is effective against the extracellular stages of *T. annulata* within the bovine host, the sporozoite, the extracellular schizont (?) and the merozoite, whereas cell-mediated immune mechanisms, in particular activated T cells, are effective against the intracellular schizont stage.
Therefore, it would seem appropriate to identify relevant antigens of the sporozoite and merozoite using antibody and possible protective schizont antigens using immune T cells. Once appropriate candidate antigens have been identified, a major challenge will be to present these antigens to the immune system in such a way as to induce protective immunity. This is not a major problem in the induction of antibody responses but very little is known about the inductive requirements for cytotoxic T-cell responses.

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Differences in cells infected by *Theileria annulata* and *T. parva* and some of the immunological consequences

R.L. Spooner and E.J. Glass

Institute of Animal Physiology and Genetics Research
Edinburgh Research Station
Roslin, Midlothian EH25 9PS, UK

*Theileria annulata* and *T. parva* have a remarkable ability to infect cells of the immune system in cattle and to transform the cells, which proliferate rapidly together with the parasite (Hulliger *et al.*, 1964). There are, however, a number of important differences between the two parasites and the diseases they cause. They are transmitted by different tick species, they do not overlap geographically and, because of other biological differences, different methods of vaccination are used. After it was shown that cells infected by either parasite could be grown *in vitro*, attempts were made to use these cells for immunization. It was possible to immunize cattle with *T. annulata*-infected cells (Pipano, 1981) and recently it has been shown that as few as $10^2$ allogeneic cells can be used (Ouhelli *et al.*, 1989). There is no evidence of a major histocompatibility complex (MHC) barrier to immunization with *T. annulata* (Innes *et al.*, 1989c). A vaccine is now in use in Morocco utilizing $10^4$ cells per animal. In contrast, it was found that very high cell numbers ($10^8$ cells per animal) (Brown, 1981) had to be used with *T. parva*, and only some animals were immune to subsequent challenge; moreover, the 'vaccine' gave rise to fatal disease in some animals. Subsequent experiments showed that cell lines would immunize against *T. parva* at lower cell doses only if the MHC of the cell line and recipient animal were the same (Teale, 1983; Dolan *et al.*, 1984).

It was not known whether the difference between the two infections depended on the inherent ability of the parasites to parasitize host cells or that only certain cells were permissive to infection. It was shown that *T. parva*-infected cell lines were positive for various T-cell markers (Lalor *et al.*, 1986) but lacked both surface immunoglobulin (Duffus *et al.*, 1978) and bovine monocyte markers (Spooner *et al.*, 1988). They were positive for MHC class I and class II antigens (Spooner and Brown, 1980). *Theileria annulata*-infected cell lines were also positive for MHC class I and class II antigens, but were negative for monocyte markers and, significantly, were negative for T-cell makers (Spooner and Brown, 1980; Spooner *et al.*, 1988).

It has been shown that *T. parva* infects T cells, B cells and null cells (Lalor *et al.*, 1986; Morrison *et al.*, 1986; Baldwin *et al.*, 1988). One report suggested that macrophages cultured for several days could be infected by *T. parva* (Moulton *et al.*, 1984) whereas others found no evidence that this parasite could infect and transform fresh macrophage/monocyte populations (Morrison *et al.*, 1986; Baldwin *et al.*, 1988). Preliminary evidence suggested that *T. annulata* might infect mammary macrophages (Musiime, 1983). Experiments were initiated in which different sub-
populations of bovine lymphocytes were separated and infected in vitro with either *T. annulata* or *T. parva* sporozoites. Monoclonal antibodies to a macrophage marker, IL-A24 (Ellis et al., 1987), sIgM, B5/4 (Pinder et al., 1980), CD4, IL-A11 (Teale et al., 1986; Baldwin et al., 1986) and MHC class II, J11 (Baldwin et al., 1987) were used to separate cells on a fluorescence activated cell sorter (FACS, Becton Dickson). *Theileria annulata* infected and transformed macrophages and B cells but not T cells, whereas *T. parva* infected T cells very efficiently and did not infect macrophages (Spooner et al., 1989; Glass et al., 1989). Although B cells were infected by both parasites, the relative efficiency of infection was considerably less. The non-permissiveness of T cells to infection with *T. annulata* was further confirmed when attempts were made to infect alloreactive cytotoxic T lymphocytes (CTL) with either *T. annulata* or *T. parva*. CTL expressing low levels of MHC class II on their surface were not infected with *T. annulata* but were very efficiently infected by *T. parva* (Innes et al., 1989b).

The significance of the different cell preferences for the pathogenesis of either disease is not clear. It has been shown that T cells infected by *T. parva* can maintain their function as alloreactive CTL at least for some time in culture (Baldwin and Teale, 1987). It appears that the important protective immune response involves MHC-restricted CTL in both *T. annulata* (Preston et al., 1983; Innes et al., 1989a) and *T. parva* infections (Goddeeris et al., 1986), thus interference with the generation or function of these cells would have implications for the outcome of either the diseases themselves or intercurrent infections.

Monocytes infected with *T. annulata* have high levels of MHC class II expressed on their surfaces. Thus, infection may affect their ability to present third-party antigens. If such ability were impaired, it might have significance with regard to the fate of secondary infections in tropical theileriosis. There are problems in studying lymphocyte proliferation with both *Theileria* species because autologous mixed lymphocytes reaction responses are generated (Pearson et al., 1979). However, these reactions do not occur with antigen specific Tₕ lines. Ovalbumin specific T-cell lines have been generated in cattle (Glass and Spooner, 1990a). Ovalbumin has been presented to these lines by uninfected antigen presenting cells (Glass et al., 1991) and *T. annulata*-infected lines from animals of defined class II genotype. *Theileria annulata*-infected cells present ovalbumin more efficiently than the uninfected cells from which they were derived (Glass and Spooner, 1990b).

Thus, cells infected by either parasite appear to maintain their original function, although in the case of *T. annulata*, augmented function is observed. The consequences of amplifying the numbers of such cells in vivo may be related to the pathogenesis of the two diseases.

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BIOLOGY
Investigations into regulation of stage differentiation in *Theileria annuiata*

B.R. Shiels*, J. Kinnaird†, J. Dickson†, L. Tetley†, S. McKellar†, J. Glascodine†, C.G.D. Brown† and A. Tait*

*Wellcome Unit of Molecular Parasitology
Department of Veterinary Parasitology
Bearseaden Road
Glasgow G61 1QH, UK

†Centre for Tropical Veterinary Medicine
Easter Bush, Roslin
Midlothian EH 9RH, UK

*Theileria annuiata*, like many protozoan parasites, undergoes major differentiation events during its life cycle in the insect vector and the bovine host. Previous work has shown that differentiation of the intracellular schizont to the extracellular merozoite can be induced by maintaining cultures of schizont-infected lymphoblastoid cells at 41 °C. We have reproduced the production of merozoites *in vitro* to analyse the events that take place. By carrying out this study, we hope to gain an understanding of how this process is regulated at the molecular level.

**REACTIVITY OF MONOCLONAL ANTIBODIES**

Using sets of monoclonal antibodies (MAbs) raised against the schizont or the piroplasm stages of *T. annuiata*, we demonstrated that differentiation results in the loss of monoclonal antibody epitopes associated with the schizont and the appearance of epitopes associated with the piroplasm. Our conclusion was that merogony is a major point of antigenic differentiation in the vertebrate phase of the life cycle, and we predicted that the merozoite is more closely related to the piroplasm than the schizont or the sporozoite (Table 1). The fact that the sporozoite and merozoite seem to be antigenically different with respect to the MAbs used, some of which are known to recognize surface polypeptides, suggests that it is unlikely that merozoites could re-invoke leukocytes and regenerate schizonts. Thus, the evidence points to a unidirectional cycle of differentiation.

The qualitative patterns of reactivity of the MAbs that detected merozoites were shown to be different. Two antibodies, 1D11 and 1C2, show a dot-like reactivity, whereas monoclonal 5E1 reacts as a halo. We postulated that this was due to different locations of the antigen detected and that the molecule recognized by monoclonal 5E1 is on the surface of the merozoite. To test this, immunoelectron microscopy on cryo-ultramicrotome sections of heat-induced culture forms was carried out. Labeling with colloidal gold clearly occurred around the periphery of the merozoite, demonstrating the surface location of the detected polypeptide. Whether this molecule
TABLE 1. Reactivity of monoclonal antibodies against different life cycle stages of *Theileria annulata*.

<table>
<thead>
<tr>
<th>Monoclonal</th>
<th>Sporozoite</th>
<th>Schizont</th>
<th>Merozoite</th>
<th>Piroplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2E4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1C7</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1C12</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1E11</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5E1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2D5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1D11</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1C2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5H2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4E5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

has any function in the merozoite invasion of erythrocytes is not known. Studies to investigate this possibility will rely upon an efficient *in vitro* assay that we are attempting to develop.

By probing Western blots with monoclonal antibody 5E1, we demonstrated that the detected epitope is present on a 30-kDa polypeptide in both merozoite and piroplasm extracts. No polypeptides were detected in extracts of schizont-infected cells or the control line BL-20. In addition, polyspecific sera also detected a polypeptide of the same molecular weight in extracts of piroplasms and merozoites. We conclude that the molecule can generate an antibody response in an immune animal and it is unlikely that the stage specificity of the MAb is due to post-translational modification but is due to the production of the complete 30-kDa molecule.

DETECTION OF NOVEL POLYPEPTIDES DURING DIFFERENTIATION TO THE MEROZOITE

Using polyspecific antisera, MAbs and a cloned schizont-infected cell line that shows a high level of merozoite production, we have investigated at which point in the differentiation process the expression of novel polypeptides takes place. Cultures of schizont-infected cells were maintained at 41 °C and samples were taken every 2 days until day 8. Samples were processed and analysed in the following manner: (a) cytospin and Giemsa-stained smears; (b) immunofluorescence assays; (c) SDS-PAGE and Western blotting; and (d) immunoelectron microscopy at selected time points after differentiation. From the results of these different procedures, we postulated the following model for the expression of the 30-kDa molecule. For the first four days after placing the culture at 41 °C, the schizont increases in size. By day 4, expression of the 5E1 polypeptide occurs on a few cells and is clearly evident by day 6. The indirect fluorescent antibody (IFA) test patterns and immunoelectron microscopy suggest that the molecule is located on the surface of the schizont, and that its expression is followed by major morphological changes to produce merozoite organelles. These organelles then bud out of the differentiating schizont and become...
surrounded by membrane containing the 5E1/30 kDa polypeptide. Merozoites are then released upon destruction of the host cell.

In addition, we have studied the expression of the molecule detected by MAb 1D11. From quantifying the number of cells reacting with this antibody (at a fixed time point) and comparing it to the number reacting with MAb 5E1, we believe that the 110-kDa molecule recognized by this antibody is expressed later than the 30-kDa antigen. This is consistent with its dot-like reactivity on merozoites, which suggests that the molecule detects a merozoite organelle.

