THE IMPACT OF THE INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

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2 Control of Pathogenesis in African Animal Trypanosomiasis: A Search for Answers at ILRAD, ILCA and ILRI, 1975–2018

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 The Impact of the International Livestock Research Institute (eds J. McIntire and D. Grace)

Executive Summary

The problem

African animal trypanosomiasis (AAT, also known as 'animal African trypanosomiasis') is a serious disease of the tropics and subtropics, adversely affecting cattle production as animals suffer from loss of condition, emaciation and anaemia, resulting in reduced meat and milk production and draught power for agricultural production. Cattle mortality can reach 50–100% within months of exposure. The disease is caused by parasites that live in the host blood plasma, body tissue and interstitial fluids. Trypanosomes are transmitted to the host by a vector, the tsetse fly. The parasite replicates within the tsetse fly and is transmitted through saliva when the fly feeds on the animals. While this disease predominantly occurs in sub-Saharan Africa, it has also been found in South America, where one AAT agent (*Trypanosoma vivax*) has been established and tabanids (biting flies) act as the mechanical vector. The most rigorous calculation of the cost of AAT in sub-Saharan Africa dates from the late 1990s (Kristjanson *et al.*, 1999). Kristjanson estimated the annual cost to be US$1.3 billion, excluding losses from potential output in regions where trypanosomes prevent livestock production and excluding the costs of foregone power and manure output.

Animal trypanosomiasis can be managed by three strategies: (i) vector control/eradication; (ii) use of trypanocides; and (iii) use of trypanotolerant breeds of cattle (see Chapter 3, this volume). Vector control includes reducing the tsetse fly population with traps and insecticides, and in areas with a high population of trypanosome-infected tsetse, animals are prophylactically administered antiparasitic drugs. To date, there is no AAT vaccine available, as discussed below.

Scientific impacts

The International Livestock Research Institute (ILRI) and its two predecessors, the International Livestock Centre for Africa (ILCA) and the International Laboratory for Research on Animal Diseases (ILRAD), have made significant contributions to trypanosomiasis research since the early 1970s.

The first contribution was the establishment of protocols for cultivation *in vitro* of bloodstream stages of *Trypanosoma brucei* and subsequently *Trypanosoma congolense*. This advance facilitated downstream investigations of *T. brucei* and *T. congolense* cell division cycles, endocytic processes, interaction with antibodies against variable surface glycoproteins (VSGs) and other trypanosome antigens, interactions with trypanocidal drugs and mechanisms of development of drug resistance.

The second contribution was recognition that trypanosome strain complexity and surface coat antigenic variation precluded the development of an effective conventional vaccine targeting the immunodominant coat antigens. Subsequent identification of required macromolecular growth nutrients and uptake pathways also did not lead to an effective vaccine because, under steady-state conditions, anti-receptor antibodies did not kill the parasites or prevent acquisition of the nutrients.

Although the vaccine approach proved unsuccessful, studies in this area at ILRAD advanced our understanding of trypanosome biology.

The third contribution was a deepening understanding of bovine immunology per se (see Chapter 4, this volume) and as it pertains to responses against African trypanosomes and other pathogens.

A fourth contribution was an understanding of the biological and genetic basis of trypanotolerance. ILRAD, ILRI and their partners were able to characterize N'Dama cattle, a small multi-purpose indigenous breed that can survive without chemotherapy in some regions of sub-Saharan Africa where AAT kills susceptible breeds.

Field research in the area of trypanotolerance established the need for complementary technologies, such as spraying to kill the tsetse vector and trypanocidal drugs.

Analysis of trypanosomiasis in trypanotolerant N'Dama cattle and their crosses with trypanosomiasis-susceptible Boran cattle found that two major indicators of trypanotolerance, namely control of anaemia and parasitaemia, are unlinked. The work introduced an additional indicator of trypanotolerance, namely the ability to generate IgG1 antibodies against buried VSG epitopes and epitopes on many common trypanosome antigens, including congopain and heat-shock protein 70 (Hsp70)/binding immunoglobulin protein (BiP). Traits that distinguished between N'Dama and Boran cattle trypanotolerance in cattle proved to be regulated by multiple unlinked genes. Mapping of the trypanotolerance genes in cattle was an integral, and initially a leading, component of the bovine genome-mapping programme worldwide. The results of the study were consistent with a single quantitative trait locus (QTL) on each of 17 chromosomes, and two on BTA16 (*Bos taurus* chromosome 16), with individual QTL effects ranging from about 6% to 20% of the phenotypic variance of the trait, weighing against use of markers for these QTLs in breeding programmes to enrich for the trait. Archives of genomic and complementary DNA (cDNA) have been established from the bovine trypanotolerance studies carried out at ILRAD/ILRI and are available for deeper analysis as technology in this field undergoes further development to facilitate linking genes to disease-resistance traits. The genetic complexity of trypanotolerance in cattle proved greater than that found in crosses between inbred strains of mice; here, three regions of host DNA were shown to be associated with trypanotolerance and a candidate resistance gene, *Pram1* (PML-RARA-regulated adaptor molecule 1), has been proposed for one of these regions.

A fifth contribution was the discovery of diagnostics and subsequent efforts to prevent and contain the development of trypanocidal drug resistance in the parasite.

ILRAD and subsequently ILRI developed computerized data management and analysis systems, geographic information systems and digital georeferenced databases to address the distribution and dynamics of AAT in the continent. Combined with measures of productivity during infection, such mapping tools have been used to evaluate trypanotolerance, to define the effect of trypanosomiasis control on land use and biodiversity, and to enhance decision making in livestock development programmes. ILRI's epidemiological research highlights include the development of a modelling technique to evaluate control options of AAT, such as: (i) chemotherapy, which remains the main parasite control option; (ii) trypanotolerant cattle, which is an important option if complementary chemoprophylaxis is adequate; and (iii) tsetse control methods, which are well established.

In terms of top-cited papers, ILRI has contributed to 36% of the global research outputs on animal African trypanosomiasis and 64% of the global research outputs on trypanosomiasis resistance, as shown in the Altmetric (www.altmetric.com/; accessed 5 February 2020) database.

Development impacts

Economic impacts

Estimates of economic losses to trypanosomiasis in sub-Saharan Africa have been made several times. Jahnke's pioneering work (1976) showed the potential output losses in East Africa. Several papers from ILCA/ILRAD (1988) showed positive economic returns to various forms of control, including spraying and trapping flies, trypanocides and use of trypanotolerant animals. Research has therefore had an economic impact outside the vaccine domain.

With respect to vaccine development, a 1990s *ex ante* assessment showed that a vaccine against trypanosomiasis in Africa would have generated a real *ex ante* rate of return of 33% (Kristjanson *et al.*, 1999). However, despite the known losses to trypanosomiasis and the potential economic gains to reducing those losses, it has not been possible to estimate indirect economic impacts arising from the main scientific impacts of trypanosomiasis research. In particular, *ex post* development impacts of vaccine research were zero because decades of investment in vaccine research failed to produce an effective vaccine.

Capacity building

ILRI has built capacity in both AAT control and farmer field training and information sharing in rational drug use. An estimated 258 scientists and students were trained through ILRAD, ILCA and ILRI on trypanosomiasis, of which 61 were specifically from the African Trypanotolerant Livestock Network (ATLN). The institution has supported 63 PhD students, 26 MSc students and four interns working on trypanosomiasis research.

Partnerships

Addressing AAT and the problems associated with trypanocide resistance has led to significant research collaborations, including: the Centre de Coopération Internationale en Recherche Agronomique pour le Développement/Département d'Elevage et de Médecine Vétérinaire (CIRAD/EMVT, France); the Centre International de Recherche-Développement sur l'Elevage en zone Subhumide, Direction Provinciale des Resources Animales (DPRA, Burkina Faso); the Food and Agriculture Organization of the United Nations (FAO, USA); Freie Universität Berlin (FU-Berlin, Berlin); the International Trypanotolerance Centre (ITC, Gambia); Justus Liebig University Giessen (Germany); the Laboratoire Central Vétérinaire (LCV, Mali); Oxford University (UK), Prince Leopold Institute of Tropical Medicine (now the Institute of Tropical Medicine, Belgium); the University of Edinburgh (UK); the University of Glasgow (UK); and University of Hannover (Germany). ILRI and its predecessors have contributed to the Programme Against African Trypanosomiasis (PAAT), which brings together agents working on this disease, ranging from rural communities to governments, research institutes and development agencies.

Introduction

In 1970, Wiley published *The African Trypanosomiases*, edited by H.W. Mulligan and W.H. Potts. Most of the contributors to the book, including A.R. (Ross) Gray, who served as Director General of ILRAD from 1982 to 1994, had gained expertise on African trypanosomes, their vectors, trypanosomiasis pathogenic processes and disease control strategies while working in Africa. As the first comprehensive work on these topics, the book was commissioned by the Trypanosomiasis Advisory Panel of the Ministry of Overseas Development of Great Britain and sponsored by the same ministry. The publication was in many respects a legacy to independent Africa from expatriate scientists who had worked to control the devastating spread of human African trypanosomiasis (HAT) and AAT during the colonial period, possibly as a result of changing ecological dynamics associated with colonial conquest and management (Ford, 1971; Scoones, 2014).

HAT and AAT are caused by tsetse-transmitted protozoan parasites that are endemic in the humid and semi-humid zones of sub-Saharan Africa. As a landmark book, Mulligan and Potts (1970) set the baseline for trypanosomiasis research programmes at ILRAD and ILCA and later at ILRI. *The African Trypanosomiases* set a standard against which progress in the field since 1970, both fundamental and practical, can be measured, because it documents the scientific investment made during the colonial period in West, Central and East Africa to control HAT, and to a lesser extent, AAT. Indeed, the decision to publish *The African Trypanosomiases* attests to the fragility of trypanosomiasis control strategies then in use, namely those based on tsetse control by environmental engineering, vector trapping and application of insecticides, and disease control by diagnosis and chemotherapy, all of which can break down in the face of infrastructure disturbance or parasite resistance to trypanocidal drugs.

The Consultative Group on International Agricultural Research (CGIAR) was formed, in

1971, shortly after the publication of *The African Trypanosomiases*. In support of its mandate, CGIAR established two livestock research institutes, ILRAD, in Nairobi, Kenya, in 1973, and ILCA, in Addis Ababa, Ethiopia, in 1974. ILRAD was to 'serve as a world center for research on ways and means of conquering, as quickly as possible, major animal diseases which seriously limit livestock industries in Africa and many other parts of the world' and to 'concentrate initially on intensive research concerning the immunological and related aspects of controlling trypanosomiasis and theileriosis (mainly East Coast fever)' with the goal of decreasing the incidence and/or severity of disease. ILCA was to 'assist national efforts which aim to effect a change in the production and marketing systems of tropical Africa so as to increase the sustained yield and output of livestock products and improve the quality of life of the people of this region'.

The mandates of these institutes were partially merged in 1977 upon establishment of ATLN, which was based on the ILRAD campus in Nairobi. The network investigated the use of trypanotolerant cattle, primarily those of the N'Dama breed, as a resource for productive livestock farming in areas of sub-Saharan Africa where AAT is endemic; this was accomplished through analyses of animal health and production databases assembled from 13 countries in West, Central and East Africa (reviewed by d'Ieteren *et al.*, 1998). N'Dama are West African taurine cattle that have been farmed in Africa in tsetse-endemic areas for several thousand years (Hassan, 2000). These multi-use livestock animals have been used to establish commercial herds and are undergoing selection for production traits. However, N'Dama cattle are still not popular in East Africa, where cattle producers favour larger breeds, despite their susceptibility to AAT. Because of their trypanotolerant phenotype, comparative analyses of N'Damas and less trypanotolerant breeds with respect to immune responses, infection-induced pathology and the genetic basis of disease control became a focus of trypanosomiasis research at ILRAD and, with respect to identification of markers for selective breeding of trypanotolerant cattle, remain so today at ILRI, which was established in 1995 by merging ILRAD and ILCA.

In the 46 years since publication of *The African Trypanosomiases*, there have been fundamental advances in trypanosome biology, biochemistry, immunology and immunopathology, including new understanding of trypanosome virulence and host resistance mechanisms. Despite these advances, the scientific community is still some way from creating tools that would help farmers in sub-Saharan Africa to improve their livestock production in trypanosomiasis-endemic areas. Examples of such tools would include an inexpensive, sensitive and specific AAT diagnostic test to validate the need for, and efficacy of, chemotherapy; new inexpensive multi-target trypanocidal drugs; a vaccine to prevent AAT or accelerate its cure in trypanosomiasis-sensitive livestock; and fully trypanotolerant livestock with production traits more closely aligned to those of improved breeds than of the smaller multi-use N'Dama cattle. However, it is still reasonable to expect eventual success in developing at least some of these tools. ILRAD, ILCA and ILRI scientists have contributed to this work in the areas of AAT diagnostics, understanding mechanisms of AAT pathogenesis, defining the trypanotolerance phenotype, identifying QTLs that govern trypanotolerance and evaluating putative vaccine antigens. These contributions are discussed after the following review of the state of knowledge in 1970 regarding AAT, which outlines the problems tackled by ILRAD/ILCA/ILRI.

Scientific Challenges

AAT causative agents and tsetse, *c.*1970

African animal trypanosomiasis (AAT) in livestock is caused by infection with any of three species of African trypanosomes, namely, *T. brucei*, *T. congolense* and *T. vivax*. The pathogenic protozoans are transmitted to their mammal hosts in the saliva of tsetse flies (genus *Glossina*) in which the parasites undergo cyclic development and of which there are more than 30 species and subspecies, with eight playing a major role in trypanosome transmission (Cecchi *et al.*, 2015). African trypanosomes can also be transferred mechanically between hosts in blood held within the proboscis of biting flies other than tsetse, predominantly horse flies (family Tabanidae) and stable flies (family Muscidae). Mechanical

transmission of the parasites by biting flies causes spread of AAT within and across herds.

African trypanosomes live extracellularly in the blood of their mammal hosts, and in the case of *T. brucei* and *T. vivax*, also in tissues. They can be detected and distinguished from each other by microscopic examination of wet, thin or thick blood films, or dried, fixed and stained thin blood films. This simple diagnostic test is effective when the level of parasitaemia is high but can be problematic at other times. Fiennes (1970) reported, 'It was standard practice to examine 600 fields of thick smear preparations (of bovine blood) but in some cases trypanosomes were only detected after weeks or even months of daily searching.' In contrast, cryptic trypanosome infections could sometimes be revealed by inoculation of putatively infected blood into laboratory mice, although this was often unsuccessful because not all trypanosomes grow in mice. Thus, definitive diagnosis of AAT in the field was not always possible in the 1970s.

Tsetse distribution and effects on cattle, *c.*1970

Tsetse fly-infested areas of Africa extend from the southern edge of the Sahara Desert to Angola, Zimbabwe and Mozambique. Of the three trypanosome species that cause AAT, only *T. vivax* is found in the western hemisphere, in approximately ten countries in the Caribbean and South and Central America. AAT is endemic in livestock maintained in tsetse-infested areas of Africa. The disease is chronic and often fatal in cattle grazed in regions that are largely free of wildlife species, although it sometimes resolves without treatment. Cattle under chemotherapeutic support can achieve immunity provided they are exposed to restricted regional stocks of *T. brucei brucei*, *T. congolense* and *T. vivax*. In contrast, AAT is typically acute and fatal in regions where livestock come into contact with trypanosomes that are transmitted from the sylvatic (wildlife) reservoir by tsetse, possibly reflecting the intensity of challenge, trypanosome strain diversity, and the presence of strains of the parasites that are highly virulent (van den Bossche *et al.*, 2011; Motloang *et al.*, 2014).