**SEGREGATION BY LIMITING DILUTION OF CLONED SCHIZONT-INFECTED LINES WITH DIFFERENT ABILITIES TO DIFFERENTIATE**

*In vitro* differentiation by culturing at 41 °C is known to be difficult to reproduce. This, coupled to the observation that prolonged culture at 37 °C can result in reduced ability to differentiate, and the fact that a population of cells can be maintained at 41 °C after merogony has occurred, led us to isolate clones from the *T. annulata* (Ankara) stock we used in our experiments. Eight different cloned parasite lines have been isolated by limiting dilution.

Initial studies have been confined to four clones. Each clone has been tested subsequently for the ability to differentiate at 41 °C. Examination of Giemsa-stained slides showed that two clones had an enhanced ability to differentiate. Merozoites were detected at day 6 and were abundant by day 7. Continued culture resulted in the complete destruction of all cells by about day 10. In contrast, the other two clones showed few merozoites by day 8, and continued culture resulted in a population of cells that survived in culture for up to six weeks at 41 °C. We conclude that the parental stock contains at least two populations of infected cells, which can be separated on the basis of their ability to differentiate.

**ANTIGENIC DIVERSITY OF THEILERIA ANNULATA MEROZOITES**

To estimate the degree of differentiation in the different, cloned schizont-infected lines, slides were prepared from cultures after seven days at 41 °C and reacted with the antibody. Two of the clones, C9 and D7, showed reactivity using monoclonal 5E1 with a range of 75-95% of the cells being positive. Clones D3 and E3, however, showed no reactivity. This result did not correlate with the Giemsa-stain morphology as clones D3 and E3 can produce merozoites. We concluded that the epitope recognized by monoclonal 5E1 is polymorphic between the different clones and that the antigenic profiles of the resulting merozoites vary. The result was confirmed by MAb 1C2, which detected differentiating cells in clones D3 and E3 but not in C9 and D7. A third MAb (1D11) detects differentiating cells in all clones, and we used its reactivity as an estimate of differentiation. Preliminary data indicates that the enhanced clone D7 has five times more differentiating cells than clone D3 after seven days at 41 °C.
ISOLATION AND EXPRESSION OF THE MAG 1 GENE

Screening a lambda gt11 genomic expression library with a set of MAbs resulted in the isolation of a clone that reacted with MAb 5E1. Subcloning the 3.0-kb Eco R1 insert and expressing a fusion protein allowed the production of a polyspecific antisera. The antisera has been shown by IFA to react with cultures undergoing merogony and piroplasms but not schizont-infected cells. Thus, the cloned gene seems to be expressed upon the differentiation of the schizont to the merozoite. To determine the size of the polypeptide coded for by the gene, Western blots were carried out. The antiserum detected multiple polypeptides, which ranged in size from approximately 130 kDa to 40 kDa in extracts of piroplasms. As the serum did not detect a 30-kDa polypeptide, we concluded that the cloned gene does not code for the polypeptide recognized by MAb 5E1. From analysis of the gene sequence and the IFA pattern, we postulate that the gene product(s) is glycosylated and has a surface location on the piroplasm. The differential expression of the Mag 1 gene has also been analysed by RNA slot-blot hybridizations. The results indicate that during merogony gene expression is regulated, at least in part, at the RNA level.

CONCLUSION AND FUTURE STUDIES

With the aim of understanding how stage differentiation is regulated at the molecular level, we have begun to analyse the events that occur upon differentiation of the schizont to the merozoite. Differentiation clearly results in antigenic alterations, and our results indicate that major changes in gene expression occur. It seems likely that such changes are coordinated, and may involve a common mechanism of regulation. For example, similar regulatory domains could exist within the promoter regions of genes whose expression is regulated at the transcriptional level. To investigate these possibilities, we have begun to clone and analyse genes that are differentially expressed.

The expression of a 30-kDa molecule has been studied and we have shown that it is expressed on the surface of the differentiating schizont. We postulate that this expression occurs quite early on, after an irreversible chain of differentiation events has been triggered, and is followed by the development of merozoite organelles and the production of merozoites by a budding process. How the triggering of differentiation is initiated and what molecules are involved in this process are unknown. Future studies will have to address early events at a biochemical level if any understanding of the triggering process is to be gained.
Proliferation of *Theileria*-infected lymphocytes

R.O. Williams

Kernforschungszentrum
Institut für Genetik und Toxikologie
Postfach 3640, 75 Karlsruhe 1, Germany

*Present Address*
IGEN, Inc.
1530 East Jefferson Street
Rockville, Maryland 20852, US

The parasite *Theileria parva* divides rapidly in the cytoplasm of bovine lymphocytes and simultaneously induces a massive lymphoproliferation, which, later in the disease, is followed by lymphocytolysis and death of the animal. The proliferating lymphocytes gradually displace the normal lymphoid tissue, resulting in a condition similar to a multicentric lymphosarcoma. Infected lymphocytes collected by lymph node biopsy carry T-cell surface markers and are easily established in laboratory cultures as permanently transformed lymphoblastoid cell lines. Infected lymphocytes can be grown in soft agar, form tumors in nude mice, and can grow in culture with wholly artificial serum supplements. Lymphocyte transformation is dependent on the continuous presence of the parasite in the cytoplasm. A unique and interesting feature of these cell cultures is that their transformed phenotype can be reversed by selective elimination of the parasite after drug treatment, leaving the host cells viable but no longer proliferating. The reversibility of cell transformation offers unusual opportunities in the study of cell proliferation and transformation.

Our approach to understanding the parasite-induced proliferation has been to characterize gene expression in the parasite as well as in infected lymphocytes. A detailed molecular analysis of the host-parasite relationship should allow the identification of important control processes used by the host and possibly modified or interrupted by the parasite that results in uncontrolled cell proliferation.

We, along with others, have shown previously that growth of *T. parva*-infected cells occurs via an autocrine mechanism (Brown and Logan, 1986; Dobbelaaere et al., 1988) involving the secretion of a growth factor with interleukin 2 (IL-2)-like activity. In addition, it was shown that cells from a cloned *T. parva*-infected T-cell line, TpM(803), constitutively express functional IL-2 receptors (IL-2R) on their surface (Dobbelaaere et al., 1988; Coquerelle et al., 1989). IL-2R expression was shown to be strictly dependent on the continuous presence of the parasite in the host cell cytoplasm, since elimination of the parasite by the theilericidal drug buparvaquone led to the rapid disappearance of IL-2R expression (Dobbelaaere et al., 1988; Coquerelle et al., 1989).

The constitutive expression of functional IL-2R on the surface of *T. parva*-infected cells probably confers a selective advantage to the parasite-infected cells.
BIOLOGY

were released from normal or infected T cells in the micro-environment near infected cells, all cells in the environment carrying IL-2 receptors potentially would be stimulated. It was therefore important to examine whether constitutive IL-2R expression was a general characteristic of all *T. parva*-infected cell lines. We analysed a range of cloned and uncloned *T. parva*-infected cell lines of different origins and phenotypes. We also tested cell lines infected with *T. annulata*, the causative agent of tropical theileriosis, which has been reported to transform a different subset of mononuclear cells. The continued growth of these cells also depends on the continuous presence of the parasite in their cytoplasm (Rintelen *et al.*, 1990). Our results show that all cells infected with *T. parva* express IL-2 receptors on their surface. We have been able to identify IL-2 transcripts in all *T. parva* cells tested. In the two lines of *T. annulata* that we have tested, we have shown that IL-2 receptors are expressed on the lymphocyte surface, although there are no identifiable IL-2 transcripts produced.

Our interests are to determine what gene regulatory processes are influenced by the presence of the parasite that result in uncontrolled cell growth. Regulatory processes in gene expression, including IL-2R gene expression, are mediated by interaction of trans-acting protein factors of transcription that bind to cis-acting elements of gene enhancers. Some of these trans-activating factors, such as steroid receptors and NF-kB, are themselves activated from pre-existing pools dormant in the cytoplasm. Recently, the nuclear factor NF-kB, crucial for expression of the kappa light-chain immunoglobulin gene, has been shown to play a role in the expression of IL-2R(α) chain as well as IL-2 during activation of T lymphocytes. NF-kB is important in the activation of several other eukaryotic genes as well. NF-kB binding sequences are found in the human immunodeficiency virus (HIV) enhancer and upstream of the major histocompatibility complex class I (Baldwin and Sharp, 1987) and class II (Blanar *et al.*, 1989) genes. NF-kB binding activity is constitutively present in mature B cells and some T-cell lines and can be induced in cells of lymphoid and non-lymphoid origin by agents such as lipopolysaccharides, phorbol esters, and DNA-damaging agents.

The established regulatory properties of NF-kB in lymphoid cells and the potential role of this factor in constitutive expression of the IL-2R gene in *Theileria*-infected T cells led us to characterize the NF-kB-like proteins in parasite-infected cells. We have presented data suggesting that the constitutive presence of the parasite in the cytoplasm of *Theileria*-infected cells results in constitutively high levels of NF-kB activity in the nucleus as well as high levels of the inactive form in the cytoplasm.

Using both transfection techniques with plasmids carrying a marker gene or an enzyme under the control of the NF-kB transcription factor as well as specific DNA-protein binding assays, we have been able to accurately detect and measure the levels and locations of NF-kB in lymphocytes infected with *Theileria*. We have been able to show that the regulatory regions of both the IL-2 and IL-2R(α) genes are constitutively activated in *Theileria*-infected lymphocytes. Using the gene segment from the HIV virus containing only the NF-kB regulatory region, we are able to show that the increase in gene transcription from an HIV plasmid as well as the IL-2 and IL-2R(α) in *Theileria*-infected cells is stimulated by the presence of NF-kB in the lymphocytes. The results of these functional studies focused our attention on the kB
sequence of the HIV enhancer. To measure directly the level and location of NF-kB-like proteins in T-cells infected by *T. parva*, we used double-stranded oligonucleotides with the HIV NF-kB sequence as radioactive probes in protein binding assays. These binding assays measure the binding of proteins to specific oligonucleotide sequences. Assays using nuclear and cytoplasmic extracts from infected lymphocytes confirm that infected cells maintain constitutively high levels of NF-kB in both the nucleus and the cytoplasm. Using ConA-stimulated lymphocytes as controls, we have been able to show both a quantitative and a qualitative difference in the infected lymphocytes. This observation suggests the indirect or possibly direct influence of the parasite on host cell gene transcription.

We have shown that the factor is localized unevenly in both nuclear and cytoplasmic fractions. The nuclear form is activated, whereas the cytoplasmic form is bound to an inhibitor. The NF-kB-like proteins in infected lymphocytes differ from those in ConA-stimulated lymphocytes and HeLa cells because, as in purified NF-kB, proteins in extracts from infected lymphocytes are stimulated to bind to their recognition motifs by GTP, while this is not true in extracts from ConA cells. We cannot distinguish in these experiments whether ConA-stimulated lymphocytes and *Theileria*-infected lymphocytes have different NF-kB-like proteins or the same proteins in different states of modification.

Having established that *Theileria*-infected lymphocytes constitutively express and activate NF-kB, we attempted to determine for what length of time the stimulatory effect of the parasite remained after removal of the parasite by drug treatment. By treating the Theileria-infected cells with buparvaquone, which selectively kills the parasite leaving the host cell intact, we determined the fate of activated NF-kB. Upon treatment with the drug buparvaquone, a marked loss of NF-kB binding in nuclear extracts over time was observed. The loss of NF-kB closely paralleled the previously documented loss of the parasite as well as the loss of parasite-specific transcripts. It also paralleled the reduction in proliferation. Loss of NF-kB from the cytoplasm occurred to a lesser extent than that from the nucleus. It appears, therefore, that killing of the parasite by buparvaquone specifically decreased NF-kB binding in nuclear fractions and at the same time decreased the size of the cytoplasmic precursor pool. We conclude that the presence of *T. parva* not only activates NF-kB but also increases the synthesis of NF-kB such that an excess of precursor is maintained in the cytoplasm. In turn, the activation of the IL-2 and IL-2R control regions by NF-kB results in rapid lymphocyte proliferation via an autocrine stimulated growth mechanism.

**REFERENCES**


Genes and vaccines in *Theileria annulata*

F.R. Hall*, S. Williamson†, C.G.D. Brown†, K. Hussain‡, A. Tait‡ and B.R. Shiels ‡

*Department of Biology
University of York
Heslington, York YO1 5DD, UK

†Center for Tropical Veterinary Medicine
University of Edinburgh
Easter Bush, Roslin, Midlothian EH25 9RH, UK

‡Wellcome Unit of Molecular Parasitology
Department of Veterinary Parasitology
Bearsden Road, Glasgow G61 1QH, UK

**INTRODUCTION**

The life cycle of organisms of the genus *Theileria* presents the biologist with a fascinating array of topics, many of which are of interest to the core of biology. Coupled with these interests is the more practical aspect of using knowledge gained from their study to provide improved control measures against the diseases they cause. Considering solely the mammalian stages of the life cycle of *T. annulata*, there are three main areas of interest, parasite/host cell recognition and invasion, schizont-induced host cell proliferation and parasite development. Our research has been aimed at understanding the molecular interactions and mechanisms involved in these areas.

**GENE STRUCTURE**

Compared to a number of parasites, our knowledge of *T. annulata* gene structure and organization is very limited, as is our knowledge of the karyotype and ploidy of each nucleus. We have no evidence supporting diploidy and assume that the nuclei are haploid. To date four genes have been isolated and characterized by sequence analysis: a sporozoite surface antigen gene (*Spag* 1), a differentially expressed merozoite/piroplasm gene (*Mag* 1), a cognate hsp70 gene and the 16s RNA structural gene. In addition, an episomal element and a large RNA species have been identified but currently await characterization. The main features of the characterized genes are briefly described below.

*Spag* 1. A 300-bp fragment was initially isolated from a genomic expression library, constructed in lambda gt11, by screening with a monoclonal antibody (1A7) recognizing the surface of the sporozoite (Williamson *et al.*, 1989). This insert was
used to screen a size selected partial Sau3A library constructed in EMBL 3. A clone, containing a 12.4-kb insert, was isolated and a 3.4-kb Eco RI fragment (containing the 300-bp sequence) sub-cloned and sequenced. In addition, a sporozoite cDNA library was constructed and a series of clones isolated (by screening with sub-clones of the 3.4-kb genomic fragment) and sequenced. Southern blots of genomic digests of the Hisar strain of the parasite, together with similar blots from cloned lines of the parasite, establish that there are three 'alleles' of the Spag I gene. Further alleles have been identified in other strains of the parasite. The three alleles in the Hisar strain show Eco RI fragments of 3.4, 4.8 and 6.0 kb in Southern blots. The genomic clone isolated and sequenced corresponds to the 3.4-kb allele, while the cDNA corresponds to one of the other alleles.

Sequence analysis of the cDNA clone shows an open reading frame containing the 300-bp sequence originally isolated. Two main features of interest shown are firstly a high proline content in portions of the sequence and secondly a series of repeats that correspond exactly to the repeats of elastin, a protein found in connective tissue (Blood, et al., 1988). The biological significance of this sequence in terms of acting as the basis of host cell recognition and in terms of co-evolution between host and parasite is of considerable interest.

Mag 1. A lambda gt11 genomic expression library was screened with the monoclonal antibody 5E1, which recognizes the surface of the merozoite (and piroplasm), and a cloned sequence isolated. The clone has a 4.5-kb insert and an internal Eco RI site. The 3.0-kb Eco RI fragment was sub-cloned in pGEM and the induced fusion protein was shown to react weakly with the MAb 5E1. Sera to the fusion protein were raised in rabbits and Western blot analyses of piroplasm and infected lymphocytes showed that the antibody recognized a complex of polypeptides that were stage-specifically (piroplasm) expressed. These antibodies recognize the merozoite and piroplasm giving a characteristic surface pattern. Surface probability plots of the translated sequence show that the repeat sequences have a high surface probability (K. Hussain and co-workers, unpublished data). By means of slot-blot analysis of RNA isolated from piroplasms, schizont-infected cell lines and merozoites, it has been shown that the steady state levels of mRNA are increased in the piroplasm and merozoite stage.

Sequence analysis of the 3.0-kb sequence shows an open reading frame of 272 bp. In the central portion of the gene, flanked by multiple Hinf I sites, is a block of complex repeat sequences. There are three motifs: a 14-bp sequence, a 75-bp sequence (K repeat) and 72-bp sequence (Z repeat); each Z or K repeat is flanked by the 14-bp repeat and a total of 9Z and 4K repeats are found within this block. Although there is some sequence divergence between repeats, in general they are highly conserved. Southern blots of restriction digests of genomic DNA show that the gene is polymorphic between strains of the parasite and that at least two 'alleles' are found within the Hisar strain. In addition, from such restriction digests, there is tentative evidence that there are two copies of this gene/haploid genome.

Hsp70. A heat shock protein gene has been isolated by screening genomic libraries of T. annulata with a Drosophila cDNA clone under reduced stringency (Mason et al., 1989). Sequence analysis shows this gene to have 65% homology with the Drosophila sequence at the amino acid level. Upstream of the transcription start (determined by S1 mapping) is a sequence (TAAATATA) similar to a TATA box and two sequences (CAGAAAGTTGCCA and GCTTAAGTGTCAA) with homology to
the heat shock element consensus sequence. Northern and slot-blot analyses show that this gene is constitutively expressed in the sporozoite, schizont, merozite and piroplasm stages of the parasite, although heat shock of schizont-infected cell lines shows a threefold increase in RNA levels.

16s RNA. The structural gene for the 16s-like RNA has been examined by PCR amplification using primers corresponding to 5' and 3' ends of eukaryotic 16s rRNAs and the resulting products cloned into M13. The coding region spans 1774 bp and has an average G/C content of 45% (Gajadhar et al., 1991). No remarkable features are apparent from the sequence; sequence comparisons using sequences from some 23 organisms show that T. annulata has a greater similarity to Sarcocystis muris than it does to either Plasmodium falciparum or P. berghei. At present, nothing is known about the overall structure of the ribosomal transcription unit or the copy number of the genes.

Extrachromosomal nucleic acid. Gel electrophoresis of high molecular weight preparations of T. annulata nucleic acid show, in addition to nuclear DNA, two smaller species of 6.5 and 2.6 kb respectively. Digestion with either RNase or DNase shows the 6.5-kb species to be DNA and the 2.6-kb species to be RNA (Hall et al., 1990). Nothing is known of the nature or structure of the latter species. The 6.5-kb element is a linear double-stranded DNA molecule found in three geographically distinct isolates of the parasite. Northern blot analysis using the 6.5-kb DNA element as a probe shows that the element has three major transcripts of 2.45, 1.05 and 0.24 kb, which are expressed in sporozoites, piroplasms and merozoites. The element does not code for sequences homologous to mitochondrial rDNA (a larger species of molecule does), but sequence analysis suggests that the element contains the structural gene for cytochrome b (F.R. Hall, unpublished), suggesting that it is an element contained within the mitochondrion. The existence of the cytochrome b gene on the element suggests that it is similar to an element described in Plasmodium.

Clearly our database of T. annulata genes is small, making it difficult to draw general conclusions. Furthermore, the Spag1, Mag1 and hsp70 genes represent a class of genes that are regulated and Spag1 and Mag1 are a further subclass representing surface antigens. Thus, the features of these genes may be specific in relation to their biological roles. Two features are striking, however; firstly, both surface antigens have repeated sequences reminiscent of similar genes from Plasmodium and, secondly, both surface antigen genes show considerable restriction fragment length polymorphism both between and within strains. The structure of the Spag1 genomic clone, with two overlapping open reading frames (one frame shifted from the other), suggest that there is RNA processing in T. annulata, which may involve a novel mechanism.

SUB-UNIT VACCINES

We have no published experimental data on the use of recombinant antigens for induction of protective immunity. Therefore, the use of such antigens can be considered only theoretically, apart from some data from Williamson (1989). From work described elsewhere in this volume and from published data (Hall, 1988; Tait and Hall, 1990), it is clear that a humoral immune response can be elicited to the sporozoite stage and that a cellular immune response is elicited to the schizont-
infected lymphocyte. These responses have been shown, in vitro, to be potentially protective. There are four main potential targets for the immune system—the surface of the sporozoite, the surface of the infected lymphocyte, the surface of the merozoite and the surface of the infected erythrocyte.

Research has been directed towards identifying molecules for all these stages, primarily with a view to understanding the interactions between host and parasite, in the case of the surface of the infected lymphocyte and erythrocyte, or the regulation of gene expression in the case of the surface of the sporozoite and merozoite, as the parasite develops from one stage to another. Much of the research effort has focused on the sporozoite surface antigen, although a series of molecules has been identified on the surface of the infected lymphocyte (Shiels et al., 1989) and the merozoite.

Experiments using the in vitro invasion blocking assay (Gray and Brown, 1981; Preston and Brown, 1985) have shown that sera from cattle immunized by irradiated sporozoites and live sporozoites have blocking activity (Williamson, 1989) as do two monoclonal antibodies (1A7 and 4B11) that recognize the surface of the sporozoite. Western blot analysis of these sera and the monoclonal antibodies (Williamson, 1989) show that there is considerable variation between individual animals in terms of the antigens they recognize: the monoclonal 1A7 recognizes a complex of antigens (54, 63, 70, 85 kDa) while 4B11 recognizes a 17-kDa antigen and most of the bovine sera recognize one or both of the antigens. Challenge experiments of animals immunized with sporozoites show no clear evidence for protection. The gene coding for the 1A7 epitope has been cloned (Spag1) and both genomic and cDNA sequences have been sequenced. The largest cDNA clone (2.4 kb) has been expressed in pGEX and the fusion protein isolated and purified. Current experiments are aimed at examining the immune response to this molecule in cattle and mapping B- and T-cell epitopes.

Surface-labelling studies coupled with surface-specific monoclonal antibodies have identified a series of infection-specific polypeptides on the surface of the schizont-infected lymphocyte. A 118–120-kDa molecule has been identified and shown to be a glycoprotein (Shiels et al., 1989). The genes coding for these polypeptides have not been isolated. Whether these molecules are involved in the recognition of the infected lymphocyte by the host immune system or are involved in the mechanism of transformation remains to be examined.