As a result of acute AAT, cattle are excluded from much of the tsetse fly habitat, which has been estimated at various times since the founding of ILRAD to cover between 8.7 million and 10.3 million km^2 of the humid and semi-humid zones of sub-Saharan Africa. Ranching of cattle is possible on the fringes of the tsetse habitat where tsetse and trypanosome challenge are relatively low, but this requires support from trypanocidal drugs and insecticides. Chemotherapeutic support is also required for ranching of the relatively trypanotolerant taurine breeds of West Africa in trypanosomiasis-endemic areas, although not to the same extent as that required for trypanosomiasis-susceptible breeds. Use of chemotherapy and vector control to manage AAT is expensive and only partially effective. In those areas where challenge is low enough to permit ranching, calf mortality is still 6–10% higher than in regions where trypanosomiasis is not endemic, death in older animals is 2–8% higher, annual calving rates are 7% lower, milk yields are 2–26% lower and oxen are 38% less efficient (Shaw, 2009). AAT-associated production losses in Africa were recently estimated to exceed US$4 billion a year (AU-IBAR, 2018).

Antigenic variation and recurring parasitaemia, *c.*1970

Gray (1970) reviewed the state of knowledge of protective, but variable, antigens on African trypanosomes in Mulligan's *The African Trypanosomiases*. Briefly, it was shown in the 1960s that cell-free serum of infected animals, and wash buffer of trypanosomes enriched by differential centrifugation, contained trypanosome material, called exoantigen, that elicited trypanosome-agglutinating antibodies, indicating that target antigens were displayed on the surface of healthy trypanosomes. Antibodies are disease-fighting proteins that are secreted by plasma cells, which are terminally differentiated B-lymphocytes or B-cells. Antibodies against exoantigen were variant specific (i.e. reacted only with the exoantigen to which they were raised) and protected against that variant but not others.

Results from several scientists showed that a single trypanosome could give rise to many different antigenic types and Gray (1967, 1970) raised the possibility that 'the total number of antigens produced in one [trypanosome-infected]

host may be limited only by the time the animal lives'. However, despite the very large repertoire of variable surface antigens expressed by bloodstream-stage parasites, there was evidence, although far from convincing, that passage of a trypanosome strain through a tsetse fly resulted in expression of a basic antigenic type by the mammal-infective forms (metacyclic parasites), and when transmitted to new hosts these gave rise to a set of bloodstream-stage parasites with a restricted set of predominant antigenic types responsible for the first few waves of parasitaemia. These findings raised hopes that a composite vaccine based on a combination of the common and predominant exoantigens would be broadly protective. Despite this optimism, Gray also commented on the lack of knowledge of the structure of exoantigens, mechanisms of antigenic variation in mammals and tsetse, and the extent of variable surface antigen diversity, knowledge that would certainly be needed to evaluate possible use of basic and predominant antigens in a combinatorial vaccine.

AAT pathogenesis, *c.*1970

A good deal of information had been assembled on AAT pathogenesis before ILRAD was established. In Mulligan's *The African Trypanosomiases*, Fiennes (1970) reported that infections of cattle with *T. brucei*, *T. congolense* and *T. vivax* give a similar disease picture, suggesting 'that the fundamental processes of pathology in all forms of animal trypanosomiasis are possibly the same'. Fiennes reported that the cardinal signs of trypanosomiasis consist of fever that spikes on clearance of trypanosome parasitaemic waves but is later sustained, anaemia, cachexia/emaciation and hypoproteinaemia/hypervolaemia. The severity of these signs of disease was observed to vary depending on the age of the infected bovid, the virulence of the infecting parasites and the stage of infection. Fiennes also noted that other pathogens ellicit similar signs of disease in cattle, e.g. *Babesia bigemina*, which causes red-water fever; consequently, the clinical signs of AAT are not pathognomonic.

With respect to morbid anatomy, Fiennes reported that the spleen and lymph nodes of infected cattle became greatly enlarged in the early stages of AAT, but during the chronic stage of disease, the spleen became small and atrophic, showing cell depletion. Furthermore, during the chronic stage of the disease, the red bone marrow of the shafts of the long bones disappeared. The infection-induced loss of erythropoiesis from the long bones may therefore affect the animal's capacity to replace red blood cells. Fiennes also reported that fatty tissues throughout the body, especially around the heart and kidneys, showed degeneration, the lungs showed marbling due to dilation of lymphatic vessels and became oedematous, the cardiac muscle also became flabby and oedematous, and exudates developed in the pleural and peritoneal cavities, all consistent with global inflammation. In addition, Fiennes reported that, as the infection progressed, parasitaemia often became cryptic, but aggregates of dead trypanosomes and areas of necrosis could be found in tissues in the case of *T. brucei*, and in capillaries often associated with small focal necroses in the case of *T. vivax* and *T. congolense*. There was also multiple organ and tissue degeneration in which kidneys became necrotic, the liver showed enlargement accompanied by central lobular necrosis in the parenchyma, and there was dilation of central veins and sinusoids and activation of phagocytic Kupffer cells. In addition, lymph nodes became fibrotic and their follicles and germinal centres were depleted of mature lymphocytes, showing that AAT-induced destruction of secondary lymphoid organs was not restricted to the spleen. Thyroid and adrenal glands were also severely affected in AAT, the former filling with colloid before disintegration and the latter becoming necrotic and fibrotic.

Anaemia is a consistent parameter of AAT and was used productively by scientists at ILRAD/ILCA/ILRI in comparative studies of AAT pathogenesis in infected N'Dama and Zebu cattle. In 1970, little was known about molecular mechanisms of anaemia in AAT, although analyses of cattle infected with *T. congolense* or *T. vivax* led Fiennes (1970) to propose four different courses of AAT in which anaemia featured differently: (i) hyperacute, which was characterized by severe haemolysis and early death; (ii) acute, which was characterized by hydraemia followed by dehydration, a haemolytic crisis and death (hydraemia is an increase in blood volume through water retention, resulting in a decrease in the specific gravity and protein concentration of blood plasma as well as a decrease in blood

packed cell volume (PCV) and red cell or haemoglobin content per unit volume); (iii) chronic, which was similar to acute but hydraemia persisted without dehydration and the haemolytic crisis was not fatal; and (iv) recovery, which was rare and typically occurred in AAT with little or no hydraemic phase. Mechanisms of hydraemia and dehydration were not defined, although Fiennes and colleagues implicated a haemolysin in trypanosome-induced anaemia showing that blood plasma from infected animals sometimes lysed red blood cells of healthy animals *in vitro* at 37°C. The identity of the putative trypanosome-derived haemolysin was not established, although it was shown to be inactivated by heating at 56°C for 30 min, consistent with involvement of a heat-labile complement factor.

Among the many pathological features of AAT, Fiennes drew attention to serum dilution and accompanying hypoproteinaemia as being mainly responsible for the progressive decline of animals during the chronic stages of trypanosome infections. Again, mechanisms of this pathology were not identified, although kidney failure leading to retention of sodium and water is a likely candidate. Fiennes cited the work of several investigators implicating kinins, which are inflammatory polypeptides, but it is unlikely that inflammation alone would cause hypervolaemia.

In summary, by the start of the 1970s, it was clear that AAT is a disease caused by three species of trypanosomes that undergo cyclic development in biting flies of the genus *Glossina* (tsetse) and are transmitted to mammalian hosts primarily in the saliva of these flies. Trypanosome isolates had been cryopreserved, shown to retain infectivity for mammals and shown in experimental systems to induce host responses, both pathological and protective, to the parasites. These early studies showed that the chronicity of infection was linked to possibly unlimited variation of trypanosome surface antigens/exoantigens and escape from immune elimination. It had also been shown that exoantigens of bloodstream-stage trypanosomes elicited protective antibody responses, but that protection was restricted to homologous parasites. The diversity of bloodstream-stage trypanosome exoantigens suggested that they could not be combined as a composite vaccine; however, there was some evidence, albeit weak, that infective trypanosomes present in tsetse saliva and the first populations of bloodstream parasites had only a few antigenically distinct exoantigens, which might therefore serve as vaccine antigens. Little or nothing was known about immune responses to parasite components other than the immunodominant exoantigens. In addition, little or nothing was known about trypanosome virulence factors or host susceptibility/resistance factors that affected the severity of the pathological processes elicited by AAT, or indeed the mechanisms of that pathology. All of these problems were solved, to various degrees, by the work of ILRAD.

Major additions to the field between 1970 and 1979 included isolation and partial characterization of the organelle that defined the order Kinetoplastida to which the genera *Trypanosoma* and *Leishmania* belong, namely the kinetoplast (Fairlamb *et al.*, 1978); localization of bloodstream-stage *T. brucei* glycolytic enzymes to a single microbody-like organelle called the glycosome (Opperdoes and Borst, 1977); isolation and partial characterization of the variant-specific surface antigen of *T. brucei* (Cross, 1975; Bridgen *et al.*, 1976), which was previously called exoantigen and is now called the variable surface glycoprotein (VSG); and cultivation of animal-infective bloodstream-stage *T. brucei in vitro* (Hirumi *et al.*, 1977). This last discovery was made at ILRAD.

During the 1970s, the pace of discovery in cell and molecular biology and immunology had greatly accelerated through advances in the manipulation of DNA and RNA, nucleotide and protein sequencing, antigen epitope targeting with monoclonal antibodies (mAbs), cell population analysis using fluorescence-activated flow cytometry and cell sorting, and data processing using personal computing, to name a few. Application of these technologies to the field of trypanosomiasis research at ILRAD/ILRI had major impacts on understanding the molecular biology of trypanosome antigenic variation and the host immune response to the parasites, as discussed below.

ILRAD's Initial AAT Mandate

The first researchers at ILRAD were challenged by the institute's mandate to 'conquer, as quickly as possible, major animal diseases [that] seriously limit livestock industries in Africa' and to make fundamental discoveries concerning the cell

and molecular biology of *T. brucei*, *T. congolense*, *T. vivax* and *Theileria parva*, and their interactions with mammal hosts.

The acquisition of knowledge of host and pathogen biology has potential application to the control of disease, but should it take precedence over more applied approaches? This question was posed by A.J.S. Davies and G.A.T. Targett in a lecture on 'Some perspectives in parasitic disease' presented at the Inauguration Symposium on Current Trends in Immunology and Genetics and Their Implications for Parasitic Diseases, a meeting held on the ILRAD campus in 1978 (Davies and Targett, 1978: p. 69). They wrote, 'Is there any way that ILRAD can create an environment in which serendipity can have its full force? What shall be the balance between fundamental and field-level investigations? In any instance, do we know enough about disease in general, and trypanosomiasis and East Coast fever in particular, to adopt any specific approaches?'

The ILRAD scientific community took a mixed view. It committed to fulfilling the mandate of the institute through the application of evidence-based research, while investigating the basic biology, biochemistry and molecular biology of African trypanosomes and their interaction with their hosts, with a view towards identifying vaccine and diagnostic antigens and increasing host resistance to AAT.

ILRAD, 1975–1979

Under the leadership of Jim Henson, who served as ILRAD Director General from 1974 to 1978, ILRAD built a modern tsetse laboratory and suites of modern open-plan laboratories, research support and administrative buildings, at Kabete in Kenya. Over the next few years, the institute was equipped with switchable power, backup generators, facilities for safely handling radioactive materials and storing radioactive waste, animal management facilities, and a flow cytometry core including highly trained staff who ran and maintained a Becton and Dickinson FACS II cell sorter. In short, in over 5 years, ILRI established standards of operation equivalent to those in reputable institutes worldwide.

By 1979, ILRAD had assembled an almost full complement of scientific support and administrative staff and, importantly, had established research focus groups with defined research goals. In addition, collaborations had been established among members of different disciplinary and focus groups, thus promoting interdisciplinary research.

In addition to assembling research teams at ILRAD, a number of questions had been identified by 1979 as important to the mission to develop AAT immunization and control strategies (Table 2.1).

Initial experimental questions on AAT vaccine development and control

By 1979, scientists had begun to answer the questions in Table 2.1.

- *T. brucei* undergoes antigenic variation *in vitro* in the absence of VSG-specific antibodies or other components of trypanosome-induced immune responses (Hirumi *et al.*, 1977; Doyle *et al.*, 1980). Thus, immune pressure was not required for antigenic variation.
- Purified messenger RNA (mRNA) encoding a *T. brucei* VSG induced synthesis of VSG in an *in vitro* translation system (Williams *et al.*, 1978) and was an early step towards characterizing the genetic basis of antigenic variation. Work at ILRAD later showed that expression of a single VSG gene could occur with or without duplicative transposition (Young *et al.*, 1983a), a process whereby a copy of a VSG gene was inserted into an active VSG gene expression site through recombination with repetitive sequence in the barren region on one side of the VSG.
- mAbs could distinguish between trypanosome VSGs (Pearson *et al.*, 1980). This technology opened the way to dissect and compare VSGs and other antigens of bloodstream-stage and metacyclic trypanosomes, of different trypanosome species and isolates.
- *T. brucei* and *T. congolense* VSGs have a common cross-reactive determinant (Barbet and McGuire, 1978). However, immunofluorescent staining of trypanosomes using sera with activity against the cross-reactive determinant showed that this determinant was not accessible on intact parasites.

Table 2.1. Experimental questions on AAT vaccine development and control.

Is the genetic information for all VSGs present in each trypanosome?
Do trypanosomes vary VSGs in response to immune pressure?
Do VSGs of bloodstream-stage trypanosomes have a common determinant or determinants that can induce a protective immune response?
Do mammal-infective, tsetse-derived (metacyclic) trypanosomes express a common VSG, and do the first bloodstream-stage trypanosomes derived from them express a limited set of predominant VSGs?
Are there conserved trypanosome antigens that can induce protective immunity against AAT or that can be used in the development of diagnostic reagents?
Do trypanotolerant animals mount protective immune responses against conserved components of VSGs and/or common trypanosome antigens?
Is trypanotolerance a genetically acquired trait, and if so, how many genes are responsible?

- Soluble *T. brucei* and *T. congolense* VSG activates complement *in vitro* (Musoke and Barbet, 1977), which raised the possibility that activation of complement by VSG released by trypanosomes might be the cause of inflammation in AAT.
- Two trypanosomiasis resistance models were established: (i) inbred strains of mice differ in their survival time after infection with *T. congolense*, which was independent of the haplotype of mouse major histocompatibility complex (MHC) expressed and which may be associated with the capacity to control parasitaemia (Morrison *et al.*, 1978); and (ii) N'Dama and Zebu cattle in The Gambia differed in their capacity to control experimental infections with *T. brucei* and *T. congolense* (Murray *et al.*, 1977b,c). These models were used, and are still being used, to identify the immunological, physiological and genetic basis for resistance to trypanosomiasis.
- *T. congolense* caused immune dysregulation in mice by inducing cells, called suppressor cells, that inhibit the responses of T lymphocytes *in vitro* (Pearson *et al.*, 1979), introducing a possible mechanism of immunosuppression in trypanosomiasis.

AAT Research at ILRAD, ILCA and ILRI, 1979–2017

This section traces the development of the research themes outlined in Table 2.1. It encompasses work on AAT at ILRAD, ILCA and ILRI up to 2015 and, where appropriate, references relevant work carried out at other institutions. This section addresses: (i) AAT vaccine antigens; (ii) AAT diagnostics; (iii) mechanisms of AAT pathogenesis; and (iv) the genetic basis of trypanotolerance. The chapter is not a comprehensive review of all work relevant to AAT carried out at ILRAD, ILCA and ILRI. For example, development of expertise in bovine immunology and of tools to dissect bovine immune responses, which were critical to AAT research, is discussed elsewhere (see Chapter 4, this volume). Important work at ILRAD on the physical nature of *T. congolense* (Rovis *et al.*, 1978) and *T. vivax* (Gardiner *et al.*, 1987) VSGs, immunocapture of mRNAs encoding VSGs (Shapiro and Young, 1981) and resolution of the puzzling disparity between VSG expression with or without an expression-linked copy (Majiwa *et al.*, 1982; Young *et al.*, 1983a,b) are not discussed here. Similarly, research at ILRAD and ILRI on trypanosome biochemistry, metabolism and death pathways (Lonsdale-Eccles and Grab, 1987; Aboagye-Kwarteng *et al.*, 1991; Bienen *et al.*, 1991; Murphy and Welburn, 1997; Welburn and Murphy, 1998; Welburn *et al.*, 1999) and *in vitro* (Borowy *et al.*, 1985a, 1985b, 1985c; Borowy *et al.*, 1988; Dube *et al.*, 1983) and *in vivo* (Peregrine *et al.*, 1987, 1991; Sutherland *et al.*, 1991; Silayo *et al.*, 1992; Mamman *et al.*, 1993; Burudi *et al.*, 1994; Mamman and Peregrine, 1994; Mamman *et al.*, 1994; Peregrine and Mamman, 1994; Waitumbi *et al.*, 1994) assays of drug resistance in trypanosomes, which also fall outside the scope of the chapter, are not discussed.

The search for AAT vaccine antigens

Metacyclic and first-generation bloodstream-stage trypomastigote VSGs

T. brucei, *T. congolense* and *T. vivax* infect and undergo specific developmental programmes in

tsetse, ultimately maturing to mammal-infective stages at sites in the tsetse that allow deposition into mammal hosts in saliva. These mammal-infective trypanosomes are called metacyclic trypomastigotes and are diploid and non-dividing, which was shown for *T. brucei* by cell-cycle analysis in the first publication from ILRAD that used the flow cytometry core (Shapiro *et al.*, 1984), and was later shown by ILRAD scientists, in collaboration with others, for *T. congolense* and *T. vivax* metacyclics using culture-derived parasites and nuclear DNA microfluorimetry (Kooy *et al.*, 1989). Upon deposition into the mammal host, the metacyclic trypomastigotes differentiate to replicative bloodstream-stage trypomastigotes, thus establishing infection. Metacyclic and bloodstream-stage trypomastigotes have been selected through evolution to express a more-or-less contiguous layer of VSG on the outer leaflet of their plasma membrane. The VSG coat protects the parasites from antibody-independent lysis by plasma complement factors (Devine *et al.*, 1986), which are innate immune effector molecules that assemble to a lytic complex on some pathogens, including uncoated trypanosomes, but not on VSG-coated trypanosomes. As discussed earlier, bloodstream-stage trypanosomes generate diverse VSGs by antigenic variation. However, investigations prior to the establishment of ILRAD (reviewed by Gray, 1970) had suggested that metacyclic and the first bloodstream-stage parasites might express only a few common and predominant VSGs, which might therefore serve as vaccine antigens.

DIRECT ANALYSIS OF VSGS EXPRESSED ON METACYCLIC AND BLOODSTREAM-STAGE TRYPANOSOMES Two lines of investigation were initially pursued at ILRAD to seek possible vaccine antigens. The first was direct analysis of the VSGs expressed on metacyclic and bloodstream-stage trypanosomes. Analyses of expressed VSGs were performed using immune sera and mAbs, and were continued with a variety of molecular genetic approaches by colleagues in and outside of ILRAD. The studies conducted at ILRAD clearly showed heterogeneity of expressed *T. congolense* metacyclic VSGs (Nantulya *et al.*, 1983) and substantial heterogeneity of VSGs expressed by initial populations of bloodstream-stage *T. vivax* arising from metacyclic parasites (Gardiner *et al.*, 1986). Studies in Scotland showed that only a small subset of specific VSGs (fewer than 28, which is 1–2% of the total VSG repertoire) are expressed by the *T. brucei* metacyclic population (Turner and Barry, 1989). Similar data are unavailable for the metacyclic VSG repertoires of *T. congolense* and *T. vivax*, but recent genome analysis indicates VSG gene repertoire diversity in these species (Jackson *et al.*, 2012), and there is no reason to think that diversity of metacyclic VSGs expressed in a tsetse infected with a clone of either parasite will be more limited than that of tsetse similarly infected with *T. brucei*, particularly in light of the *in vivo* infection-and-treatment studies reported below. Although the repertoire of *T. brucei* VSGs expressed by metacyclic trypanosomes is smaller than that expressed on the bloodstream-stage parasites, it is still substantial, is unstable over time (Barry *et al.*, 1983) and, as discussed below, differs among different serodemes of *T. brucei*, *T. congolense* and *T. vivax*, thus showing that trypanosomes from different serodemes have different metacyclic VSG repertoires.

INDUCTION OF IMMUNITY BY THE INFECTION-AND-TREATMENT METHOD The second line of investigation involved induction of immunity by the infection-and-treatment method. Cattle, goats and mice were subjected to cyclic infection using *Glossina morsitans submorsitans* infected with *T. brucei*, *T. congolense* or *T. vivax*, cured by treatment with diminazene aceturate (Berenil; Hoechst AG, Frankfurt, Germany), and subsequently exposed to tsetse infected with homologous or heterologous trypanosomes (Nantulya *et al.*, 1980; Akol and Murray, 1983; Nantulya *et al.*, 1984; Akol and Murray, 1985; Nantulya *et al.*, 1986; Vos *et al.*, 1988; Taiwo *et al.*, 1990). These studies showed that trypanocidal treatment of an established infection resulted in protective immunity against homologous but not heterologous cyclic infection. Protection was associated with parasite control at the level of the tsetse bite (i.e. in the skin), because a chancre that results from an immune response induced by parasites at the bite site did not develop in the immune animals.

Immunity, at least in the case of infections with homologous strains of *T. brucei* and *T. congolense*, correlated with the presence of neutralizing serum antibodies specific for the VSGs of their metacyclic trypanosomes, whereas immunity in the case of infections with *T. vivax* resulted from accumulation of antibodies specific for

VSGs of homologous bloodstream-stage *T. vivax*. Disappointingly, from the perspective of immunoprotection, attempts to elicit protective immunity in individual animals against several serodemes of trypanosomes by simultaneous, or sequential, cyclic infection of hosts followed by trypanocidal treatment were unsuccessful (Dwinger *et al.*, 1987). This resulted from failure to establish mixed infections in the hosts, or from an inability of the infected and treated hosts to develop protective immune responses against the wider range of metacyclic and bloodstream-stage VSGs of the mixed populations.

One important observation made in sequential cyclic infections with bloodstream-stage trypanosomes was that the superimposed heterologous parasites did not establish an infection (Morrison *et al.*, 1982). Similarly, superimposed infections with metacyclic parasites did not elicit a chancre (Dwinger *et al.*, 1986), indicating that the heterologous parasites were killed in the skin or did not establish replicating bloodstream-stage parasites in the skin. This phenomenon was called 'interference'. Its maintenance required sustained infection, and it was not equally effective against all superinfecting trypanosomes (Dwinger *et al.*, 1989), all suggesting against interference as a manageable strategy of AAT control. Interference was hypothesized to result from elevated microbicidal responses by innate effector cells in the skin of infected cattle, but the mechanism was not studied at ILRAD. It is now well established that trypanosomes are killed by proinflammatory products of macrophages/monocytes, including reactive oxygen and nitrogen species, amphiphilic peptides/cathelicidins, and, in the case of some trypanosomes, the cytokine tumour necrosis factor (TNF). It is also established that interferon-γ (IFN-γ), which is produced by type 1 T-helper cells, greatly increases in trypanosome-infected animals and activates macrophages to produce these microbicidal products.

A NEW VACCINE APPROACH It has recently been proposed that activation of the innate defence system of the skin should be a strategy for AAT vaccination (Tabel *et al.*, 2013). Thus, while immunization and infection-and-treatment regimens that induce immune responses against highly variable components of metacyclic VSGs are considered unlikely to have an impact on the control of AAT, it is possible that induction of type 1 T-helper cell responses against conserved components of these VSGs, such as the C-terminal-domain conserved peptides of *T. brucei* and against other conserved antigens that are accessible in trypanosomes present in the chancre and other tissue sites, might expedite the development of protective innate immune responses in these regions. This approach is currently under investigation (Black and Mansfield, 2016).

THE VSG CROSS-REACTIVE DETERMINANT Rabbit antisera prepared at ILRAD against VSGs of antigenically distinct clones of *T. brucei* and *T. congolense* contained antibodies specific for epitopes that were unique to the immunizing VSG as well as antibodies specific for a cross-reacting determinant (CRD) common to all VSGs (Barbet and McGuire, 1978). The latter antibodies did not bind to intact trypanosomes. Thus, the CRD target epitope was masked, or cryptic, on membrane-bound VSGs, suggesting that antibodies against this epitope are unlikely to be host protective. Subsequent studies at ILRAD showed that the CRD was 'located within oligosaccharides linked to the VSG through *N*-glycosidic and other unidentified types of linkages' (Rovis and Dube, 1981) and that these were added to VSGs in the *trans*-Golgi region during export to the surface (Grab *et al.*, 1984) and were present at the C-terminal portion of the molecule and close to the trypanosome plasma membrane when attached to the parasites. Subsequent studies at Cambridge University, UK, showed that the CRD was exposed upon release of soluble VSG from the trypanosome membrane as a result of cleavage of the dimyristoyl glycosylphosphatidylinositol (GPI) lipid anchor, which attaches the VSG to the plasma membrane, by an endogenous phospholipase C (PLC)-like hydrolase (Cardoso de Almeida and Turner, 1983). Work at ILRAD showed that little or no soluble VSG is released by healthy bloodstream-stage trypanosomes (Black *et al.*, 1982), indicating that access of the PLC to the membrane-form VSG is tightly regulated. The possibility of disturbing this regulation to cause spontaneous release of VSG and exposure of naked trypanosomes to destructive complement components in host plasma spurred further research on the location and regulation of the VSG GPI-PLC.

Later work by Grab *et al.* (1987) at ILRAD showed that the VSG GPI-PLC was associated with flagellar membrane fractions of disrupted trypanosomes, and more recently it has been shown at Trinity College Dublin and Cambridge University to be present as a linear (patchy) array on the face of the flagellar membrane, between the paraflagellar rod and the cell body and close to the flagellar attachment zone (Hanrahan *et al.*, 2009; Sunter *et al.*, 2013). Despite exposure on the outer leaflet of the flagellar membrane (Sunter *et al.*, 2013), the GPI-PLC is not accessible on intact trypanosomes to specific antibodies and is unlikely to serve as a vaccine antigen. Nevertheless, VSG GPI-PLC may be the key to understanding the pathogenesis of AAT. It was shown that deletion of the gene encoding PLC from the trypanosome genome did not prevent the capacity of the parasite to infect and grow in tsetse or mammals but did substantially decrease trypanosome virulence (Webb *et al.*, 1994, 1997), implicating the PLC, or VSG GPI cleavage products, in dysregulation of immune control of the parasites. Thus, what began as a search for a conserved, possible vaccine, component of VSG at ILRAD was part of the path to the discovery of the only known trypanosome virulence factor, the VSG GPI-PLC.

Candidate vaccine antigens other than VSGs

In addition to characterizing VSGs of the AAT parasites (Rovis *et al.*, 1978; Gardiner *et al.*, 1987), scientists at ILRAD began the isolation and characterization of four antigen systems that might yield vaccine antigens: (i) conserved plasma membrane proteins (Rovis *et al.*, 1984); (ii) receptors for macromolecular nutrients (transferrin and serum low-density (LDL), intermediate-density (IDL) and high-density (HDL) lipoproteins) required by the parasites to grow (Black and Vandeweerd, 1989; Vandeweerd and Black, 1989; Grab *et al.*, 1993); (iii) trypanosome endosomal compartments (Webster, 1989; Webster and Fish, 1989; Grab *et al.*, 1992), including clathrin-coated endocytic vesicles (Shapiro and Webster, 1989; Shapiro, 1994), which would be expected to contain molecules involved in receptor-mediated endocytosis; and (iv) peptidases that might be released from living or dying trypanosomes and hence might contribute to pathogenesis (Knowles *et al.*, 1987; Lonsdale-Eccles and Grab, 1987; Authie *et al.*, 1993a, 2001).

CONSERVED PLASMA MEMBRANE PROTEINS The first immunization studies at ILRAD involved plasma membranes purified from bloodstream-stage *T. brucei* and also an 83-kDa protein that was present in lysates of *T. brucei*, *T. congolense* and *T. vivax*. Immunization of rabbits and goats with these materials elicited high-titre antibody responses but did not alter the course of the disease in the immunized animals following subsequent infection (Rovis *et al.*, 1984), suggesting that none of the target antigens was exposed at a high enough concentration on the surface of trypanosomes to support antibody-mediated killing, clearance or growth inhibition, and thus were not candidate vaccine antigens.

MACROMOLECULAR NUTRIENT RECEPTORS Groups at ILRAD and elsewhere turned their attention to receptor-mediated endocytosis in trypanosomes, which might yield immunoprophylactic targets. Using axenic culture systems developed at ILRAD, Black and colleagues obtained definitive proof that the parasites required LDL, IDL or HDL to progress through their cell division cycle, and transferrin for sustained replication (Black and Vandeweerd, 1989; Morgan *et al.*, 1993, 1996). Studies by Coppens and colleagues in Belgium implicated an 86-kDa *T. brucei* protein in uptake of LDL, and suggested that uptake of lipoproteins and parasite growth could be inhibited by antibodies to this molecule (Coppens *et al.*, 1988). However, collaborative studies (unpublished data) between Coppens and Black carried out at ILRAD showed that the antibodies did not affect growth of culture-adapted bloodstream-stage trypanosomes, and further work on the putative LDL receptor did not yield a candidate vaccine. The trypanosome receptors for bovine serum LDL, IDL and HDL have not yet been identified.