The merozoite is found relatively transiently in infected animals. In T. annulata it is an important stage due to the high erythrocyte parasitaemia and the associated anaemia. Little is known of the immune response to this stage, although, in principle, if protective immunity could be induced against it, it would prevent erythrocyte invasion and therefore anaemia. Experiments are currently under way to isolate the gene coding for the 30-kDa merozoite surface antigen (Glascodine et al., 1990) and one putative surface antigen gene (Mag1) has been isolated. With these genes and the ability to generate merozoites in vitro (Glascodine et al., 1990), it is now possible to examine the role of the merozoite and its surface antigens in protective immunity; however, no such experiments have been undertaken.

Another possible target for a protective immune response, by analogy with studies on Plasmodium, is the surface of the infected erythrocyte. If the surface of the erythrocyte is altered by infection, antigens could be isolated and examined for their role in protection. No major changes have been detected using a range of surface-labelling and other techniques (Glascodine, 1989), although some tentative evidence
for the presence of parasite-derived molecules was obtained. Further research is required to consolidate these findings.

Overall, some of the reagents (antibodies and gene clones) for examining the role of surface antigens of the sporozoite and merozoite stages in protective immunity have been generated together with the culture methods that allow examination of the merozoite. A number of other surface proteins of both stages await further characterization. These results will allow future studies on the role of the surface antigens of these two stages in protective immunity.

REFERENCES


CONCLUDING DISCUSSION
Concluding discussion

The concluding discussion was chaired by Dr. J.J. Doyle and organized around the reports of the chairmen of each session from the summaries prepared by their rapporteurs. Other issues that the participants felt required further discussion were listed and are shown in Figure 1. The summaries of the sessions and the discussion contain many references to work on Theileria parva that was presented in short papers by ILRAD scientists in parallel with T. annulata papers. These have not been included in the proceedings, but the main points are included in the summaries presented below.

1) Diagnosis
2) Early vaccination (against T. annulata and use of buparvaquone)
3) What is attenuation?
4) Carrier state:
   (a) duration
   (b) maintenance:
      i) schizonts
      ii) piroplasms
5) Genetic resistance
6) Epidemiology
7) Distribution
8) Mild strains

FIGURE 1. Issues listed by Workshop participants for further discussion.

The Chairman (Dr. Doyle) opened the session by calling for the summaries from the sessions on Country Reports, Current Vaccines and Immunology.

Country reports—summarized by Dr. C.G.D. Brown

Reports were presented from China, India, Morocco, Turkey, Sudan and the USSR, representing a broad cross-section of countries in which tropical theileriosis is endemic. In addition, two important papers from Israel and Iran were submitted in written form. The consensus from these reports indicated that:

- the disease was only a problem in imported or improved cattle, predominantly in European dairy breeds;
- disease control could be pragmatically and effectively implemented using a live tissue-culture vaccine of schizont-infected lymphoid cells attenuated by long-term passage;
CONCLUDING DISCUSSION

• of the eight countries reporting, seven now produce their own vaccines with which they have vaccinated between thousands and millions of cattle; and
• to date little emphasis has been placed on other control measures such as vector control, chemotherapy, the selection and use of resistant cattle, management procedures or through an awareness of tick ecology and novel chemotherapeutics. Buparvaquone has proved highly effective in the treatment of the clinical disease throughout the *T. annulata*-endemic zone, while primaquin is used in China. Chemotherapy might have a role in protecting the calf too young to immunize.

Priorities for future work would include a detailed epidemiological study of the disease and its transmission patterns as well as cost-benefit analyses of control measures in different husbandry and production systems. Novel vaccines would, one hopes overcome the cold-chain and delivery problems of the current live vaccine, but these are not expected to improve on the protection afforded. The long-term approach may well prove to be the selection of genetically resistant, moderately productive cattle for the smallholder dairy farm.

Current vaccines—summarized by Drs. S. Williamson and P.R. Spooner

A general discussion was held to summarize the methods of vaccine preparation and use against *T. annulata* in the different countries represented at the meeting. These are summarized in Table 1.

<p>| TABLE 1. Details of cell dose, passage, method of delivery and duration of immunity for different <em>Theileria annulata</em> cell lines. |
|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Dose (number of cells)</th>
<th>Attenuation days in culture (medium changes per week)</th>
<th>Duration of immunity (tick-free conditions)</th>
<th>Delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>India (D.K. Singh)</td>
<td>5 × 10⁶</td>
<td>675 (3)</td>
<td>18 months</td>
</tr>
<tr>
<td>China (Z.H. Zhang)</td>
<td>1 × 10⁶</td>
<td>120-320 (2)</td>
<td>&gt;13 months</td>
</tr>
<tr>
<td>USSR (V.T. Zablitsky)</td>
<td>0.5 × 10⁶</td>
<td>Not known</td>
<td>42 months</td>
</tr>
<tr>
<td>Turkey (F. Sayin)</td>
<td>10 × 10⁶</td>
<td>Not known</td>
<td>Not investigated</td>
</tr>
<tr>
<td>Morocco (H. Ouhelli)</td>
<td>1 × 10⁴</td>
<td>912 (2)</td>
<td>&lt;6 months</td>
</tr>
</tbody>
</table>

STORAGE

Dr. Singh found that vaccine kept for two months at 4 °C produced variable results, although it was agreed that short-term storage at 4 °C (1 month) was acceptable, especially where high cell numbers are used for immunization (1–5 × 10⁶).
CROSS-IMMUNITY

Each of the countries represented used one stock in all areas without any indication of problems with breakthrough infection. Dr. Singh had carried out cross-immunity trials challenging the vaccine stock with six other stocks without breakthrough, although Prof. Grewal mentioned that in one area of India one stock, known as *T. annulata* Parbani, broke through the vaccine stock. Thus, there is a need for some further cross-immunity studies.

DEFINITION OF ATTENUATION

According to Prof. Pipano’s paper, complete attenuation has been achieved when, after inoculation of the vaccine dose, there are no clinical signs and no schizonts, that piroplasms are detected and that there is no transmission to susceptible cattle through ticks. Most participants agreed that piroplasms, and occasionally fever, are observed in vaccinated animals and that a carrier state is established.

CARRIER STATE

Since piroplasms are observed by many workers in vaccinated animals, they are potentially able to infect ticks. One question raised was whether the parasite transmitted to ticks feeding on carrier cattle (cattle carrying piroplasms from the vaccination stock) is infective and virulent. Prof. Grewal reported the transmission of infection from vaccinated to susceptible cattle resulted in infection but not clinical disease. Salivary gland dissection confirmed low infection rates in ticks fed on the vaccinated cattle.

CHALLENGE DOSE

Dr. R.L. Spooner pointed out the importance of defining the challenge dose used to test vaccines since this often varied between research groups. Similarly, the method used to detect carriers should be defined since the sensitivity of detection varies between methods.

Current vaccines for *Theileria parva* (Dr. T.T. Dolan)

East Coast fever (ECF) is presently controlled by the application of acaricides to control the tick vector. This method is becoming less reliable because of the high cost of acaricides, civil unrest, poor management and illegal cattle movements. Therefore, newer strategies are sought to control ECF and other tick-borne diseases. The method of immunization available and applicable for ECF is infection-and-treatment in which live sporozoites (stabilate) are inoculated and the animals...
are treated simultaneously with oxytetracycline. Immunized animals become infected, develop inapparent or very mild disease and immunity and in many instances become carriers.

Once this method has been accepted for use within a country, a decision has to be made whether to use a locally isolated parasite (e.g., Kenya, Zanzibar, Zambia, Zimbabwe) or to use a centrally prepared vaccine that may be a combination of several stocks. This means that a new stock of parasite may be introduced into an area (e.g., Malawi, Zambia). South Africa prevents the spread of *T. parva* from the buffalo population by operating a slaughter policy should any cattle become infected.

The most comprehensive and recent costing of an ECF vaccine (Mukhebi *et al.*, 1990, *Preventive Veterinary Medicine* 9: 207–219) estimates the cost per animal as being US$0.89 for the stabilate dose and US$2.37 for the delivered vaccine. This was estimated on the basis of a 30-year program to vaccinate 2.9 million cattle.

Tools have been developed that demonstrate the antigenic diversity among *T. parva* stocks, e.g., monoclonals and DNA probes, but cross-immunity remains the definitive test in the selection of vaccine stocks as the in vitro tests are not correlated with the immunogenic nature of the stock.

ECF immunization is being implemented with success in Burundi as a national program and in Zambia with donor input. Trials are currently in progress in Kenya, Malawi, Uganda, Zanzibar (Tanzania) and Zimbabwe.

**QUESTIONS**

Dr. R.L. Spooner queried the low number of vaccine doses that had been applied to date and Dr. Singh suggested that farmers were apprehensive because disease reactions occur during immunization. Although few of cattle may react following vaccination, this was not thought to be a main reason for reluctance to implement ECF immunization. Dr. Dolan considered that increased application of ECF immunization depended on acceptance by both governments and farmers and would require a change in attitude concerning strict tick control policies. Dr. McHardy pointed out that costing of ECF immunization should be more a balance between cost of disease and cost of vaccination.

**Diagnosis of Theileria annulata** (Dr. A. Grewal)

The indirect fluorescent antibody (IFA) test is the standard serological test used for detection of antibodies in most countries. A DOT-EIA has been developed in India based on piroplasm antigen and appears both specific and sensitive; anti-*T. annulata* antibodies appeared three to four weeks after immunization. A dip stick method has been developed using the same antigen. The standardized antigen is distributed to research centres throughout India. The test is used for epidemiological purposes in cattle and buffalo to assess the need for immunization and to monitor immunization.
Prof. A. Grewal also summarized his experimental work on *T. annulata* immunization. A novel approach in immunizing calves, designed to overcome the possibility of field infection being contracted before effective immunity could be established, was described. This involved simultaneous inoculation of tissue culture vaccine and buparvaquone (Butalex) at 2.5 mg/kg.

Experiments attempting to immunize with purified piroplasms or infected erythrocytes, with or without adjuvant, were unsuccessful.

**QUESTIONS**

Dr. Brown raised the concern that piroplasms occurring in an animal after buparvaquone treatment could generate a buparvaquone-resistant strain of parasite. The cost of the drug was also mentioned—it is likely to be comparable to parvaquone (Clexon, Coopers Pitman Moore) and would depend on the marketing chains of the companies in individual countries.

**Immunology—summarized by Drs. N. Flynn and J. Naessens**

Dr. Innes summarized the immune responses to the different stages of *Theileria annulata*.

**Tick:** Whilst an inflammatory response has been described at the bite site, the actual role that this response plays in the generation of immunity is poorly understood.

**Sporozoite:** Although this stage of the parasite is exposed to the immune system for only a short period of time, immune serum from cattle exposed to multiple challenges has been shown to inhibit the infection of PBMs *in vitro*. The involvement of humoral factors such as TNF or IFN may also have a role in inhibiting the development of trophozoite to schizont stages in infected cells, but this is poorly understood.

**Schizont:** Once the parasite has matured into a schizont, immunity is not mediated by antibody but by MHC class I-restricted cytotoxic lymphocytes (CTLs). These cells peak around day 9–10 following challenge of immune animals and around day 16 in naïve animals challenged with autologous infected cells. In animals challenged with allogeneic cells, an anti-class I CTL response is observed, peaking around day 9. This response does not prevent immunization, but reduces pathogenicity of the cell line. By day 23, schizont-infected cells have the BoLA phenotype of the recipient, indicating transfer of the parasite from donor to recipient host cells. It is not known how these parasite-specific CTLs are induced, which antigens are recognized, or the role of accessory cells and helper factors.

**Cytokines:** The possibility of the potential role of cytokines, produced by the infected cells, in pathogenicity and development of immunity was mentioned.

**Merozoites/piroplasms:** Immune sera contain antibodies to merozoite/piroplasms and may be involved in blocking transmission. No antibodies to the surface of infected red blood cells have been identified.
CONCLUDING DISCUSSION

The responses to Theileria parva (Dr. P. Toye)

Sporozoites: Sera from immune animals block in vitro sporozoite infectivity. The major antigen recognized is a 67-kDa surface protein, which does not show heterogeneity among different T. parva stocks.

Schizont: The response to the schizont stages in T. parva is also mediated by CD8+ class I-restricted CTLs. The arguments in favour of a major role for these specific CTLs in protective immunity are that no correlation exists between antibody titres and immune status, transfer of immunity can be achieved with lymphocytes, but not with serum, appearance of CTLs coincides with remission and a correlation between in vitro parasite specificity and ability to withstand parasite challenge in vivo.

The major task ahead is to identify the parasite antigens recognized by the CTLs. At present no methodology exists to do this, due to the processing required of the parasite antigens into immunogenic peptides which are presented in association with MHC class I. It may be possible to mimic the processing by co-transfecting bovine MHC and parasite genes into mouse L-fibroblasts, and checking them with relevant CTLs. The choice of appropriate parasite genes could involve all schizont genes or a selection of putative candidates: those that are recognized by antibody or T helper cells, those present on the schizont surface. Evidence was presented that preferential combinations of MHC products and parasite peptides drive the immune responses, and this will complicate the search for relevant antigens.

Dr. Williams mentioned that a further complication could be the existence of different CTL repertoires in different animals. Dr. Toye said that he was not looking for an epitope that drives a CTL response during an in vivo infection, but for any epitope present in all parasites.

The 67-kDa sporozoite antigen has been shown to protect against sporozoite needle challenge in two cattle.

Dr. Spooner talked briefly on comparative aspects of T. annulata and T. parva. Theileria annulata preferentially infects macrophages and B lymphocytes in contrast to T. parva which infects T cells and to a lesser extent B cells, but not macrophages. In an attempt to assess the effect of T. annulata-infection of macrophages on their ability to process and present third party antigens, ovalbumin-specific T-cell lines were generated. Theileria annulata-infected cells presented ovalbumin more efficiently than uninfected antigen presenting cells at a constant cell ratio.

POINTS RAISED DURING DISCUSSION

In response to a question from Prof. Tait on the ability of CTLs to protect, Dr. McKeever said that although CTL responses were 'light and transient' in the peripheral blood, they were quite dramatic in draining lymph. Parasite-specific CTL-precursor frequencies rose to 1/40 cells. Transfer of these cells to non-immune recipients gave protection from sporozoite challenge.

Dr. Brown suggested that the cellular response may be more biased to helper cells rather than CTLs in T. annulata. He mentioned the difference in pathogenesis in the two diseases. Dr. Dolan drew attention to the importance of humoral immunity in T. sergenti, for which a good blood vaccine exists.
Dr. Morzaria suggested that merozoite-blocking vaccines could disturb epidemiological stability by removing the constant re-boosting in a field situation.

Dr. Ole-MoiYoi highlighted that by selecting putative antigens for CTL recognition, one might omit potentially important molecules, such as the nuclear proteins in influenza.

Dr. Williams said that there was growing evidence that processing of antigen and association with MHC products can also occur on the membrane. Therefore, he found it worthwhile to use a random pool of peptides with the right binding properties to screen with immune CTLs.

Prof. Tait asked whether immune precipitation of class I could be used to identify associated parasite peptides, as has been done for class II. Dr. Iams commented that low quantities and many different peptides would be obtained.

Dr. Doyle asked what the role of the carrier state was in maintaining immunity to *T. annulata*. According to Dr. Brown, immunity lasts at least 15 months after infection-and-treatment, and there was some evidence for a schizont-carrier state. Dr. Doyle emphasized the importance of selecting CTLs important in maintenance of the carrier state.

Dr. Williams said that no seroepidemiological studies have been carried out in *T. annulata*-endemic areas; only experimentally infected animals had been studied. A further discussion ensued on the mechanism of schizont transfer. No concrete data could be given. According to one hypothesis, macrophage phagocytosis of parasite material could explain the higher transfer in *T. annulata*.

Discussion of country reports, current vaccines and immunology

*Drs. Doyle and Dolan raised the issue of early vaccination and the observations of Dr. Grewal in which very young cattle could be treated with buparvaquone (Butalex) and immunized with cell-culture vaccine at the same time. The drug did not appear to interfere with the take of vaccine and the animals were immune. Dr. Grewal confirmed that this was the case, that calves of three weeks of age had been successfully immunized and that protection had been demonstrated two weeks later (calves were then five weeks old). These experiments used only small numbers of calves and were often complicated by intercurrent disease, particularly diarrhoea. More data was available on the success of this method in calves of four to six weeks of age—still below the generally recommended age for immunization of two months (Table 1). This work suggests a significant finding for situations where calves are at risk to disease exposure from very early in life and Dr. Brown recommended that this approach should be explored with different parasite stocks in different countries. This could obviate the need to attempt to modify vaccines to cater for very young calves in high challenge environments. Dr. Singh pointed out that in his experience calves immunized below two months of age were susceptible on challenge. The meeting recognized that the drug might be expensive, or unavailable, and that in a practical immunization scheme, calves of various ages would be presented for immunization. Nonetheless, Dr. Grewal's 100-day vaccine stock, the drug and the immune response*
interacted in this circumstance and if it is reproducible it could greatly enhance the effectiveness of vaccination.

Dr. Doyle then referred the workshop to the items attenuation, carrier state and genetic resistance in Figure 1. Dr. Brown mentioned that the work of Prof. Sayin's group in Turkey addressed some of these questions, which would be critical in understanding the epidemiology of T. annulata. He believed that the parasite carrier state in cattle was maintained by the schizont, although there was no hard data to confirm this. Prompted by Dr. Doyle, Dr. Brown agreed that he recognized an association between cell dose (vaccine dose) and duration of immunity. In addition, the longer a parasite was held in culture, the more cells were required to immunize. Dr. Singh had found that when groups of animals were immunized and held under tick-free conditions, then subjected to challenge at 6, 12 and 18 months, immunity was undiminished at 18 months. Dr. Ouhelli explained that in Morocco they visit farms before the start of the theileriosis season to vaccinate calves and newly introduced cattle, but for convenience they vaccinate all cattle. Dr. Singh followed a similar strategy.

Dr. Doyle summarized the discussion to this point. There was likely to be a limit on the duration of immunity and the passage and cell numbers might influence that duration. He wondered what revaccination might do in adult immunized cattle. Dr. Innes explained that she had encountered difficulty with revaccination and suggested that, if the same cell line was being used, a problem of rejection might arise. Dr. Grewal agreed that this was likely to happen. Other participants thought that this might be the case but had no experimental evidence to support it. This was clearly an area in which further work was required, not only to address the duration of immunity following immunization with a particular cell line, but also to examine declining immunity in relation to the seasonality of challenge, tick control and pick up and transmission of the vaccine stock.

Prof. Tait outlined the variables that might be involved in attenuation. One was the transfer of the schizont from donor to recipient cells which might be influenced by the expression of peptides presented on the surface of the infected or recipient cells. The second was the rate of proliferation of cells following transfer—this might lead to attenuation. The third might be the rate of production of merozoites—could this be down-regulated? And finally, it could be in the invasiveness of the merozoites for erythrocytes. He did not think that anyone had looked at these factors and wondered if it should be done. Dr. Singh commented that there was a cellular (blasting) reaction following inoculation of the immunizing cells and a delay (incubation period) before the immunizing infection could be detected. When animals were revaccinated, the cellular response was much reduced. He believed that there was a population of immune cells responding on revaccination and that piroplasms were very few. Dr. Doyle wanted to know if he was rejecting all of Prof. Tait's variables that might influence attenuation but Dr. Singh thought that the rate of multiplication might be affected. Dr. Ouhelli observed that following vaccination, few parasites are seen and they persist for only about two months compared with a year following natural infection (Dr. Pipano's vaccine line was attenuated to the point where it did not produce piroplasms and could not be picked up and transmitted by ticks).

Dr. Doyle then asked if it was worthwhile investigating the kinetics of the vaccine infection, the initial delay before linear transformation of host cells. Prof. Tait thought
that the main question was to work out what was actually happening in attenuation. Dr. Doyle compared the immune response to that in T. parva, where there are probably very similar cytotoxic T-cell responses. He then asked how many of the million or so cells were functional in the vaccine and what they did. Dr. Singh thought that 25–30% might be all that survived. Dr. Williams asked what if just 100 cells survive—what then? Dr. Doyle wondered if these cells could be identified and why they transfer the infection. Dr. Williams asked what you would do with that information and Dr. Doyle replied that you could see how it relates to cell type, the numbers of cell types or clones you have transferred infection to, then how it affects carrier state and the duration of immunity. It might also reduce the number of cells needed to vaccinate—What do people who use these vaccines think about these possibilities? Dr. Morzaria thought that by maintaining cultures for a long time, you might select parasite strains that do not produce merozoites. Dr. Doyle agreed, but most of the people at the workshop who use vaccines reported the development of piroplasms. He asked if the technologies are available to look at what is happening in the attenuation process and if there were some kind of recommendation from the meeting as to whether those using vaccines wanted to know the mechanism(s) of attenuation. Dr. Ouhelli thought that it was academically interesting although he agreed that if we knew and could use the process, it might speed up attenuation, which in some cases had taken three to four years to develop. Dr. Osman considered this a viable area for research.

The Chairman then asked the following questions of those countries which might plan to use vaccination in the future. Would they use existing vaccines from other countries as the evidence for antigenic differences in T. annulata was not strong, or would they envisage political problems? If they are to produce their own vaccines, should they not start with mild strains that already exist in their country so that they would not have to address the problem of attenuation either by passage, mutagenesis or by the use of technologies to understand the genetic changes that are taking place? Dr. Singh recalled literature reports that suggested that virulent strains were more likely to provide broad protection than mild strains but he had no experience to support this. Dr. Brown considered the political implications of live vaccines as important. While it is relatively easy to produce a 'new' vaccine, the question of safety in moving a live vaccine from one country to another, or even within one country, might raise important disease control issues.

Dr. Doyle summarized the three sessions and the discussion. There is a vaccine that is effective but there are things we don't know about it at various levels and people are working on these, either in the countries themselves or in collaboration with the Edinburg and Glasgow groups. There seems to be consensus that this work is justified and should continue. The political issues and questions of safety regarding live vaccines suggest that countries will continue to produce their own vaccines and this work should be funded. Regarding the flow of knowledge or technologies from work on T. parva to T. annulata, there is no advantage or special knowledge in one parasite or the other as to why potentially susceptible cells remain uninfected in carrier cattle. We do not know where T. annulata-infected cells are likely to survive, although sites of low immunological surveillance have been identified for T. parva. Therefore one can only encourage communication in these areas where progress with one parasite might be translated to the research effort of the other.