Studies at ILRAD to identify the *T. brucei* components that bind transferrin revealed a 90-kDa holotransferrin-binding *T. brucei* protein (Grab *et al.*, 1993). This protein was isolated from parasites that had been grown in rats and when injected into rats induced production of specific antibody that inhibited growth of bloodstream-stage *T. brucei* in axenic cultures; further

vaccine tests were not carried out *in vivo*. Isolation of the *T. brucei* transferrin receptor (TbTfR) was subsequently achieved by Steverding *et al.* (1995) in Germany, who showed that it was a heterodimer of proteins encoded by VSG expression site-associated gene 6 (*ESAG6*) and *ESAG7* whose products had molecular masses of 50–60 kDa and 42 kDa, respectively, and thus were distinct from the material isolated at ILRAD. Interestingly, uptake of transferrin by *T. brucei* was poorly inhibited by TbTfR-specific IgG but strongly inhibited by fragments of these antibodies comprising their antigen-binding sites, which may have easier access than intact antibodies to the transferrin receptor that is embedded in VSGs of the trypanosome flagellar pocket. The investigators stated that attempts to protect mice from *T. brucei* AAT by immunization with the receptor were unsuccessful. Subsequent studies showed that: (i) the different VSG expression sites in *T. brucei* contain different copies of *ESAG6* and *ESAG7*; (ii) their products differ in binding affinity for different mammal transferrins; and (iii) culture of bloodstream-stage trypanosomes in medium containing transferrin to which their TbTfR had low affinity resulted in selection of parasites that had switched to a VSG gene expression site with *ESAG6* and *ESAG7* encoding a higher-affinity transferrin receptor for that transferrin (Gerrits *et al.*, 2002). Based on studies on the TbTfR to date, it seems unlikely that it will serve as a vaccine antigen. *T. congolense* also expresses *ESAG6* and *ESAG7*, but *T. vivax* lacks these genes (Jackson *et al.*, 2013). In addition, work at ILRAD showed that the *T. vivax* VSG is smaller than that of *T. brucei* and the surface coat is more diffuse (Gardiner, 1989); consequently, it would be of interest to determine how *T. vivax* acquires iron and whether this mechanism could be blocked by a specific antibody to the detriment of the parasite.

EXTRACTS OF TRYPANOSOME ENDOCYTIC VESICLES

An attempt was made at ILRAD to induce antibodies that interfere with endocytosis of essential molecules by trypanosomes. Rabbits were immunized with proteins isolated from purified *T. brucei* clathrin-coated vesicles hypothesized to be 'putative parasite receptors for adsorptive endocytosis'. The resulting antibodies recognized many parasite proteins, including epitopes on the parasite's endocytic surface, but did not stimulate *in vitro* lysis of the parasites, inhibit their growth *in vitro* or improve control of infection in the immunized rabbits (Shapiro, 1994). Similar results were obtained with endosomal proteins, purified by tomato lectin affinity chromatography, reported below.

T. CONGOLENSE CYSTEINE PEPTIDASE (CONGOPAIN)

A negative correlation was found at ILRAD between the titre of IgG1 antibodies specific for a *T. congolense* cysteine protease (CP), congopain, in post-infection bovine serum and the severity of AAT (Authie *et al.*, 1993a). To determine whether these antibodies have a protective role in AAT, cattle were induced to generate antibodies against the catalytic domains of two families of related *T. congolense* CPs, called CP1 and CP2, by immunization with recombinant truncated proteins that had been expressed in a baculovirus system and that lacked the trypanosome-specific C-terminal extension (Authie *et al.*, 2001). The immunized cattle were subsequently infected with *T. congolense* by the bites of eight infected tsetse flies. Cattle subjected to the same immunization regime with ovalbumin served as controls. Immunization with truncated CP1 or CP2 did not affect either the time to patency or the levels of parasitaemia throughout infection, or the rapid decline in blood PCV during the first 40 days after infection, but did accelerate weight gain and recovery of blood PCV and of residual leukocyte counts following the initial rapid decline in these parameters. Furthermore, cattle immunized with CP2 mounted rapid and higher-titre IgG antibody responses against a VSG (IL-C49) unrelated to that of the infecting parasites. This last result is difficult to interpret because the IL-C49 VSG-specific antibodies were not assayed for cross-reactivity with the VSG, or any other antigens, of the infecting parasites. Despite some difficulty in interpreting this study, it is quite clear that immunization with truncated CP2 alleviated some aspects of AAT pathology. This work was not continued at ILRAD, and there have been no reports (to mid-2018) on how the truncated CP2 vaccine accelerates recovery from AAT.

Other vaccine studies

The search for AAT vaccine antigens was not restricted to ILRAD. Other investigators reported inducing partial protection (reviewed by La Greca and Magez, 2011), achieved by immunizing with:

(i) trypanosome flagellar pocket fractions; (ii) DNA encoding an invariant surface glycoprotein; (iii) trypanosome cytoskeletal proteins; (iv) *Trypanosoma evansi* actin or tubulin; or (v) plasmid containing the catalytic and N-terminal domain of *trans*-sialidase. The primed animals were typically boosted with the priming antigen and infected shortly thereafter with a low number of trypanosomes (500–1000). This regime resulted in 40–60% of recipients showing sterile immunity (i.e. they did not become infected in contrast to the uniform infection achieved in control unimmunized animals). The opinion of the review authors was that these immunization regimes elicited short-lived innate immune responses that killed infecting organisms. This interpretation is consistent with data obtained in the author's laboratory in collaboration with Noel Murphy of ILRI and Derek Nolan of University College, Dublin, on the vaccine potential of *T. brucei* tomato lectin binding (TL) antigens (Nolan *et al.*, 1999), which encompass most if not all trypanosome endosomal proteins and macromolecular growth-factor receptors. Mice were immunized via the peritoneal cavity with TL antigens in the presence or absence of the antimitotic drug cyclophosphamide, which inhibits antibody production (Table 2.2). Irrespective of the generation of TL antigen-specific antibodies, 50–66% of the immunized mice resisted subsequent infection by the intraperitoneal route. Subsequent studies showed that the protective response was lost 3 weeks after the last immunization and could not be boosted using soluble TL antigens. It was concluded that infecting parasites were killed by a microbicidal innate immune response in the peritoneal cavity induced by the immunization regime.

Alternatives to an AAT vaccine

As research at ILRAD, and subsequently ILRI, on AAT vaccines waned, during the 1980s three AAT research themes emerged and were continued at ILRI. These themes are summarized in Table 2.3 together with pros and cons (with respect to ILRAD/ILCA/ILRI mandates) that were considered at the time. Perhaps it would have also been reasonable to take a drug-discovery approach (i.e. to interrogate axenic cultures of pathogenic trypanosomes developed at ILRAD with every compound made or extracted by humankind in search of new effective trypanocidal compounds). While this could have been done in collaboration with Big Pharma, which was equipped with robotic testing centres and vast libraries of testable compounds, ILRAD and ILRI did not have the resources to discover new drugs by brute force and that route was not followed. High-throughput *in vitro* drug-screening approaches for African trypanosomiasis have been taken up by other researchers in recent years resulting in more than 20 papers cited in PubMed on this topic during the decade 2008–2018.

Improved AAT Management through Use of Trypanotolerant Stock

ILRAD's interest in AAT diagnosis, treatment and management was linked to the decision to develop a field/epidemiology programme that would strengthen the institute within Africa and

Table 2.2. *T. brucei* TL antigens induce a protective innate immune response. (unpublished data, ILRAD).

TL antigen	Ovalbumin	Cyclophosphamide (200 mg/kg body weight)	Challenge	Animals protected (%)
+	−	−	+	66
+	−	+	+	50
−	+	−	+	0
−	+	+	+	0

Groups of mice (n = 6 per group) were primed by intraperitoneal injection of 20 µg of TL antigen (*T. brucei* clone ILtat 1.4; Nolan *et al.*, 1999) or with 20 µg of ovalbumin, emulsified in Freund's complete adjuvant, boosted after 1 month with the same amount of antigen emulsified in Freund's incomplete adjuvant, and challenged with exponentially growing trypanosomes (500 *T. brucei* strain GUTat 3.1) 8 days later. Tail-blood parasitaemia was assayed at 4, 8 and 12 days post-challenge, and mice in which parasites were not seen on any occasion were designated as protected. Antibodies specific for TL antigens reached a titre of 1:1600 in a TL-antigen enzyme-linked immunosorbent assay, but were not detected in mice that were given TL antigens plus cyclophosphamide, which kills dividing B-cells, and were not detected in the control ovalbumin-immunized mice. TL, tomato lectin.

Table 2.3. AAT research at ILRAD/ILRI in the post-trypanosomiasis vaccine era. (Constructed by author.)

Theme	Pros	Cons
Improve productivity of N'Dama cattle in large herds under natural tsetse/trypanosome challenge by diagnosis and chemotherapy	Helps producers Involves ILRAD with ILCA in workforce training and management of AAT in N'Dama Develops diagnostic tools to monitor AAT Identifies indicators of trypanotolerance expressed in a managed herd and generates data on the heritability of some of these indicators	Involves ILRAD in large-scale epidemiology studies that address the phenotype of trypanotolerance, not the mechanisms of trypanotolerance
Identify the cell and molecular basis of AAT pathogenesis by comparative analyses of innate and acquired immune responses of trypanotolerant and susceptible hosts	Adds to our understanding of the bovine immune system under AAT stress and adds reagents to the bovine immunology tool chest May identify molecular triggers of pathology in AAT and thus contribute to the elucidation of the genetic basis of trypanotolerance and its exploitation	AAT pathogenesis might be multifactorial and of unfathomable biological complexity
Identify genes linked to trypanotolerance through comparative analysis of post-infection phenotypes and genome linkage maps of F_2 progeny of N'Dama × Boran crosses	Involves ILRAD in the global effort to map the bovine genome May identify host genes that control anaemia, parasitaemia and mortality in AAT and expedite marker-assisted selection to enrich trypanotolerance	Trypanotolerance may be a polygenic trait with small contributions from each of many unlinked genes, precluding mapping

that would address mechanisms of disease resistance in the taurine breeds of cattle in West and Central Africa, namely the N'Dama and West African Shorthorn. These taurine cattle, particularly N'Dama, were reputed to be trypanotolerant, i.e. to have the capacity to survive and be productive in tsetse-infested areas without treatment (Camille-Isidore, 1906; Murray *et al.*, 1982). Trypanotolerance is thought to have arisen within the taurine breeds of West Africa during several thousand years of selection in tsetse-infested areas. The degree of trypanotolerance varies within the breed and may therefore be open to improvement through selective breeding. It may also be possible to introduce the trait into other cattle breeds by marker-assisted selection, creating a domestic bovid ideally suited to African agriculture.

African Trypanotolerant Livestock Network

The African Trypanotolerant Livestock Network (ATLN) was established in 1977 by John Trail (ILCA) and Max Murray (ILRAD) to identify indicators of trypanotolerance in N'Dama cattle under natural tsetse/trypanosome challenge and to improve management of N'Dama cattle under such challenge. ATLN was an unusually collaborative effort involving the United Nations Environment Programme (UNEP), FAO, the ITC and the Centre International de Recherche-Développement sur l'Elevage en zone Subhumide (CIRDES), as well as national entities of Côte d'Ivoire, Ethiopia, Gabon, Kenya, Nigeria, The Gambia, Togo and what was then Zaire (now the Democratic Republic of the Congo).

Importation of N'Damas to ILRAD for on-site study, which had been (reasonably) discouraged by the then-Director General of ILRI, Jim Henson, and by the Kenya Veterinary Service for fear of accidental disease spread, was eventually realized in 1984 when frozen N'Dama embryos were imported and implanted in surrogate Boran mothers, through the expertise of Ivan Morrison, Geoff Mahan and Torbin Jordt (Jordt *et al.*, 1986a,b). In 1984, 10 N'Dama calves were born at ILRAD. These animals and their progeny

were used extensively in studies of mechanisms of resistance to AAT and in linkage genome-mapping studies reported later in the text.

ATLN had aimed to 'improve livestock production in tsetse-infested areas of Africa by achieving a better understanding of genetic [-ally acquired] resistance, environmental factors that affect susceptibility and the efficacy of present control measures, and by ensuring better application of existing knowledge and recent research findings' (ILRAD, 1985). In 1985, the network was coordinating investigations at sites in nine countries of West and Central Africa. Five of these study sites were well established, (Gabon, Ivory Coast, Nigeria, Togo, Zaire), two were under development (Senegal, The Gambia) and two were under consideration (Benin, Congo).

ILRAD was to provide supervision of animal health, infection status, and tsetse evaluation, while ILCA was responsible for animal production, nutrition and data processing. Data collection was rigorous. 'Field operations involved the simultaneous collection of data on infection, health and productivity. Staff at all sites recorded data on simple pre-printed forms for transmission to Nairobi every month. These were checked for completeness, verified and entered into a computer file in Nairobi. Major analyses that were carried out on field data in Addis Ababa involved the computation of productivity indices based on reproductive performance, viability, calf growth, and cow weight, as well as the assessment of possible factors affecting animal performance at different sites (ILRAD, 1985). Data analysis programmes used for these studies had been developed and tested using detailed records of matching animal health, animal productivity and trypanocidal drug treatments kept for many years at two large ranches in East Africa, namely Kilifi Plantations, Kenya, and Mkwaja Ranch, Tanzania, with which ILRAD had a long-term involvement.

The dark-ground buffy coat (DGBC) phase-contrast diagnostic technique

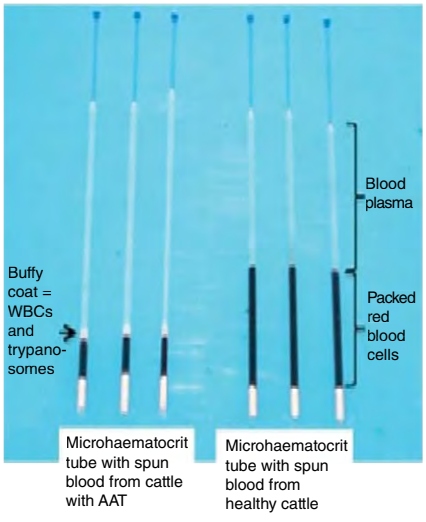
A dual anaemia/infection diagnostic test that was developed by Murray *et al.* (1977a) played an important role in ATLN field studies. This test was specific for blood-borne trypanosomes, and was sensitive, scalable for high-throughput testing, could be used in a proximal field laboratory, provided an estimate of parasitaemia (number of trypanosomes/ml of blood) and allowed identification of trypanosome species determined by morphology. The test also provided matched data on the blood PCV of the test animal. Briefly, blood was drawn by capillary action into a heparinized haematocrit tube, centrifuged in a haematocrit centrifuge and the blood PCV recorded as a percentage of total blood volume, as shown in Fig. 2.1. The tube was nicked with a diamond pen 1 mm beneath the buffy coat layer, which is composed of white blood cells and trypanosomes and subsequently snapped to remove the red cell layer. It is then nicked 1 cm above the buffy coat (i.e. in the blood plasma layer) and snapped and the white blood cell/trypanosome plug is then expelled on to a glass slide, covered with a 22 × 22 mm coverslip and the preparation scanned by microscopy for the presence and prevalence of trypanosomes, which can provide an estimate of parasitaemia. This technique was used by ILRAD to train legions of veterinary technicians throughout Africa. The relationship of parasite prevalence in the buffy coat and estimated parasitaemia was established by seeding samples of blood with known numbers of trypanosomes.

With respect to animal health data, although the AAT-associated decrease in PCV might arise as a result of red blood cell lysis or phagocytosis, or through haemodilution, as discussed by Fiennes (1970), the decline in PCV denotes a detrimental change in subject health and therefore informs on disease. With respect to infection status, one drawback of the DGBC technique is that it provides an estimate of trypanosomes circulating in the peripheral blood only, and hence may underestimate infections where trypanosomes adhere to blood vessels (*T. congolense*) or inhabit tissues (*T. brucei* and to a lesser extent *T. vivax*). Thus, the DGBC diagnostic technique was supplemented with antigen-detection tests as discussed below.

Antigen enzyme-linked immunosorbent assays, isoenzymes and polymerase chain reaction

Expertise in mAb production at ILRAD, and in molecular genetic techniques, including the polymerase chain reaction (PCR) and

(a)



(b)

Number of trypanosomes	Score	Estimated parasitaemia tryps/ml
Swarming > 100 per field	6+	$>5 \times 10^6$
> 10 per field	5+	$>5 \times 10^5$
1 – 10 per field	4+	$10^4 - 5 \times 10^4$
1 per 2 fields–1 per 10 fields	3+	$5 \times 10^3 - 5 \times 10^4$
1 – 10 per preparation	2+	$10^3 - 10^4$
1 per preparation	1+	$10^2 - 10^3$

Fig. 2.1. Microhaematocrit and estimation of parasites in the buffy coat. (a) The percentage of blood packed cell volume (PCV) is calculated as: PCV = packed red blood cells/(packed red blood cells + plasma) × 100. WBCs, white blood cells. (b) The blood buffy coat layer + 1 cm column of plasma, collected by cutting the microhaematocrit tube shown in (a) 1 mm below and 1 cm above the buffy coat, is expelled on to a glass slide, dispersed beneath a 22 × 22 mm cover slip, observed under dark-ground illumination with a Phaco2 NPL25/050 objective and a 10× eyepiece. Observations as shown in column 1 are scored as shown in column 2, providing an estimate of trypanosomes/ml blood as shown in column 3. The test can be used with all species of pathogenic African trypanosomes. (unpublished data from ILRAD.)

orthogonal-field-alternation gel electrophoresis (OFAGE), made it possible to design additional diagnostic tests based, respectively, on trypanosome antigen capture from host blood or lysates of tsetse, and analysis of DNA extracted from the blood buffy coat, or from tsetse or purified trypanosomes. These tests provide an estimate of parasite material present in the sample and thus provide evidence of both an ongoing infection and the intensity of that infection. The mAbs used were generated at ILRAD against *in vitro*-propagated procyclic forms of *T. congolense*, *T. brucei* and *T. brucei rhodesiense* (the cause of East African HAT) and *T. vivax* (Nantulya *et al.*, 1987), and react with antigens in the corresponding bloodstream-stage trypomastigotes. PCR primers designed at ILRAD to amplify trypanosome species-specific nucleotide sequences (Kukla *et al.*, 1987; ole-MoiYoi, 1987; Dirie *et al.*, 1993a,b; Masake *et al.*, 1997) were also powerful tools for parasite analysis, allowing investigators to dissect trypanosome diversity more fully than could be achieved by morphological and mAb analysis. Similarly, OFAGE, which separates DNA of different lengths and hence resolves the numbers and lengths of chromosomes and minichromosomes in preparations from isolates and clones of trypanosomes, contributed to parasite characterization (Majiwa *et al.*, 1985; Masake *et al.*, 1988; Kihurani *et al.*, 2000), as did isoenzyme analysis, which detects polymorphisms that result in changes in activity, molecular weight and isoelectric point of a panel of enzymes detected by coupled coloured dye precipitation reactions after gel electrophoresis or isoelectric focusing (Gibson *et al.*, 1983; Knowles *et al.*, 1988; Fasogbon *et al.*, 1990). These studies, performed at ILRAD and in other institutions, contributed to the development of a molecular taxonomy of trypanosomes and a better understanding of patterns of their coevolution (Hamilton *et al.*, 2007; Adams *et al.*, 2010).

The trypanosome antigen enzyme-linked immunosorbent assay (Ag-ELISA) is based on capture of trypanosome antigen from blood or another material using an antigen-specific mAb immobilized on a microtitre plate. Unbound material is removed by washing, and bound antigen is revealed by the addition of a second antibody specific for the captured antigen, tagged with an enzyme so that it gives a colour reaction. This

reaction is proportional in intensity to the amount of enzyme-tagged antibody bound and thus to the amount of captured antigen. This basic format was modified to facilitate tube capture and agglutination reactions for field analyses of infections.

Vinand Nantulya was the driving force for developing, validating and field testing trypanosome Ag-ELISA diagnostic tests at ILRAD. These included tests for HAT caused by *T. b. rhodesiense* (Nantulya, 1988, 1997; Komba *et al.*, 1992) and *T. b. gambiense* (Nantulya *et al.*, 1992a; Nantulya, 1997); for AAT caused by *T. brucei*, *T. congolense* or *T. vivax* in cattle, goats and horses (Nantulya *et al.*, 1992b; Nantulya and Lindqvist, 1989; Trail *et al.*, 1991b; Kihurani *et al.*, 1994; Masake *et al.*, 1995); and for surra caused by *T. evansi* in camels (Nantulya *et al.*, 1989; Waithanji *et al.*, 1993; Nantulya, 1994). Many of these tests were carried out in collaboration with scientists from institutes throughout Africa, including the University of Nairobi, Kenya; the Kenya Trypanosomiasis Research Institute, Kikuyu; the National Institute for Medical Research, Tabora, Tanzania; and the Noguchi Memorial Institute for Medical Research, Accra, Ghana; and with scientists from ATLN, discussed below.

As with the DGBC test, there are some operational limitations of the trypanosome Ag-ELISA. The trypanosome species-specific tests used the same capture and detecting antibody; hence, their sensitivity might be diminished by competition for the same antigenic epitopes. For these reasons, next-generation tests focused on generating capture and detection antibodies that recognize distinct epitopes on the same antigen. In addition, the capture antibodies are competing with endogenous host antibodies for the same antigens and quite possibly the same antigenic epitopes (the part of an antigen bound by an antibody), which will also diminish test sensitivity. Next-generation tests are being developed with nanobodies – recombinant antigen-combining sites of single heavy-chain camelid antibodies (Muyldermans *et al.*, 2009) – which, because of their antigen-combining site and size, bind antigenic epitopes distinct from those recognized by conventional antibodies and therefore do not compete with endogenous antibody for antigen capture.

Despite these concerns, the Ag-ELISA provides additional information to the DGBC diagnostic test and, when combined with parasitological data generated using the latter, allows infection classification as follows: (i) parasitaemic (DGBC positive); (ii) antigenaemic (Ag-ELISA positive); (iii) parasitaemic but not antigenaemic (DGBC positive but Ag-ELISA negative); or (iv) antigenaemic but not parasitaemic (Ag-ELISA positive but DGBC negative). These distinctions were of importance to field studies. For example, using a combination of the DGBC and Ag-ELISA tests on samples from N'Dama, Zebu and N'Dama × Zebu crosses subjected to sequential experimental *T. congolense* infections, colleagues at the ITC, in Banjul, The Gambia, found that during the course of the first parasitaemia wave, the overall percentage of positive cases detected by DGBC in blood was higher ($p<0.0001$) than that obtained by Ag-ELISA in tested samples of the three cattle populations. However, although the Ag-ELISA was less than 50% sensitive in detecting circulating antigens during the first 2 months of the primary infection, during the second infection, the overall number of infections detected by DGBC was lower in N'Dama ($p<0.005$) and the F_1 population ($p<0.001$) than that obtained using the Ag-ELISA (Mattioli and Faye, 1996). Similarly, Trail *et al.* (1991b), in ATLN, while studying infection of N'Dama cattle in Gabon, Central Africa, observed that of the animals detected as parasitaemic, 90% were also positive by Ag-ELISA; however, 40% of the animals with negative parasitological findings were found to be antigenaemic. Therefore, the tests should be used in combination wherever possible.

The trypanosome antigen-specific mAbs generated at ILRAD were also used to develop dot ELISAs for detecting trypanosome antigens in extracts of tsetse, i.e. for diagnosing infections of tsetse (Bosompem *et al.*, 1995a,b, 1996). The assay was performed by lysing tsetse or tsetse tissues in detergent (Triton X-100), adhering extract to a localized spot on a nitrocellulose membrane and treating with hydrogen peroxide to colour bleach the tsetse material, followed by membrane blocking with irrelevant protein to prevent non-specific sticking of the detection antibody to the membrane. An enzyme-tagged detection antibody was then added and an enzyme-linked colour reaction was carried out to detect the bound antibody. Thus, over time, ILRAD/ILCA/ILRI developed tools to map trypanosome infections of cattle and tsetse throughout Africa.

Combined with measures of productivity during infection, such mapping tools could be used to evaluate trypanotolerance and hence the possibility that N'Dama cattle were an African solution to an African problem.

Ranching with chemoprophylaxis and chemotherapy: the N'Dama advantage

In the absence of chemoprophylaxis and chemotherapy, the impact of AAT is proportional to tsetse challenge, with the killing of all trypanosomiasis-susceptible Boran cattle in areas of high challenge (Blaser *et al.*, 1979). In the absence of chemoprophylaxis/chemotherapy, AAT also has a substantial impact on trypanotolerant N'Dama cattle under natural tsetse/trypanosome challenge. In this respect, data from ATLN showed that productivity of N'Dama herds in The Gambia decreases by 11% in areas of low tsetse prevalence (determined by tsetse trapping), by 42% in areas of moderate tsetse prevalence and by 53% in areas of high tsetse prevalence, relative to that in areas without tsetse, based on analyses of the total weight of a 1-year-old calf and the live weight equivalent of milk produced by 100 kg of cow per year (Murray *et al.*, 1984). However, the presence of tsetse/trypanosome challenge does not necessarily preclude productive ranching of cattle, even that of trypanosomiasis-susceptible Boran cattle. Thus, ATLN analyses of 20,000 calving records for grade Boran beef cattle collected over 10 years at Mkwaja Ranch, located in a coastal region of Tanzania under high tsetse challenge, showed that these animals could be as productive as pure Boran cattle maintained under trypanosomiasis-free conditions in Kenya as long as they were supported by a chemoprophylactic regime based on the use of isometamidium chloride (Samorin; May and Baker Ltd, London) and occasional use of the therapeutic drug diminazene aceturate (Berenil; Hoechst). In fact, the animals required an average of 4.4 treatments with Samorin and 0.6 treatments with Berenil each cow-year to sustain their productivity (Trail *et al.*, 1985). In the absence of treatment, the level of tsetse challenge was such that cattle could not survive (Blaser *et al.*, 1979). Trypanosomiasis drug resistance did not pose a problem to this strategy at that time, although it has since been reported for trypanosomes present in East and West Africa (Clausen *et al.*, 1992; d'Ieteren *et al.*, 1997).

Parameters of trypanosome infection and animal health under natural tsetse/ trypanosome challenge in N'Dama cattle

This section summarizes studies performed by the ILCA/ILRAD ATLN that established parameters of trypanotolerance under natural tsetse/ trypanosome challenge, thus providing quality control for analysis of trypanotolerance in experimental infections in the ILRAD laboratory setting. In separate tests carried out in Gabon, Central Africa, between 1987 and 1989, a total of 436 1-year-old N'Dama cattle were maintained for periods of 12, 18 or 24 weeks under a medium natural tsetse/trypanosome challenge, and infection and health parameters measured. Infection prevalences were 25%, 31% and 9%, respectively, and anaemia, measured by the average packed red cell volume (PCV, measured as a percentage) over the test period, or by the lowest PCV reached, was found to be more closely associated with animal performance than when measured by average PCV when detected as parasitaemic (Trail *et al.*, 1990). The possibility that maintenance of PCV under tsetse/trypanosome challenge might serve as a measure of trypanotolerance was supported by studies of N'Dama cows maintained for 3.5 years under a high natural tsetse challenge in Zaire. Thus, a simultaneous evaluation of the effects of three possible criteria of trypanotolerance – namely, time detected as parasitaemic, parasitaemia score and PCV – showed that the latter had the major effect on reproductive performance, calf weaning weight and cow productivity (Trail *et al.*, 1991a). Additional studies of N'Dama cattle in Gabon under natural tsetse/trypanosome challenge showed that 'animals capable of maintaining PCV values, even when [trypanosome antigenaemic] on a high number of occasions, grew at the same rate as uninfected animals' whereas 'animals that could not maintain PCV values when infected had poorer growth' (Trail *et al.*, 1992), supporting the hypothesis that maintenance of PCV during AAT is an indicator of trypanotolerance and indicating variability in trypanotolerance within members of an N'Dama herd.

Trail *et al.* (1993) evaluated the impact of prophylactic and curative drug treatments on

the productivity of N'Dama cattle at the government ranch of the Office of Gabonais d'Amélioration de Production de Viande, in south-eastern Gabon. In one study, curative drug treatments were given to 13.7% of cows over the calendar year and to 40% of calves from birth to weaning. The mean number of curative treatments was 0.16 per cow per year and 0.52 per calf from birth to weaning (where 1 signifies that curative treatments administered are the same as the number of animals in the study, although not necessarily that all animals receive one curative treatment). Treatments were on the following basis: cattle that were parasitaemic and had PCV values of less than 25% were routinely given a curative treatment with Berenil, as were animals with PCV values below 20%, whether they were parasitaemic or not (antigenaemia was not measured). The kinetics and levels of recovery of PCV were similar in both groups of animals, indicating that, even though parasites were not detected in blood, animals with low PCVs in this study were nevertheless infected with trypanosomes.

Both calf and cow average PCVs were linked to calf weaning weight, 'there being a 0.91 kg increase for each 1% increase in calf average PCV, and a 0.95 kg increase in weaning weight for each 1% increase in cow average PCV' (Trail *et al.*, 1993). Similar studies by Trail *et al.* (1991a) in Zaire where the prevalence of cow and calf infection was similar to those in the Gabon study showed that a high average calf PCV increased weaning weight by 9.9 kg (7.7% of total weaning weight) and a high average cow PCV value increased calf weaning weight by 8.8 kg (6.1%). Hence, maintenance of N'Dama PCV through curative drug treatment is an important production strategy. Half of the cattle in the Gabon study (Trail *et al.*, 1993) received a prophylactic treatment with Samorin at 6 and 18 months into the study to determine its impact on prevalence of infection. This treatment regime decreased the number of curative treatments required in cows from an average of 0.25 per cow-year in unprotected cows to 0.06 per cow-year in protected cows. Although there are no data on the relative tsetse/trypanosome challenge at the Gabon study site versus the Mkwaja Ranch in Tanzania cited earlier, it is noteworthy that grade Boran cattle on the Mkwaja Ranch required 4.4 prophylactic treatments and 0.6 curative treatments per cow-year (Trail *et al.*, 1985), which is much greater than the treatments required by N'Damas and is consistent with the lesser ability of the Borans to survive and be productive under natural tsetse/trypanosome challenge.

The above studies support the idea 'that the ability to control anaemia during infection as indicated by average PCV might be one reliable criterion of trypanotolerance with which to identify trypanotolerant animals' (Trail *et al.*, 1993). However, it is not the sole criterion. Thus, Trail *et al.* (1994), working with data from 255 N'Dama under high natural tsetse challenge in Zaire over a 2-year period from weaning at 10 months of age, showed that the species of infecting trypanosome, length of time parasitaemic, intensity of parasitaemia and anaemic condition had approximately equal effects on the final performance trait of daily live weight gain, and thus information on all of these is essential for assessing an animal's overall trypanotolerance phenotype. As evident from the number of animals studied and the number of parameters measured, these were demanding and comprehensive studies.