The Chairman then called for the summary from the session on Biology.
CONCLUDING DISCUSSION

Biology—summarized by Drs. P. Gardiner and N. Murphy

Because of the extensive polymorphism within isolates of the *T. parva* species complex at the monoclonal antibody and immunization level, it was decided to investigate the genetic basis of this diversity (presented by Dr. S.P. Morzaria). In restriction digests using rare cutting enzymes, it was noticed that there was a limited number (approximately 30) of fragments. There was some correlation between *SfiI* restriction patterns and previous methods of classification by immuno-sitivity of stocks. It was further thought that such fragments could be used to determine the variability in antigenicity. Thus the goal was to determine the basis of the *SfiI* polymorphisms, provide a set of mapped markers for genetic recombination studies and locate the antigen genes that may be responsible for protection or stock characterization. The generation of a map should allow for the generation of linkage groups and then facilitate the mapping of unknown genes.

The problems in map generation were co-migrating *SfiI* fragments (resolved by the use of stocks that were polymorphic at that site) and partial *SfiI* digestions with preferential digestion of some sites. The use of probes helped generate the map and determine chromosome number, which was confirmed through the use of a telomeric probe. Polymorphisms were located predominantly in telomeric regions.

The genome size of *T. parva* is $1 \times 10^7$ bp. There are four chromosomes, generating 30 *SfiI* fragments and 7 *NotI* fragments. The linkage map has been generated but fragment 12 is still to be assigned. Mapped genes include identified antigen genes, the actin gene, heat shock genes and cDNAs of unknown function.

*Theileria annulata* also has four chromosomes with a small number of *SfiI* fragments and, by analogy to the above, a map of the *T. annulata* genome could be generated quite readily. It was stated that from measurements of DNA content by cytofluorimetry of sporozoites and piroplasms of *T. annulata*, these life cycle stages were likely to be haploid, and this is also implied by the mapping data.

The differentiation of *T. annulata* in vitro from the schizont stage to merozoites has been examined. Using a number of monoclonal antibodies (MAbs), stage-specific antigens have been identified and the timing of their appearance determined in the above differentiation sequence. Cloning from established cell lines infected with *T. annulata* yielded two types of clones: one that showed an enhanced ability to transform to merozoites at 41 EC, and another that showed a reduced ability. The interest will be in the stage-specific expression of genes governing this transformation in the two types of cloned line. The phenotype of the original, infected cell was unknown, but both types of cell lines responded by the induction of the hsp70 transcript by temperature stimulation. Although the high-producing line could not be induced to produce piroplasms regularly in vitro, infection of animals with these lines produced piroplasms very quickly, even before schizonts were detected.

This area has not been investigated in *T. parva*, partly because the production of piroplasms is of lesser importance in the pathogenesis of disease caused by this organism. Studies, if they were initiated, would probably be different in serological reagents, although it is possible that at the DNA level useful homologies may exist.

Dr. Bishop, complementing the early approaches to differentiate parasite stocks of *T. parva*, described DNA probes that have been developed and that recognize a
variety of moderately or highly repetitive sequences. These repetitive DNA probes (the same sequences have been identified at ILRAD and Cambridge) have been able to distinguish *T. parva* from *T. annulata* and *T. taurontragi*. The highly repetitive sequence is extremely polymorphic amongst *T. parva* stocks and gives each a distinctive fingerprint. However, it is difficult to identify immunological relationships among these stocks with these probes.

A second generation of probes was generated using primers to the original sequence in a polymerase chain reaction. These have been used for carrier state detection and stock-specific probes have been generated. These are now being used to examine the occurrence of sexual recombination in *T. parva*. The probe sequences detect large open reading frames (ORFS) arranged in tandem arrays and it is of interest to identify the encoded protein and determine its function since this molecule is so polymorphic among stocks.

Further probes have been developed that give simpler patterns on restriction digests of various theilerial DNAs: e.g., small ribosomal subunit pattern (three RFLPs have been identified); antisense probes to ribosomal RNA subunits have distinguished stocks and species (including *T. annulata* and buffalo-derived *T. parva*); and telomeric sequences (conserved in sequence, but differing in hybridizing restriction fragments in all stocks examined). The latter probe may be useful in examining relatedness of isolates.

To date, such analyses have not seemed important for tropical theileriosis because of the widespread applicability of the attenuated vaccine for *T. annulata*. However, if recombinant vaccines were to be contemplated, given the individual phenotypic heterogeneity of *T. annulata* isolates (see below), characterization by similar methodologies would become important. At present the reagents available for distinguishing stocks of *T. annulata* are MAbs, two stage-specific genes (*Mag* 1 and *Spag* 1) and glucose phosphate isomerase (GPI) type. The *Spag* gene reveals eight alleles and when all characteristics are combined, all isolates examined show high variability.

At ILRAD, to avoid the problems associated with (i) multiple infections of lymphocytes with sporozoites and (ii) more than one infected tick acinar cell, scientists have adopted the following cloning technique for *T. parva*. Sporozoites are titrated and cultures established from a dilution of one sporozoite to at least 100 lymphocytes. Infected lymphocytes are doubly cloned by limiting dilution, then used to infect the autologous cell donor and stabilates are made from ticks after pick-up of the infection. The time-scale for this protocol is at least a year. It was suggested that sufficient markers were available at ILRAD to distinguish if lymphocytes infected with more than one schizont might contribute to inaccuracies in the more rapid cloning of schizont-infected lymphocytes.

Differences in cloning methodology were discussed and later work (see the discussion of the *Spag* 1 gene) demonstrates the necessity of efficient cloning procedures when analysing polymorphic genes. In stocks of *T. parva* that proved to be genotypically heterogeneous, one clonal type tended to predominate in the stock. Despite high variability in the *T. annulata* stocks, cross-protection was still noted amongst the majority of stocks except the Parbani, Razi and Tara isolates.

The subject was raised as to whether studies of differentiation, as it affected parasite attenuation *in vitro*, could be valuably extended (for both species but particularly for *T. annulata*). Passage number and other factors affecting attenuation...
should probably be identified for the more efficient production of the *T. annulata* vaccine material. The Tova (virulent) isolate could be fruitfully examined vis-a-vis *T. parva* and mild *T. annulata* isolates to identify such factors and possibly attenuation markers.

Dr. Williams considered the identification of parasite factors that may govern the transformation of the host cell by theilerial parasites upon infection. Examination of parasites for oncogene products yielded negative results. However, cells were responsive to cytokines IL-2, IL-4 and (erratically) γ-interferon. The IL-2 receptor was found to be constitutively expressed. Similarly, a protein regulating factor for expression of the IL-2 receptor has been identified and found to be constitutively expressed.

Reasoning that other inductive factors are either secondary or membrane proteins of the parasite, an assay has been developed to identify such proteins as direct products of a plasmid library of *T. parva*. Ten such products have been identified (many thousands of plasmids remain to be screened), but none has yet been associated with the transformation process.

Dr. ole-MoiYoi reported that *T. parva*-transformed cells express elevated levels of bovine casein kinase-II, which can phosphorylate both endogenous and exogenous substrates, but also, probably, a similar parasite enzyme. The latter was identified through its similarity to the *Drosophila* β-subunit gene, but the α-subunit has not been detected. The β-subunit of the parasite enzyme has a unique 78 amino acid tract, but whether this is concerned with membrane binding is unproven.

It is not clear whether the induction of the host and parasite enzymes, which may have the ability to phosphorylate oncogene products, are related to the initiation of the proliferative state of host cells or to the maintenance of that state. However, phorbol-esters can activate casein-kinase II and Dr. Williams has shown that in the presence of phorbol-esters and IL-2, the cell lines generated following the removal of the parasite can be made to proliferate for up to six months.

In the case of *T. annulata*, infected-PBLs have been shown to secrete growth factor(s) but these have not been identified. There are also infection-specific tyrosine and serine/threonine kinases produced by the BL-20 cell line infected with this parasite. The Tova (virulent) isolate of *T. annulata* has a single endogenous target of 116–120 kDa for casein kinase II rather than the five such molecules seen in *T. parva*. These studies could continue in both parasites, but it might be more fruitful to choose a single system for such studies. The fundamental difference between the two parasites might be either in their selection of target host cells or in their fundamental transformation capacity in vivo; *T. parva* is generally found in T cells, *T. annulata* in B cells and macrophages. Other cell types can take up *T. parva* sporozoites, but development of the parasite in "unusual" cells is rare. B cells may develop more slowly and it has been noted that *T. parva* infection of B cells leads to milder reactions on injection into cattle.

The discussion of the points indicated that definition of host cell type and transformation of those cells both in vitro and in the host were all areas that would be worth studying. Similarly, it would be of interest to know whether the two parasites both generate and react to cytokines in the same way, and whether those induced by the other species helped determine growth and transformation functions.
Prof. Tait identified genes in *T. annulata* that were of current interest and detailed the structure of the merozoite (*Mag* 1) and sporozoite-specific (*Spag* 1) genes as far as they are known.

The *Mag* 1 gene, which is expressed in merozoites and piroplasms but not in other stages, contains three types of repeat motif and is reminiscent of some plasmodial genes. It may code for a surface (glyco) protein.

*Spag* 1 was identified from a genomic library generated from an uncloned *T. annulata* parasite. The cDNA and genomic nucleotide sequences so far determined differ, suggesting that the sequenced cDNA is derived from another, closely related allele of the *Spag* 1 gene. Nothing is known about RNA processing of the transcript, but the existence of an intron within the gene suggests that this occurs in repeats with high homology to that found in the elastin gene. It is speculated that *Spag* 1 is the *T. annulata* surface molecule responsible for sporozoite binding to host mononuclear cells and macrophages, but not T cells expressing elastin receptors.

The *T. annulata* merozoite antigen is not simply a product of *in vitro* cultivation as it is recognized by infected sera. The relationship of merozoites produced *in vitro* to those *in vivo* is not known.

Dr. Iams described antibodies that identify the major *T. parva* sporozoite antigens. A 104-kDa molecule, the antibody to which does not neutralize sporozoite infectivity, is a component of rosettes. A 67-kDa molecule that induces a good neutralizing antibody is encoded from two open reading frames separated by a small intron and it occurs on the surface of the sporozoite.

An incomplete clone that is thought to define a 30-kDa and a 44/48-kDa sporozoite protein is presently being sheared so that the immunogenicity of peptides derived from fragments can be analysed for binding of antibody raised to the larger molecules.

The gene for an 85-kDa antigen that is immunodominant in the induction of 'infection' sera and monoclonal antibodies has eluded numerous library screens.

No discussion was held of possibly common 'house-keeping' genes or the mitochondrial elements found in both species. The identified antigen genes would appear to be quite different and this may accurately reflect the two parasites' selection of different host cells.

Given the differences in host cell choice and production of piroplasms (and subsequent pathology), fundamental differences in surface antigens and maturation antigens might be expected between the two theilerial species, but it is recommended that both immunologically and biologically based investigations be continued in efforts to identify alternative targets for vaccination. Such antigens are required now for *T. parva* and may ultimately be required for *T. annulata* when recombinant vaccines produced in bulk may supplement the attenuated vaccine for this parasite and might surmount the problems of the cold chain requirements and the problems of immunizing young animals.