The ATLN findings have been summarized in a book (ILCA/ILRAD, 1988) and in several review articles (Trail *et al.*, 1989; Murray *et al.*, 1990; d'Ieteren *et al.*, 1998). Those findings jointly validate the trypanotolerant status of N'Dama and identify indicators of the trait under natural challenge. These studies show that trypanotolerant cattle can be productive with low-level chemoprophylactic/therapeutic intervention in areas of moderate to high tsetse/trypanosome challenge, thus establishing N'Dama as a gene pool for use in tsetse-infested sub-Saharan Africa. In the period of broad and systematic studies of trypanotolerance, done jointly by ILRAD and ILCA in the 1970s and continued by ILRI after 1994, the three institutions generated more than 175 papers on various aspects of trypanotolerance, averaging four papers annually (24 mean citations per paper) from 1975 to 2018. Notable achievements were Murray *et al.* (1982) on host susceptibility, which remains at the 99th percentile of citations in its field within Scopus; the ILCA/ILRAD (1988) book on the achievements of the ATLN; Trail *et al.* (1993, 1994) on productivity; Hanotte *et al.* (2003) on genetic mechanisms of trypanosomiasis; and the later review article of Naessens (2006) summarizing more than 30 years of research on the subject.

The cell and molecular biology of trypanotolerance in cattle

T. congolense *cyclic infection and homologous cyclic reinfection of N'Damas and Borans*

N'Dama cattle produced by embryo transplants that were raised at ILRAD until 13 months of age were subjected to four sequential cyclic infections (*Glossina morsitans centralis*) with *T. congolense* derived from four different serodemes (Paling *et al.*, 1991a). After each infection, the cattle were treated with a curative dose of Berenil and rested for 1 month before the next cyclic infection. Boran cattle, a trypanosomiasis-susceptible breed of *Bos indicus*, were similarly infected and these animals were treated with a curative dose of Berenil as required. Irrespective of the infecting *T. congolense* serodeme, there was no significant difference between Boran and N'Dama groups in the number of chancres that developed at tsetse bite sites or in the kinetics or magnitude of their development, indicating that there was no pre-programmed (innate) component in either host breed that prevented infection by killing parasites in the skin.

All cattle became parasitaemic with similar kinetics irrespective of breed or trypanosome serodeme used for cyclic infection, indicating that there was no pre-programmed response in either breed that prevented migration of the parasites from the skin to the bloodstream, which occurs via the lymph (Akol and Murray, 1986). With three of the four test serodemes, levels of parasitaemia and the kinetics of parasitaemic wave remission were similar in N'Dama and Boran cattle for 30 days or so after infection; with the other serodeme, they were similar for the first 15 days after infection. These data showed that host protective immune responses were initially similar in the infected N'Damas and Borans. Thereafter, breed-related differences in parameters of infection became evident, replicating, in the case of N'Dama cattle, indicators of trypanotolerance observed with natural tsetse/trypanosome challenge, as discussed above. Thus, irrespective of the infecting serodeme, most infected Boran cattle, but no N'Dama cattle, rapidly developed life-threatening anaemia (PCV of 15% or less) by around 40 days after infection and were treated with a curative dose of Berenil. In addition, all N'Dama cattle suppressed the level of parasitaemia in an incremental manner until it became cryptic (less than 10^2 trypanosomes/ml of blood), whereas those few infected Borans that did not require curative Berenil treatment were still presenting high levels of parasitaemia until all animals were treated with a curative dose of Berenil to terminate infection.

In a follow-up study, cattle that had been sequentially exposed to four cyclic heterologous infections were cured after the last infection, rested for 3 months and subjected to homologous cyclic reinfection with the first infecting serodeme, which was approximately 2 years after their first exposure to this *T. congolense* serodeme (Paling *et al.*, 1991b). Although five of the eight Borans and all eight N'Damas had neutralizing anti-metacyclic VSG antibodies present in their serum, determined by incubating the parasites in serum and their injection into mice, these antibodies did not prevent infection of the cattle and presumably were present in serum at much higher concentrations than in the skin. As with primary infections, neither the kinetics nor the size of the chancres that developed at tsetse bite sites following homologous reinfection differed significantly between N'Damas and Borans. However, infected N'Damas subsequently developed a single parasitaemic wave, which was two orders of magnitude lower than that developed on primary cyclic infection, and rapidly suppressed parasitaemia to a cryptic level, whereas the reinfected Borans developed multiple waves of parasitaemia, albeit of lesser magnitude, than those developed on primary cyclic infection, and these decreased in amplitude as infection progressed, thus tending towards cryptic infection. It is unclear whether these cattle, and in particular the N'Damas, had serum antibodies against bloodstream-stage *T. congolense* serodeme-1 VSGs that suppressed parasitaemia on homologous cyclic reinfection or had a recall adaptive response against these VSGs or both. It is clear that Borans had a lesser advantage in this respect. Importantly, N'Dama cattle that were reinfected by homologous cyclic infection did not show signs of anaemia, whereas similarly reinfected Borans had an acute drop in PCV, although less acute than Borans on the primary cyclic infection, and 50% of these animals required curative Berenil treatment by 50–70 days post-infection to avoid death.

Following curative treatment, the N'Dama and Boran cattle were again subjected to homologous cyclic reinfection, this time with tsetse transmitting *T. congolense* serodeme 2 (Williams *et al.*, 1991). The results were similar to those seen in the first homologous cyclic infection and, together with those for sequential cyclic infections, are summarized in Table 2.4.

Thus, cyclic *T. congolense* infections carried out at ILRAD showed that N'Dama cattle have a greater capacity to sustain blood PCV levels during infection than similarly infected Borans, in which PCVs dropped from a mean of 33% to 16% over a period of about 40 days after infection. Given that the lifespan of a bovine red blood cell is about 160 days, a drop in PCV of 50% over 40 days most likely reflects a substantial destruction of red blood cells. Thus, if the drop in PCV resulted solely from a shutdown of erythropoiesis and no other process, PCVs would have dropped by only 25% during the 40-day period, resulting in a PCV of more than 24% at 40 days after infection. Furthermore, a shutdown of erythropoiesis does not occur in *T. congolense*-infected Borans; it was shown at ILRAD that *T. congolense*-infected N'Damas and Borans have an erythropoietic response characterized by peaks above pre-infection levels of early and late erythroid progenitor cells (burst-forming units–erythroid and colony-forming units–erythroid, respectively) in the bone marrow of the fourth, fifth and sixth sternebrae (Andrianarivo *et al.*, 1995, 1996), and presumably in red bone marrow elsewhere in the animals, which agrees with conclusions from other investigators (Dargie *et al.*, 1979).

AAT-induced anaemia

Erythrophagocytosis by monocytes, macrophages and neutrophils has been demonstrated in cattle infected with African trypanosomes (reviewed by Murray and Dexter, 1988). While quantitative comparisons of this process were not made in infected N'Damas and Borans at ILRAD, colleagues at the University of Ibadan, Nigeria, who include ILRAD alumni Victor Taiwo and Victor Anosa, showed, using an *in vitro* assay, that erythrocyte phagocytosis and lysis by splenic plastic adherent cells (predominantly macrophages) from *T. congolense*-infected Borans was greater than that of splenic macrophages from similarly infected N'Damas and correlated dynamically with the degree of anaemia developed by these animals (Taiwo and Anosa, 2000). In addition, researchers at ILRAD showed that acute anaemia in *T. vivax*-infected cattle correlated with the production of TNF (Stijlemans *et al.*, 2016), a cytokine that increases the phagocytic activity of bovine neutrophils, by blood monocytes (Rainard *et al.*, 2000). In support of a role for erythrophagocytosis in AAT-induced anaemia, studies using a mouse model of *T. brucei* AAT showed that the acute drop in

Table 2.4. Characteristics of experimental cyclic *T. congolense* infections in N'Dama and Boran cattle. (Data from Paling *et al.*, 1991a,b; Williams *et al.*, 1991.)

Infection	N'Damas	Borans
Primary cyclic infection (four serodemes)	Drop in PCV but not life-threatening Parasitaemic waves decrease in amplitude incrementally over time to a cryptic level (<10^2 *T. congolense*/ml of blood), which is reached by 100–130 days post-infection Fast recovery of PCV as parasitaemia is suppressed	Acute drop in PCV: 75–100% of animals require curative treatment by about 40 days post-infection Repeating waves of parasitaemia do not decrease in amplitude over time Slow recovery of PCV in those animals that did not need curative treatment
Homologous cyclic reinfection	No change in PCV Rapidly suppress parasitaemia to a cryptic level with occasional low-amplitude spikes of parasitaemia	Drop in PCV, although less so than in primary cyclic infection: 50% of animals require curative treatment by 50–70 days post-infection Repeating waves of parasitaemia that decrease in amplitude over time in those animals that do not require curative treatment

PCV after infection results from phagocytosis of erythrocytes by activated liver monocytic cells and neutrophils and by splenic macrophages (Stijlemans *et al.*, 2015) and is induced by IFN-γ produced by natural killer (NK) cells, NK T-cells and $CD8^+$ T-cells. Furthermore, the drop in PCV is prevented by the deletion of these cells and of the gene encoding the receptor for IFN-γ (Cnops *et al.*, 2015).

Dargie *et al.* (1979) noted a positive correlation between the severity of anaemia in cattle infected with *T. congolense* and the level and duration of parasitaemia. While this may be the case within a breed, it is not the case between breeds. Thus, work at ILRAD showed that *T. congolense*-infected Boran cattle developed anaemia more rapidly than similarly infected N'Dama cattle at ILRAD, despite initially similar levels of parasitaemia (Paling *et al.*, 1991a). It is possible that the number of *T. congolense* in the entire vascular system, rather than that of peripheral blood only, will correlate with the level of anaemia. In this regard, earlier work at ILRAD showed that *T. congolense* were not uniformly distributed in the host vasculature and that many more parasites were present in the microcirculation than were free in the cardiac blood, and some adhered to vessel walls (Banks, 1978). In addition, Trail *et al.* (1993) showed in their studies on N'Dama cattle in Gabon that some animals judged to be aparasitaemic by the DGBC technique developed low PCVs that were restored by a curative dose of the trypanocidal drug Berenil.

Whether or not differences in parasite load and distribution have an impact on AAT-induced anaemia in N'Damas and Borans, it is certainly not the only factor to do so; there is a clear impact of the genotype of the haematopoietic system. In a remarkable set of studies carried out at ILRAD, Naessens *et al.* (2003a) analysed levels of anaemia and parasitaemia in infected Borans and N'Damas exposed to cyclic infection with the *T. congolense* serodeme 1 ('IL 1180'). The cattle were either intact or modified experimentally to have a chimeric haematopoietic system, which in the case of N'Damas was 70–94% of Boran origin, depending on the individual, and in the case of Borans was from 30 to less than 5% of N'Dama origin, depending on the individual, based on analysis of a polymorphic T-cell marker, CD5, using mAbs that distinguish between cells of *Bos taurus* and *B. indicus* origin. The development and severity of anaemia was significantly less in intact N'Damas than in Borans, chimeric Borans and chimeric N'Damas, all of which developed a similar degree of anaemia. Strikingly, levels of parasitaemia were lower in the N'Damas, whether intact or chimeric, than in the Borans, whether intact or chimeric. These results show that control of anaemia, which in the chimeric N'Damas was similar to that of infected Borans and is thus determined by the genotype of the haematopoietic tissue, is quite separate from control of peripheral blood parasitaemia, which in the chimeric N'Damas resembled that of Borans and is thus determined by non-haematopoietic elements although most likely acting on immune responses mediated by progeny of haematopoietic stem cells.

The haematopoietic chimeras were created by implanting an N'Dama and a Boran embryo at the late-morula stage into the same surrogate Boran mother, which results in anastomoses of chorioallantoic vessels in the placenta of the twin foetuses in early fetal life and haematopoietic chimerism in which the Boran component dominates. Haematopoietic stem cells give rise to erythroid, myeloid and lymphoid lineages, and hence, while it is clear that susceptibility to AAT-induced anaemia is a property of the Boran haematopoietic system and not other aspects of host physiology, it is unclear which component(s) of the haematopoietic system predispose(s) to this pathology.

Production of erythrocytes from precursor cells in the bone marrow is stimulated by erythropoietin, which is produced by kidney fibroblasts. Work at ILRAD showed that, while the transcript for erythropoietin was similarly increased in the kidneys of *T. congolense*-infected N'Damas and Borans at 35 days after infection, there was higher expression of the transcript encoding the erythropoietin receptor (epoR) in the bone marrow of the infected N'Damas than in the Borans (Suliman *et al.*, 1999). Assuming a direct association between expressed gene and protein, this finding suggests that haematopoietic progenitor cells in the bone marrow of the N'Damas would be more responsive to ambient erythropoietin than those of Borans, consistent with their resistance to AAT-induced anaemia. However, unexpectedly, this did not result in a stronger reticulocyte response, which is an

indicator of erythropoiesis, in the N'Damas. Rather, the reticulocyte response of the infected Borans during the early stages of infection was greater than that of the N'Damas (Andrianarivo *et al.*, 1996), despite the more profound anaemia that developed in the Borans. It is perhaps noteworthy that the epoR transcript analyses were performed with whole bone marrow from infected N'Damas and Borans and not from purified erythroid progenitor cells (Suliman *et al.*, 1999); hence, it is unclear which cell types in the former have the increased epoR transcript. This may be important because erythropoietin is now known to have pleiotropic effects on the immune system, where it inhibits macrophage functions (Nairz *et al.*, 2012), among other processes. Consequently, higher expression levels of epoR in bone marrow macrophages of infected N'Damas compared with infected Borans at an equal concentration of erythropoietin might result in a lower level of activation and thus a lower level of *in situ* phagocytosis of reticulocytes and erythroid progenitor cells consistent with haematopoietic-tissue intrinsic regulation of anaemia. Along similar lines, a significantly greater increase in transcript for the macrophage-activating cytokine IFN-γ, and the pyrogens interleukin (IL)-1α and IL-1β, was found in the bone marrows of infected Borans compared with those of the N'Damas (Suliman *et al.*, 1999), consistent with a greater potential for macrophage activation in the Borans. It is not known whether an N'Dama background would affect these properties of the Boran haematopoietic tissue; hence, we can only speculate that it would not, thus accounting for the inability of N'Damas bearing the Boran haematopoietic system to control anaemia.

A second haematopoietic-system intrinsic mechanism that could lead to greater destruction of red blood cells in animals with the Boran haematopoietic system affects expression on red blood cells of important surface antigens, including blood group antigens. Red blood cells of N'Damas have been shown to have significantly higher levels of sialic acids on their surface than red blood cells of Borans (Shugaba *et al.*, 1994), and hence are less affected by trypanosome *trans*-sialidases (Buratai *et al.*, 2006), which cleave sialyl groups from surface glycoproteins and glycolipids and directly promote phagocytosis of the red blood cells (Nok and Balogun, 2003; Guegan *et al.*, 2013). In addition, it has been shown that AAT sometimes causes production of antibodies that react with antigens on healthy red blood cells, or against antigens exposed by infection-related processes, or bound as immune complexes with trypanosome VSG (Kobayashi *et al.*, 1976; Assoku and Gardiner, 1989; Rifkin and Landsberger, 1990), and thus promotes phagocytosis of the antibody-coated red blood cells. However, while this occurs in both N'Damas and Borans infected with a haemorrhagic *T. vivax*, as shown at ILRAD (Assoku and Gardiner, 1989; Williams *et al.*, 1992), there is no evidence that is the case in the *T. congolense*-infected intact and chimeric N'Damas and Borans. Indeed, Naessens and colleagues considered it unlikely that an adaptive immune response is responsible for anaemia in the *T. congolense*-infected cattle, because disruption of immune responses in N'Damas and Borans by complete deletion of $CD4^+$ T-cells, $CD8^+$ T-cells and γδ T-cells from the blood and peripheral organs using specific mAbs (Naessens *et al.*, 2003b; Sileghem and Naessens, 1995; Naessens, 2006) did not affect their distinct levels of anaemia, although the anti-CD4 treatment severely decreased host antibody responses (data not published, but summarized in Naessens *et al.*, 2002).