It is clear from the work presented at this session that the approaches taken to identify antigen genes of interest and to study parasite differentiation and transformation are similar in both parasite species. The reagents and technologies are also similar although the states of knowledge in certain areas are different. In this context, four areas that can be considered are genome mapping, differentiation, antigen genes and transformation.
CONCLUDING DISCUSSION

There is a range of *T. annulata* (gene clones and monoclonals) recognizing isolates and clones that could be developed as diagnostic and epidemiological tools. The contributions that ILRAD could make to these studies therefore are primarily in genome mapping and transformation, but, perhaps as importantly, ILRAD could promote improved exchange of personnel, material, reagents and information between the 'annulata' and 'parva' fraternities.

The Chairman thanked Prof. Tait and then called for the reports on Epidemiology and Modelling.

Epidemiology—summarized by Dr. J. Delehanty

Dr. Perry described the mandate and the work of ILRAD’s Epidemiology and Socioeconomics Program. This Program was established quite recently as an adjunct to ILRAD’s basic biological research on animal diseases and has concentrated, up till now, on the epidemiological, socioeconomic and environmental contexts of *T. parva* and its control.

The basic assumption underlying the Program’s research to date is that theileriosis affects different regions and different production systems differently. We do not really know the magnitude of this variable effect in different African systems, yet we ought to. Even where we have a good idea of severity, our knowledge of disease impact, particularly economic impact, tends to be, at best, anecdotal. It follows then that in some systems a new method of controlling theileriosis will have profound and positive effects while in other systems a given method will have little effect, or actually be deleterious in one way or another. We do not really know where, in Africa, a given control method could be applied to positive effect, and where it would be inadvisable, yet we ought to. For this reason, much of the work of the Program, thus far, has been explicitly geographic. Relying heavily on geographic information systems, an attempt is being made to assemble data and compile maps that will describe spatial variability in disease risk, disease effect, and the potential impact of better control. Not all variability is spatial. An integral part of this work is to index non-spatial considerations or factors within the spatial matrix.

Work is being conducted at three levels of resolution. At the smallest scale are farm- and district-level studies, all in Kenya. Here, detailed descriptions of disease epidemiology, farm management, farm economics and the social context of cattle production are being assembled in the expectation that the dynamic descriptions assembled, and the control recommendations derived, can be projected to similar regions elsewhere. At a middle level of resolution, country-level studies have been undertaken in Zimbabwe to assess both the biological and the economic efficacy of current tick-borne disease control strategies, and to determine where, and under what conditions, these could be modified to positive effect. Finally, at a continental scale, various geographic models of vector and disease suitability (such as CLIMEX and BIOCLIM) have been run in order to test their predictive powers and assess their utility for determining where improved control is warranted. These latter activities are just a first step in a broad campaign to assemble and analyse, for Africa as a whole, a range of data, including economic and environmental data, that will allow us to assess patterns of current disease impact and the potential impact of control.
Three major points of discussion were raised in response to Dr. Perry’s presentation. First, concern was raised over research costs. Can the local-level studies described here be replicated cost-effectively in all areas, or are we not better off abandoning concern for small-scale variability in disease risk and vaccinating everything? These concerns are legitimate, but Dr. Perry stressed in response that studies of this sort are not contemplated on a broad scale. Projections from a few carefully chosen regions to a broader universe is precisely the point of the exercise.

Second, it was pointed out that the databases and analytical procedures being developed in conjunction with this work will be widely applicable to studies of the control of a host of livestock diseases. Fundamentally, these data and procedures are not specific to theileriosis or to *T. parva*.

Modelling—summarized by Mr. J. Scott

A wealth of information exists on East Coast fever, which has been reported in the literature for over 80 years. Relationships and rules can be extracted from empirical findings and it is possible to use them to construct a model of the interactions among ticks, cattle and *T. parva*. Using meteorological and other data, a model can explain the pattern of tick population dynamics and disease outbreaks in different areas. For example, results of computer experiments using the ECFXPERT model indicate that carrier state is necessary to maintain disease. Without carrier state, ticks did not develop infection. It was noted that superinfection is poorly reported in the literature and further information is essential for inclusion in the model.

Models can be classified according to the type, the site, the biological level and function and precision of the model. The use of index models and artificial intelligence models should be explored for other theilerioses. Looking into the future, geographical information systems may be combined with these classical techniques to give rise to integrated geographical information systems that might provide a high prediction power. Another type of mathematical model might be chaos, whereby small changes in inputs to the model ripple through and produce very different outputs from what we might expect.

Four main points were raised in the discussion: carrier state, models, chaos and parasitaemia.

1) Carrier state is important in the model and wildlife provide large reservoirs for the disease. These now appear to be accepted dogmas in the epidemiology of East Coast fever.

2) Regarding chaos, one way to determine whether a system was chaotic or not is to vary inputs and to see what kind of output is produced.

3) Regarding models, one way to improve them is to adjust parameters to try to improve the fit to data that we have to work with. With complex models, it is also noted that one of the major problems with ECFXPERT, and other types of models, is that if we want to make radical changes in the model, we have to change the computer code, which is time-consuming and there is no guarantee that it will work. It is hoped that the new generation of expert systems, artificial intelligence and
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simulation models will make such changes a lot easier and that models can be generated a lot faster.

4) Parasitaemia in relation to tick infection needs to be much better understood for incorporation within infection models.

Finally, how do the geographic models described by Dr. Perry fit with the dynamic disease models developed and described by Dr. Gettinby? To which approach should researchers turn? On this question, Dr. Gettinby and Dr. Perry concurred. These are two approaches to the same questions, coming from opposite directions. Ultimately, they are reconcilable and may prove mutually dependent.

Dr. Gettinby’s models of disease dynamics are driven by the site-specific determinants of parasite behaviour. Dr. Perry’s models attempt to outline the variable contexts of disease occurrence and disease cost; in other words, what goes to determine site-specificity. Each approach uses, and may require, the other. The question remaining is this: what are the minimum data sets necessary to do both well?

Dr. Gettinby commented that epidemiological data from \textit{T. annulata} can be input into a model such as ECFXPERT and many of the questions discussed such as the consequences of carrier state or of different forms of vaccination in relation to transmission could be addressed. He believed that it would be short-sighted to concentrate on epidemiology and not on both epidemiology and vaccination. The relationships of these two have to be put into empirical models if we are to link the life cycle of the tick to the transmission of disease.

\textit{The Chairman} opened the discussion on epidemiology by asking those involved in vaccination how much they wished to know about the epidemiology of the disease. He had heard questions raised about the detection of disease in water buffalo and indigenous cattle. What more did people wish to know about the susceptibility of these animals and how did they plan to get it?

Dr. Grewal thought that PCR-based DNA probes would be extremely useful for studying carrier state parasitaemias. Dr. Singh commented that the extent of the disease is not known and wondered if it would be possible to distinguish between immunizing and natural infections. Dr. Brown thought that some very detailed studies, such as those described by Dr. Perry for \textit{T. parva} (in Kaloleni in Kenya) would be important. Such an exercise might address the problem as it affects the poorest farmer and provides economic data. If it is shown that these very poor farmers cannot afford vaccination, what could be done to provide vaccination for their cattle? This could be done effectively only with efficient epidemiological and immunological tools. Dr. Ouhelli considered that epidemiological studies should include other tick-borne diseases and that sensitive specific tests for \textit{Anaplasma} and \textit{Babesia} were essential in investigating and controlling \textit{T. annulata}.

Dr. Doyle agreed with the importance of all the points raised, particularly the identification of vaccine strains in the natural pool of parasites. This of course was more difficult where locally produced local isolates (strains) were used for vaccination. Nonetheless, the technologies for determination of genetic changes in vaccine strains were available. It was apparent also that many reagents were available which might prove to be efficient epidemiological tools and the expertise was available to develop them in a practical period of time. All that is required is a decision that it is important and that it should be done. In Dr. Brown’s opinion,
CONCLUDING DISCUSSION

these were very difficult components to write up within a three-year project. These were reasonably long-term ambitions and the question was how to persuade donors to provide long-term support. Dr. Doyle agreed that this was a problem, but clearly those countries applying vaccination appreciate the priority need in developing technologies to sustain vaccine development and application.

Dr. Singh raised the suspicion that there is some degree of immunosuppression following vaccination. It was pointed out that this could be addressed by direct experimentation. The relationship of age, nutritional status, intercurrent disease and vaccination with other vaccines such as rinderpest and foot-and-mouth could be experimented upon.

Dr. Doyle then asked Dr. Spooner (R.L.) for his views on genetic resistance. Dr. Spooner remarked that there is some evidence for genetic resistance to disease and much more for resistance to ticks. Disease resistance may be more important in the case of T. parva. As these diseases are dose dependent, tick resistance may have a significant effect if ticks are not heavily infected. Together with Dr. Brown, he had been reviewing the literature on resistance to theileriosis, which he believed was very limited. They had made proposals to do specific experiments to look at epidemiology and genetic resistance and to try to separate disease and vector resistances. Dr. Brown reported that they had just completed a small experiment with Sahiwal animals. This breed is being crossed with high-producing breeds, particularly Jerseys, in New Zealand and then being exported to Southeast Asia. This type of animal is what World Bank projects are planning to use for the long term in countries such as India. The results show that Sahiwal are more resistant than Ayshire or Holstein breeds. The literature from India reports that indigenous cattle are resistant to tropical theileriosis but there are an equal number of reports that describe their susceptibility. This needs to be defined for planning breeding programs where T. annulata is a problem. Dr. Dolan pointed out that there is clear evidence of decreased susceptibility to T. parva in endemic areas and that in the country reports delivered at this workshop most countries reported that the cattle at risk to T. annulata were the imported high-producing stock.

Dr. Doyle felt that this area required much more study using the best epidemiological tools available. The suggestion that buffalo and indigenous cattle are susceptible, or at least not as resistant as previously thought, means that the problem may be much greater than has been assumed. The selection for resistance and identification of the resistance trait, away from any environmental noise, will be expensive and time consuming. ILRAD's trypanotolerance program is an example. The work being undertaken on mapping the bovine genome over the next five to seven years will identify linkages which may be useful—but that is not available now. The epidemiological research at ILRAD, developing improved epidemiological tools, using GIS and beginning to look in depth at theileriosis in eastern and southern Africa in different epidemiological regions and in different farming systems, will establish methodologies that will be applicable in other disease situations, particularly tropical theileriosis. However, our knowledge of disease impact and its economic cost is extremely poor and ILRAD's approach using these newer technologies is justified in attempting to put real costs on these very significant problems.

The Chairman then called for the summary of the session on novel vaccines
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Novel vaccines—summarized by Dr. M. Sileghem and Mr. N. MacHugh

The need for alternative vaccine strategies has arisen because of the disadvantages of the existing methods of immunization against *T. parva* and *T. annulata*, namely, the viability of cells and sporozoites, the need for a cold chain system, the parasite heterogeneity, the use of live parasites and the induction of a carrier state. In immune cattle, both antibodies to the sporozoite stage of the parasite and cytotoxic T cells specific for schizont-infected cells can be demonstrated. Subunit vaccines could be used to exploit either or both of these immune systems.