The pro-inflammatory cytokine TNF-α also has a role in the induction of anaemia, at least with some species/strains of African trypanosomes. Thus, studies in mice have shown that levels of anaemia were similar in *T. congolense*-infected TNF-α knock-out mice and similarly infected wild-type mice, suggesting that anaemia in this infection is TNF-α-independent. In contrast, the anaemia induced by *T. b. rhodesiense* (Naessens *et al.*, 2005) and *T. b. brucei* (Magez *et al.*, 1999) infection was significantly lower in TNF-α knock-out compared with intact mice, suggesting that the infected intact mice have a TNF-α-dependent mechanism that exacerbates anaemia. The highly virulent haemorrhagic East African strain of *T. vivax* may also induce this TNF-α-dependent mechanism of anaemia in infected cattle, including N'Damas, whereas *T. congolense* does not (Williams *et al.*, 1992; Sileghem *et al.*, 1994). Furthermore, N'Dama cattle proved not to be tolerant against an infection with the haemorrhagic *T. vivax*, suffering as much as or more so than susceptible cattle. Thus, N'Dama cattle may have evolved a trait that protects against TNF-α-independent

anaemia, but that is less, or not, effective against TNF-α-dependent anaemia.

AAT-induced lymphopenia and possible role of haemophagocytic syndrome in AAT

Studies at ILRAD on peripheral blood leukocyte dynamics in N'Damas, Borans, chimeric N'Damas and chimeric Borans following cyclic infection with *T. congolense* showed the same pattern as that of anaemia. Thus, while all groups of cattle had an initially similar acute decrease in numbers of total white blood cells, lymphocytes and neutrophils in the peripheral blood during development of the first parasitaemic wave (Ellis *et al.*, 1987; Williams *et al.*, 1991; Naessens *et al.*, 2003a), these values recovered quickly in intact N'Damas but not in intact Borans, chimeric Borans or chimeric N'Damas (Naessens *et al.*, 2003a). There was also a trend in gain of body weight to suggest that it, too, followed the pattern of anaemia and leukopenia in the infected intact and chimeric cattle, but these results were less clear cut than anaemia and leukopenia. Nevertheless, the correlation between anaemia, leukopenia and decrease in weight gain in intact and chimeric *T. congolense*-infected Borans, chimeric Borans and chimeric N'Damas is consistent with the possibility that these pathological processes are co-regulated, leading Naessens to consider them as a pathogenic syndrome and to seek other disease states in which these indicators of pathology are similarly linked. The result was recognition that AAT and haemophagocytic syndrome (HPS) share many clinical and pathological features (Naessens, 2006). HPS is 'a severe and often fatal syndrome resulting from potent and uncontrolled activation and proliferation of T lymphocytes, leading to excessive macrophage activation and multiple deleterious effects. It is associated with defects in cytotoxic granule-dependent cytotoxic activity of lymphocytes … thus highlighting the determinant role of this function in driving the immune system to a state of equilibrium following infection' (reviewed by Menasche *et al.*, 2005). While little is known about cytotoxic effector cells in AAT in cattle, the author's laboratory has recently shown that NK cells are globally activated in a murine model of AAT, deleting splenic B2 B-cells (Frenkel *et al.*, 2016) and $CD8^+$ T-cells (D. Frenkel and S.J. Black unpublished data, 2019) in the spleen, and as yet unresolved target cells in the liver and lymph nodes. Perhaps this strangely aberrant behaviour of the NK cells is a futile attempt to dampen excessive immune system activation, an excessive application of the very response that is defective and inactive in HPS. Analysis of NK and other cytotoxic cell activation in AAT is warranted, as this might inform on the severe depletion of spleen and lymph node cells in late-stage infection, described by Fiennes (1970).

Antibody responses of trypanosome-infected N'Dama and Boran cattle

Studies by the ATLN clearly showed that a curative dose of Berenil restored PCV, body weight and productivity in cattle with AAT, thus showing that AAT-induced pathology is utterly dependent on the continued presence of trypanosomes in affected hosts. The goal of ILRAD scientists was to determine which aspects of the host immune response to trypanosomes promotes self-cure and recovery versus sustained infection and severe pathology by comparing immune responses that arise in similarly infected N'Dama and Boran cattle. Because African trypanosomes are extracellular parasites that are killed by VSG-specific antibody-dependent processes, researchers addressed antibody responses. Two antigenic variants of *T. congolense* were cloned from the first peak of parasitaemia arising in cattle that received the first cyclic infection (Paling *et al.*, 1991a). These clones were shown by VSG analysis to be present in first-wave parasitaemias of both N'Damas and Borans. Analysis of clone-specific antibodies in host serum was performed up to 35 days after infection, which corresponds to a major dip in parasitaemia in both host breeds (Paling *et al.*, 1991a). The studies showed that the clone-specific antibodies comprised both IgM and IgG classes and were similarly increased in both breeds of cattle, reflecting the similar patterns of parasitaemia in both breeds up to 35 days after infection. Both classes of antibody were shown to bind to VSG on intact trypanosomes and mediate their attachment to phagocytes (predominantly neutrophils and monocytes) from N'Damas and Borans *in vitro*, with attachment to adherent cells from N'Damas exceeding that of adherent cells from Borans, and with IgG1 being the most efficient antibody at facilitating this attachment (Kamanga-Sollo

et al., 1991). Clearance of antibody-coated African trypanosomes from the bloodstream in mouse models of AAT is mediated by phagocytic cells in the liver, spleen and other organs (Macaskill *et al.*, 1980; Black *et al.*, 1985); consequently, the superior capacity of phagocytes from N'Damas compared with Borans to bind antibody-coated trypanosomes *in vitro* might be expected to be associated with more efficient clearance of the parasites from infected N'Damas than from Borans *in vivo* and thus lower levels of parasitaemia in the former at equivalent levels of specific antibodies. Because this was not observed up to 35 days after infection of the cattle (Paling *et al.*, 1991a), it is possible that a disparity in binding of antibody-coated parasites to phagocytes from N'Damas and Borans does not arise in infected animals, perhaps as a result of activation of phagocytes, which is known to increase expression of IgG1 receptors on bovine monocytes (McGuire *et al.*, 1979).

In a related study of the same serum samples, an analysis was performed to determine the fine specificity of serum antibodies that recognize *T. congolense* clone ILNat 3.1 VSG (Williams *et al.*, 1996), a variant that arises in the first parasitaemic wave of infected N'Damas and Borans. This analysis is of interest because VSG is tightly packed on the surface of intact trypanosomes; as a result, only antibodies specific for antigenic epitopes present on the N-terminal domain of VSGs (exposed VSG epitopes) can bind to intact trypanosomes. An antigenic epitope is that small portion of the antigen that is bound by a specific antibody; a single antigen can have several different antigenic epitopes. Antibodies against other epitopes on the same VSG molecule (buried VSG epitopes) cannot bind to coated parasites but can bind once the VSG is released from the parasites, for example as a result of cleavage with the VSG GPI-PLC. Binding of antibody to soluble VSG and its clearance by phagocytes might be important because this material had been shown by investigators at ILRAD to activate bovine complement (Musoke and Barbet, 1977), which can elicit a cascade of pro-inflammatory components and thus might contribute to systemic inflammation. This investigation also showed that infected N'Damas and Borans made IgM and IgG1 antibodies against exposed VSG epitopes on infecting parasites, and in addition showed that IgG2 antibodies were made (Williams *et al.*, 1996), which had not been assayed in the previous study. The kinetics and magnitude of *T. congolense* ILNat 3.1 VSG exposed-epitope-specific IgM, IgG2 and IgG1 responses by N'Damas and Borans were close to identical, as determined by fluorescent antibody-binding assays on intact parasites, consistent with similar control of the first-wave parasitaemia. However, this was not the case for antibodies specific for buried VSG epitopes, against which Borans made IgM antibodies and little IgG1, while N'Damas made IgG1 antibodies and little IgM. Analyses of splenic antibody-secreting cells from these animals showed a similar disparity in IgM:IgG ratios of plasma cells in infected N'Damas and Borans secreting antibodies that bind to soluble VSG (Taylor *et al.*, 1996b). Unfortunately, we know very little about how antibody responses against VSG on intact trypanosomes versus that of soluble VSG are regulated (Black *et al.*, 2010). However, it is possible that antibody responses against exposed VSG epitopes on parasite-attached VSG, versus those against epitopes on VSG that are buried on intact trypanosomes but accessible on soluble VSG, are made by different sets of B-cells that are differently regulated. Indeed, as a result of work at ILRAD, we know that a substantial portion of the antibody response to VSG is made up of low-affinity antibodies that react both with VSG and with a variety of cross-reacting proteins, including β-galactosidase and autoantigens (Naessens and Williams, 1992; Williams *et al.*, 1996). These antibodies are predominantly produced by a subset of B-lymphocytes that express the differentiation antigen CD5 (Williams *et al.*, 1991) and, at least with respect to antibodies that react both with ILNat 3.1 VSG and β-galactosidase, are a feature of the response of infected Borans but not of N'Damas (Williams *et al.*, 1996).

The concentration of IgM and IgG2 antibodies specific for VSG epitopes of *T. congolense* ILNat 3.1, and of IgM antibodies specific for buried VSG epitopes present in the blood plasma of Borans and N'Damas declined to baseline by 40 days after infection. In contrast, IgG1 antibodies in the blood plasma of the infected N'Damas and Borans that was specific for both exposed and buried VSG epitopes of ILNat 3.1 VSG remained at close to peak concentrations 40 days after infection in both breeds of cattle. Data were not obtained for later time points. At

appropriate concentrations, antibodies specific for exposed VSG epitopes neutralize trypanosomes expressing that VSG and therefore prevent recurrence of trypanosomes expressing that or a cross-reacting VSG. In this regard, the accumulation of trypanocidal/trypanostatic antibodies specific for exposed epitopes of multiple VSG types in the serum of infected Cape buffalo (*Syncerus caffer*) correlates with, and may be responsible for, maintenance of cryptic parasitaemia in this extremely trypanosomiasis-resistant bovid (Guirnalda *et al.*, 2007). It would therefore be interesting to learn how long the ILNat 3.1-specific IgG1 remained in the plasma of the infected N'Damas and Borans and whether similarly long-lived IgG1 antibody responses arise against later VSGs in both breeds of cattle. This would inform the relative effectiveness of host protective antibody responses over time in the trypanosomiasis-susceptible and trypanotolerant breeds.

Infected N'Damas were also found to differ from Borans by producing IgG1 antibodies against a greater number of proteins than are common to different trypanosome serodemes (Shapiro and Murray, 1982; Authie *et al.*, 1993b), including the 33-kDa congopain (Authie *et al.*, 1993a), which was discussed earlier with respect to its possible contribution to AAT pathogenesis and as a possible target for an anti-AAT vaccine. As with the response against buried VSG epitopes considered above, the more diverse antibody response against trypanosome-common antigens and the lesser production of antibodies against irrelevant antigens in infected N'Damas compared with Borans (Williams *et al.*, 1996) suggests functional differences in their immune responses. The possibility that this reflects differences in infection-induced T-cell-dependent B-cell responses in the infected cattle is considered next.

AAT-induced T-cell responses and immunosuppression

Infection with *T. congolense* and *T. vivax* compromises primary and recall vaccine responses in Boran cattle (Rurangirwa *et al.*, 1978, 1979, 1980, 1983; Whitelaw *et al.*, 1979; Ilemobade *et al.*, 1982; Sharpe *et al.*, 1982). This manifests as a substantial decrease in production of vaccine-specific IgM and IgG antibodies in the infected compared with the uninfected vaccinated animals (Ilemobade *et al.*, 1982). While AAT can dramatically affect primary and recall antibody responses to vaccines in cattle, it may not have an equally profound effect on recall antibody responses to early-arising VSGs, evidenced by occasionally recurring spikes of antibody against these VSGs during sustained infection in some infected Borans (Nantulya *et al.*, 1979; Musoke *et al.*, 1981; Vos and Gardiner, 1990). Similarly, processes that compromise antibody responses to vaccine antigens do not appear to prevent the immune responses that control newly arising trypanosome antigenic variants, reflected in repeating peaks of parasitaemia, each of which is cleared by antibodies specific for exposed VSG epitopes. These observations suggest that immune responses against exposed VSG epitopes differ in some important respect from primary and secondary immune responses against conventional antigens. Responses to conventional antigens are dependent on cognate interactions between B-cells, which produce the antibodies, and $CD4^+$ T-cells, which provide critical stimuli (by cell contact and by secretion of helper cytokines) that direct B-cell proliferation and differentiation to antibody-secreting cells. To determine whether T-cells are required for antibody responses to exposed and buried VSG epitopes, Naessens deleted $CD4^+$ T-cells from N'Dama and Boran calves with CD4-specific mAbs prior to their cyclic infection with *T. congolense* (unpublished data, summarized in Naessens, 2006). The antibody responses 'were found to be markedly reduced and delayed in the depleted animals. . . . This was the case for IgG and IgM antibodies to surface-exposed and internal trypanosome epitopes, as well as for natural IgM antibodies that react with non-trypanosome antigens.' Thus, help from $CD4^+$ T-cells was required for these responses.

T-cells provide help for B-cell responses to two types of antigens, called T-cell-dependent (TD) and T-cell-independent type 2 (TI-2). TD antigens are soluble proteins without multiple repeating (identical) epitopes on each molecule. TI-2 antigens are usually polysaccharides that lack direct mitogenic activity and have multiple repeating epitopes. The B-cell response to TD antigens requires a direct interaction between: (i) B-cells that have endocytosed the antigen, processed it in an endosome and placed peptides derived from the antigen on their surface in complex with MHC class II (MHC-II); and

(ii) T-cells with receptors specific for the MHC-II antigen peptide complex. This response develops in germinal centres of lymphoid follicles and generates B- and T-memory cells, as well as antibody-producing plasma cells and memory plasma cells. The memory cells mount rapid immune responses upon re-encountering the same antigen. The plasma cells secrete lots of antibody specific for the antigen that simulated the B-cell, and the long-lived memory plasma cells migrate to niches in the bone marrow and continue to secrete this specific antibody, often for years. In contrast, the B-cell response to TI-2 antigens occurs in the absence of MHC-II-restricted T-cell help, although it can be facilitated by cytokines produced by activated T-cells (Mond *et al.*, 1995), does not require a germinal centre, and yields short-lived antibody-secreting plasma cells but not memory cells or memory plasma cells. Responses against TI-2 antigens, because they do not require complex interactions of T- and B-cells, arise faster than those against TD antigens and play an important role in protecting against pathogens (Vos *et al.*, 2000).