**SUBUNIT VACCINES**

The idea of constructing subunit vaccines that affect different life cycle stages of the parasite and eventually trigger different components of the immune system was proposed by different speakers. Immunity to sporozoites can be achieved by antibodies; however, high titres are required and a low number of escaping sporozoites can initiate infection. Therefore, a subunit vaccine affecting both sporozoites and merozoites was proposed by Prof. Tait for *T. annulata*. With this vaccine approach, the few sporozoites escaping destruction could still differentiate into schizonts but would be unable to differentiate into piroplasms. Immunity to both sporozoites and merozoites are mainly mediated by antibodies specific for surface molecules.

Another subunit vaccine directed to sporozoites and schizonts was proposed by Dr. McKeever for *T. parva*. The immunity to sporozoites would be achieved through antibody induction whereas the immunity to schizonts would be achieved through activation of cytotoxic T lymphocytes (CTLs).

**DELIVERY SYSTEMS**

It is well known that T cells do not recognize native antigen but are triggered by a processed molecule which is co-presented with MHC molecules. The processing of antigens that associate with the MHC class I molecules and trigger CTLs is thought to take place in the cytosol. To trigger T cells, it is thus essential to deliver the antigen in the correct way. This can be achieved by the use of pH-sensitive liposomes, which dissolve after internalization and guarantee a cytoplasmic delivery, or by the use of ISCOM particles. ISCOM particles are constructed of cholesterol, lecithin and Quil A and have been shown to induce a strong CTL activity in other systems. Another suitable system is the construction of lipopeptides by conjugating the peptide antigen to a lipid ‘tail’. Finally, the gene encoding the antigen can be delivered after integration in recombinant viruses or bacteria. The best known systems for this type of delivery are vaccinia, herpes, adenoviruses, *Salmonella* and *Escherichia coli*.

Dr. McKeever gave an example of antigen delivery using a recombinant virus. The gene encoding the 67-kDa surface protein from *T. parva* sporozoites was cloned and inserted into a vaccinia virus. Rabbits and guinea pigs were challenged with $10^6$ and...
5 x 10^7 plaque-forming units, respectively. In both cases, a strong response to the p67 was observed as determined by Western blotting. Both antisera were capable of blocking the in vitro infection of lymphocytes by sporozoites. This blocking effect, however, was only seen at high antiserum titres.

In a preliminary experiment, cattle were challenged with the recombinant virus. The antiserum was positive for p67 recognition and blocked in vitro sporozoite infection at a 1:4 dilution. Out of two animals, one also manifested a strong T-cell proliferative response to the purified p67.

Both Prof. Tait and Dr. Musoke presented data on the identification and cloning of genes encoding antigens that induce sporozoite neutralizing antibodies in cattle. In the *T. annulata* study, the gene encodes a polypeptide of approximately 104 kDa. Immunization with a recombinant antigen produced using this gene conferred protection against challenge with *T. annulata* in one animal. It failed to protect another. In a further study, animals immunized with lysed sporozoites injected with either saponin or Freund's adjuvant showed delayed onset of disease when challenged. Dr. Musoke presented data on immunization with recombinant antigen produced using a gene encoding a 67-kDa polypeptide. Two animals were immune to challenge with a 1/100 dose of *T. parva* stablate 3087, but one animal had a severe response to a tenfold higher dose while the other showed a less severe reaction.

POINTS RAISED IN DISCUSSION

Prof. Tait suggested that the 104-kDa recombinant could be used to map potential B- and T-cell epitopes. Evidence in *Leishmania* suggests that single epitopes can be protective.

Closing remarks

*Dr. Gray, Director General, ILRAD, thanked all the participants for their vigorous commitment during the three days of the workshop. He felt that ILRAD had obtained a thorough update on the extent of the tropical theileriosis problem and the current state of research and control. This will help significantly in ILRAD's planning for the quinquennium 1993–1997. He recalled an EEC-funded meeting in Edinburgh a few years ago which had addressed many of the problems discussed this week. He thought that it would be useful for the authors of the report on this workshop to review the conclusions of that meeting to see what progress had been made and if the recommendations for future work remained the same. He was impressed with expertise on *T. annulata* that existed in and around Nairobi. He thanked Dr. Doyle whom he felt chaired the concluding discussion particularly well and he thanked the participants once again and wished the visitors an enjoyable continuation of their stay in Kenya.*
CONCLUSIONS
AND RECOMMENDATIONS
Conclusions and recommendations

- Cell-culture vaccines for use in immunization against *Theileria annulata* are effective and widely used and any novel vaccine that is developed will have to be of comparable efficacy and as economical to produce.

- Standards are required for assessing the immunizing capacity of individual vaccine cell lines (strains) and for ensuring that vaccine lines are free from other bovine pathogens.

- The widespread protection provided by individual isolates suggests that antigenic diversity is not a significant problem with *T. annulata*. Collaborative cross-immunity studies could identify the most widely protective cell lines and the use of these would assist in standardization, quality control and safety testing.

- The duration of immunity varies between vaccine cell lines and may be related to passage number, cell dose, individual parasite idiosyncrasy and age or nutritional state of the animal. Further work is required to define the duration of immunity with important immunizing stocks, to identify the need for, and frequency of, re-immunization and to investigate the possible rejection of the immunizing cell line following repeated immunization.

- The mechanism(s) of attenuation is unknown and an understanding of the molecular basis of this process could facilitate the rapid production of non-virulent parasites that would meet the strictest standards for vaccines related to immunizing capacity, carrier state and transmissibility.

- The chemotherapeutic drug buparvaquone has an important place in integrated control of *T. annulata* and its potential in protecting calves below the age of two months from natural disease challenge, while apparently facilitating the development of the cell-culture-vaccine-induced immunity needs thorough investigation.

- The nature of the immune responses to all stages of *T. annulata* requires further elucidation to assist in the identification of the major protective antigens for exploration in novel vaccines that will provide effective, long-lasting immunity that rivals or exceeds that of the current live-cell-culture vaccines.

- Current research on the antigens of the sporozoite and merozoite stages of *T. annulata* should be pursued and a comparison of the sporozoite surface antigen, *Spag*., of *T. annulata* and the p67 antigen of *T. parva* should be undertaken.

- The epidemiology of *T. annulata* is poorly understood and work needs to be undertaken to define the extent of tropical theileriosis, to measure the susceptibility of cattle of different types and ages and Asian buffalo, to identify immunogenic types (strains) of the parasite circulating in the field, and to study the persistence of infection in animals and ticks and the antigenic nature and transmissibility of these parasites.
CONCLUSIONS AND RECOMMENDATIONS

- Although tropical theileriosis is known to extend from North Africa to China, few studies have been undertaken of the economic importance of the disease, on the mortality and production losses or on the cost of control. This is an important area of study that must be addressed to determine the economic impact of tropical theileriosis and to direct conmensurate resources towards more effective control.

- ILRAD's immunological expertise and approaches to molecular vaccines, its geographical information systems and epidemiological and socioeconomic skills are all of potential benefit to research and control of *T. annulata* and should be exploited in collaboration with, and in the transfer of relevant technologies to, national and international laboratories working on tropical theileriosis.
APPENDIX:
LIST OF PARTICIPANTS
Workshop participants.
List of participants

Brown, C.G.D.  
Center for Tropical Veterinary Medicine  
University of Edinburgh  
Easter Bush  
Roslin, Midlothian EH25 9RH  
UK

Delehanty, J.  
International Laboratory for Research on Animal Diseases  
P.O. Box 30709  
Nairobi  
Kenya

Dolan, T.T.  
International Laboratory for Research on Animal Diseases  
P.O. Box 30709  
Nairobi  
Kenya

Doyle, J.J.  
International Laboratory for Research on Animal Diseases  
P.O. Box 30709  
Nairobi  
Kenya

Flynn, N.J.  
International Laboratory for Research on Animal Diseases  
P.O. Box 30709  
Nairobi  
Kenya

Gardiner, P.R.  
International Laboratory for Research on Animal Diseases  
P.O. Box 30709  
Nairobi  
Kenya

Gettinby, G.  
Department of Mathematics  
University of Strathclyde  
Livingstone Tower  
26 Richmond Street  
Glasgow G1 1XH  
UK

Gray, A.R.  
International Laboratory for Research on Animal Diseases  
P.O. Box 30709  
Nairobi  
Kenya

Grewal, A.S.  
Department of Immunology  
Tick-Borne Diseases Research Laboratory  
College of Veterinary Science  
Punjab Agricultural University  
Ludhiana 141 004  
India

Iams, K.  
International Laboratory for Research on Animal Diseases  
P.O. Box 30709  
Nairobi  
Kenya

Innes, E.A.  
International Laboratory for Research on Animal Diseases  
P.O. Box 30709  
Nairobi  
Kenya

MacHugh, N.  
International Laboratory for Research on Animal Diseases  
P.O. Box 30709  
Nairobi  
Kenya
LIST OF PARTICIPANTS

McHardy, N.
Coopers Pitman-Moore
Berkhampsted Hill
Berkhampsted Hertfordshire
HP4 2QE
UK

McKeever, D.J.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Morzaria, S.P.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Murphy, N.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Musime, J.
Organization of African Unity/Inter-African Bureau for Animal Resources
P.O. Box 30776
Nairobi
Kenya

Musoke, A.J.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Naessens, J.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Nderitu, C.G.
Kenya Agricultural Research Institute
P.O. Box 57811
Nairobi
Kenya

Nene, V.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Ngulo, W.
Ministry of Science & Technology
P.O. Box 30568
Nairobi
Kenya

ole-MoiYoi, O.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Osman, O.M.
Veterinary Research Administration
P.O. Box 8067
Khartoum
Sudan

Ouhelli, H.
Institute Agronomique et Vétérinaire Hassan II
B.P. 6202
Rabat-Instituts
Morocco

Sayin, F.
T.C. Ankara Universitet Vetiner Fakultesi Protozoologi Entomologi
Irfan Bastug
Caddesi Dikapi
06220 Ankara
Turkey
LIST OF PARTICIPANTS

Scott, J.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Shiels, B.R.
Wellcome Unit of Molecular Parasitology
Department of Veterinary Parasitology
Beardsden Road
Glasgow G61 1QH
UK

Singh, D.K.
Animal Diseases Research Laboratory
National Dairy Development Board
Anand 338 001
Gujarat
India

Spooner, P.R.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Spooner, R.L.
Edinburgh Research Station
Institute of Animal Physiology and Genetics Research
Roslin Midlothian EH25 9PS
UK

Tait, A.
Wellcome Unit of Molecular Parasitology
Department of Veterinary Parasitology
Beardsden Road
Glasgow G61 1QH
UK

Toye, P.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Wamukoya, J.P.O.
Ministry of Livestock Development
P.O. Box 34188
Nairobi
Kenya

Williams, R.O.
Kernforschungszentrum
Institut für Genetik und Toxikologie
Postfach 3640
75 Karlsruhe 1
Germany

Williamson, S.
Kenya Agricultural Research Institute
P.O. Box 32
Kikuyu
Kenya

Zablostsky, V.T.
The All-Union Institute of Experimental Veterinary Medicine
Viev
Kuzminki 109472
Moscow
USSR

Zhang, Z.H.
Science and Technique Development Company of Ningxia
Animal Husbandry and Veterinary Institute of Ningxia,
Academy of Agricultural and Forestry Sciences
Yinchuan, Ningxia
People’s Republic of China