Trypanosome-common antigens such as congopain and Hsp70/BiP are most likely TD antigens, and soluble VSG has formally been shown to be a TD antigen in cattle, stimulating T-cells that require antigen-presenting cells (McKeever *et al.*, 1994). VSG epitopes exposed on the surface of intact trypanosomes could jointly be considered as TI-2 antigens. Thus, although each independent VSG molecule lacks repeating antigenic epitopes, the assembly of VSGs on the parasite surface is an array of repeating, antigenically identical epitopes. Therefore, the development of IgG antibody responses against congopain, Hsp70/BiP and buried VSG epitopes in *T. congolense*-infected N'Damas but not Borans, discussed above, suggests that the capacity to mount immune responses against TD antigens is, or becomes, compromised in Borans during infection but not, or to a lesser extent, in N'Damas. In support of this, Flynn and Sileghem (1991) at ILRAD showed that *T. congolense*-infected Borans were unable to generate T-cell responses to soluble VSG of an infecting trypanosome (cloned from the first parasitaemic wave) and developed only short-lived responses to other trypanosome antigens, whereas *T. congolense*-infected N'Damas generated long-lived T-cell responses to both VSG of an infecting trypanosome and common trypanosome antigens, although these decreased somewhat with time after infection, and despite repeated curative treatments and reinfections, retained memory T-cells against these antigens for years after infection.

Infections with AAT parasites in mice are known to induce unresponsiveness of lymph-node T-cells to mitogens *in vitro*, which results from inhibition of secretion of the T-cell growth factor IL-2 and its receptor on T-cells, is mediated by nitric oxide and prostaglandin production by macrophages, which is stimulated by trypanosome components, IFN-γ and TNF-α (Sileghem *et al.*, 1991; Darji *et al.*, 1992; Schleifer and Mansfield, 1993; Sternberg and Mabbott, 1996; Gomez-Rodriguez *et al.*, 2009). Work at ILRAD showed that suppressive monocytes/macrophages that inhibited T-cell proliferation in response to a mitogen (concanavalin A) *in vitro* arose in the peripheral blood, lymph nodes and spleen of *T. congolense*-infected cattle (Flynn and Sileghem, 1991) and simultaneously suppressed production of IL-2 and IL-2 receptor expression (Sileghem and Flynn, 1992b) by the T-cells but did not suppress their production of IFN-γ (Sileghem and Flynn, 1992a). Unlike the mouse AAT-induced suppressor cells, those in cattle were not inhibited by indomethacin, which prevents production of prostaglandins (Flynn and Sileghem, 1991), and did not produce nitric oxide in response to IFN-γ (Taylor *et al.*, 1996a), possibly because of a potent IL-10 response (Taylor *et al.*, 1998; O'Gorman *et al.*, 2006). The mechanism through which macrophages from the infected cattle inhibit T-cell proliferative responses *in vitro* was not resolved nor was a relationship explored between this *in vitro* T-cell suppressive response and the impaired responses of infected Borans and to a lesser extent N'Damas to TD antigen *in vivo*.

A loss in confidence that immunological differences between infected N'Damas and Borans could be resolved in any way that would improve productivity of either breed under natural tsetse/trypanosome challenge was perhaps a factor in the decision by ILRI to withdraw from this area of investigation and to diversify into studies of other pathogens. Nevertheless, it is noteworthy that this programme showed that two major indicators of trypanotolerance – control of anaemia and parasitaemia – are unlinked, and

introduced an additional indicator of trypanotolerance, namely the ability to generate IgG1 antibodies against buried VSG epitopes and epitopes on many common trypanosome antigens, including congopain and Hsp70/BiP. One possibly practical finding from the programme was that infected N'Damas retain responsiveness to TD antigens throughout infection, whereas Borans do not. This suggests that N'Damas, more so than Borans, would benefit from priming with putative anti-AAT vaccine antigens such as congopain (Lalmanach *et al.*, 2002) and conserved peptides of VSGs (Black and Mansfield, 2016). A second possibly practical outcome is that the magnitude and diversity of IgG1 responses against trypanosome-common antigens and VSG buried epitopes should be used together with data on anaemia and body weight gain in infection-based screening for trypanotolerance.

The genetic basis of trypanotolerance

Selection for trypanotolerance has occurred in certain breeds of *B. taurus* (humpless) and *B. indicus* (humped) cattle (Dolan, 1987), such as the N'Dama, a small humpless long-horn from West Africa, and the Orma Boran, the smallest breed of the humped short-horned Zebu cattle from East Africa. Obviously, trypanotolerance is a desirable trait in cattle to be ranched in tsetse-infested areas, which includes most of sub-Saharan Africa. Despite data from ATLN showing that N'Dama cattle can be productive under low tsetse/trypanosome challenge without any treatment and can be productive under medium tsetse/trypanosome challenge when managed by diagnosis-based application of prophylactic and curative drugs, there is reluctance among producers in East Africa to adopt this breed because it is small, feisty and has a low milk yield. Producers would prefer the trypanotolerant phenotype to be introduced by introgressive hybridization into larger breeds of cattle, including improved European breeds, which would be straightforward if disease resistance was due to the presence of one or a few genes only, but considerably less so if a large number of loci are controlling the trait. Selection of breeds with high expression of trypanotolerance within a herd could be based on their high expression of indicators of trypanotolerance following natural or experimental infection (Dolan, 1987) or, in the absence of infection, using markers with high fidelity to the trypanotolerance trait, such as polymorphic loci that affect or are responsible for trypanotolerance, if such were available.

An ILRAD/ILRI Trypanotolerance Gene Mapping Programme was established in the hope of identifying markers of trypanotolerance for marker-assisted selection and also of providing insights into biological processes that control parasitaemia, anaemia and the efficacy of immune responses. Figure 2.2 (Ullmann *et al.*, 2005) outlines the approach taken and explains the mapping process, assuming that a single gene controls the trait; the legend additionally considers traits governed by expression of many genes in combination or multiple unlinked genes.

QTLs and murine trypanotolerance

ILRAD started a Bovine Trypanotolerance Mapping Programme in 1990. Its leaders, A.J. Teale and S.J. Kemp, found that while the principle behind the mapping strategy is simple, the practice is extremely demanding (Kemp and Teale, 1998). Mapping required: (i) multiple ovulation families of full-sibling F_2 N'Dama × Boran; (ii) challenge of about 200 F_2 animals with *T. congolense* under carefully defined conditions of challenge and monitoring of responses; (iii) development of a genomic map of high marker density (with input from the global scientific community); and (iv) genotyping of the three generations of cattle (Kemp and Teale, 1998)

Clearly, this was a major undertaking, particularly given the absence, at the time of initiation, of a high-marker-density bovine genome map, the age of cows at first calving (about 3 years) and calving intervals (time between birth of a calf and the birth of a second calf from the same mother – about 12–14 months). Given these constraints, it would take several years to generate the F_2s. Mapping studies were also conducted in crosses of inbred mouse strains that had been shown at ILRAD to differ in susceptibility to infections with *T. congolense* following needle challenge (Morrison *et al.*, 1978). Survival of inbred mice after infection with *T. congolense* might not be analogous to trypanotolerance in N'Dama cattle but nevertheless provided a robust phenotype for mapping. Thus, infected C57BL/6 mice

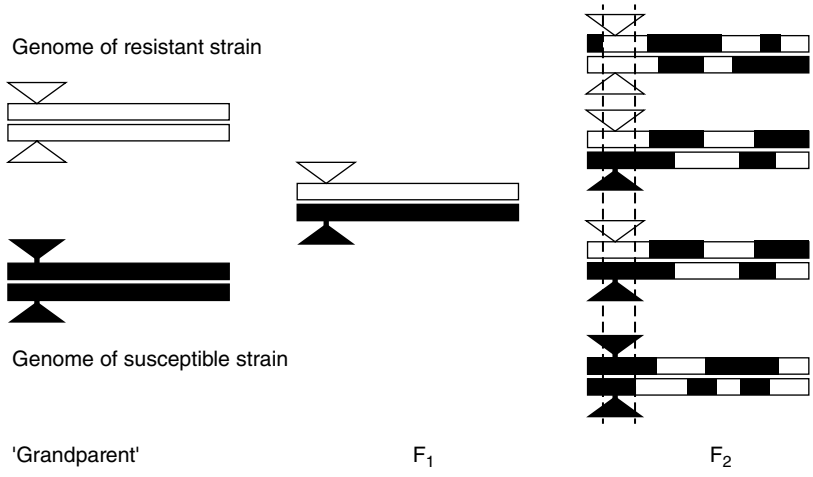


Fig. 2.2. Linkage mapping to identify genes responsible for trypanotolerance in N'Dama cattle. A single gene is used as an example. N'Damas are homozygous for a resistant allele (open triangle) and Borans for a susceptible allele (filled triangle). Their F_1 offspring obtain the resistant allele from the N'Dama parent and a susceptible allele from the Boran parent. When the F_1s are crossed, the F_2 generation contains animals with all possible combinations of alleles of the trypanotolerance gene. When infected with trypanosomes, individuals are expected to differ in expression of indicators of trypanotolerance. A correlation of indicators of trypanotolerance with areas of the grandparents' genomes in each animal allows mapping of areas of the grandparents' genome responsible for the trypanotolerance trait. The principle is the same for creating a linkage map for a trait that is controlled by many genes if these are arranged as clusters. Traits that are controlled by multiple unlinked genes that are dispersed throughout the genome are not good candidates for linkage mapping. (Constructed by author.)

survived for 110.2 days after infection and similarly infected BALB/c mice survived for 49.5 days, while few infected A/J mice survived longer than 20 days after infection. Inbred strains of mice have several advantages over cattle with respect to mapping studies. In each inbred strain, all genes are homozygous, a genome map had been developed using microsatellite markers (Dietrich *et al.*, 1996), the age of mice at first pupping is about 11 weeks, the mice have several offspring from each mating and there is a short interval between births. It is therefore unsurprising that mapping studies progressed somewhat faster in crosses between inbred strains of mice than in N'Dama × Boran crosses.

Loci (sites of genes) controlling the duration of survival of mice after needle challenge with *T. congolense* were mapped using C57BL/6, BALB/c and A/J grandparents and F_2 generations of the C57BL/6 × A/J F_1 and the C57BL/6 × BALB/c F_1 crosses. Survival and marker data were subjected to parametric linkage analysis, whereby the probability that a gene important for a disease is linked to a genetic marker is determined by a statistical method, which yields a LOD score (logarithm of the odds to the base 10). This assesses the probability that a pedigree where the disease and the marker are co-segregating is due to the existence of linkage or to chance. For example, an LOD score of 3 means the odds are 1000:1 in favour of genetic linkage. Regions on mouse chromosomes 5 (maximum LOD score 4.6) and 17 (maximum LOD score 11), within large genomic intervals of 20–40 cM (1 cM contains on average 50 genes), were found to be important in determining resistance in both crosses, while a region on chromosome 1 (maximum LOD score 5.7) showed evidence of involvement in only the cross of C57BL/6 × BALB/c (Kemp *et al.*, 1997). The impact of these loci on survival times after infection was of large effect, accounting for most of the genetic variation in both F_2 populations. The three loci on chromosomes 17, 5 and 1 were designated, respectively, *Tir1*, *Tir2* and *Tir3* (for trypanosome infection response). *Tir1* represents the major trypanotolerance QTL in

mouse with an additive effect of 31 days on survival time. Following these initial QTL mapping results, an advanced intercross line approach was taken, which involves random and sequential intercrossing of F_2s, infection to assess phenotype and linkage mapping. Using F_6 crosses, *Tir1* was mapped to a 95% confidence interval of 1.3 cM, *Tir2* was mapped to a 12 cM region and *Tir3* resolved into three QTLs (*Tir3a*, 10 cM; *Tir3b*, 1.8 cM, and *Tir3c*, 8 cM) (Iraqi *et al.*, 2000), necessitating higher resolution mapping for positional cloning of genes underlying the QTL. An attempt to do this using F_{12} generations fixed for the susceptibility or resistance alleles at *Tir1* mapped *Tir2* to less than 1 cM but was less successful in narrowing the positions of *Tir3a*, *Tir3b* and *Tir3c* (Nganga *et al.*, 2010). An issue arising from F_{12} generation mapping was that the map positions of *Tir2* and *Tir3a*, *Tir3b* and *Tir3c* were different from the F_6 mapping study. Further mapping of congenic mice carrying the C57BL/6 *Tir1*, *Tir2* and *Tir3* resistance alleles on the A/J background partially resolved this disparity, supporting the F_6 location of *Tir2* and the F_{12} location of *Tir3a*. These studies, conducted by an international research team including ILRI scientists, showed that survival after infection was increased in *Tir1* and *Tir2* but not *Tir3* congenics (mice that are genetically identical except for the loci of interest) and that survival was negatively correlated with parasitaemia (i.e. mice with lower parasitaemia survived longer) but positively correlated with alanine aminotransferase levels in serum, suggesting that inflammatory responses in the liver were beneficial (Rathkolb *et al.*, 2009). Using a wide variety of techniques to identify candidate genes for murine tolerance to infections with *T. congolense*, it was postulated that *Pram1* (an adaptor protein used in T-cell receptor signalling) was the most plausible candidate QTL gene in *Tir1* and that Cd244 (NK cell receptor 2B4) was a strong candidate QTL gene at the *Tir3c* locus (Goodhead *et al.*, 2010). Thus, contrary to concerns that trypanotolerance in mice might be regulated by multiple unlinked genes, this proved not to be the case, at least with respect to events that control early mortality in *T. congolense*-infected mice. There have been no further publications in this area, so a contribution of a *Pram1* polymorphism to infection-induced early mortality remains to be confirmed.

QTLs and bovine trypanotolerance

Twenty-three groups of N'Dama × Boran F_2 animals, each containing between three and 13 calves, together with parental groups of N'Damas and Borans, were challenged with *T. congolense*-infected tsetse flies. Infections were followed for 150 days with respect to 16 phenotypic traits (Hanotte *et al.*, 2003). Twenty-eight of the F_2 offspring needed curative treatment (minimum day 14, maximum day 146), and the last value taken for these animals for all traits studied was taken as the value of the traits for the remainder of the challenge period. The traits measured informed infection-induced decreases in PCV, recovery of PCV, infection-induced loss in body weight, recovery of body weight, levels of parasitaemia between days 11 and 150 post-infection and the number of times an individual is parasitaemic by the DGBC technique, and thus jointly informed QTLs for parasitaemia, body weight and anaemia.

The animals were genotyped at 477 molecular marker loci covering all 29 cattle autosomes (i.e. any chromosome that is not a sex chromosome), covering 82% of the bovine genome. Putative QTLs were mapped to 18 autosomes. The results were consistent with a single QTL on each of 17 chromosomes, and two on BTA16. Individual QTL effects ranged from about 6% to 20% of the phenotypic variance of the trait. Alleles for resistance to trypanosomiasis originated from the N'Dama parent at nine QTLs and from the Kenyan Boran at five QTLs, and at four QTLs there was evidence of an overdominant mode of inheritance, when the heterozygote lies outside the phenotypic ranges of the parents (Hanotte *et al.*, 2003).

Noyes *et al.* (2011) tried to obtain short lists of candidate genes by focusing on polymorphisms within the bovine trypanotolerance QTL by transcriptome analysis of gene expression in the liver, spleen and precrural lymph node of N'Damas and Borans after infection with *T. congolense*. In this work, they assessed QTL regions and candidate loci for evidence of selective sweeps (the reduction or elimination of variation among the nucleotides in neighbouring DNA of a mutation as the result of the recent fixation of a beneficial allele due to strong positive natural selection). The gene expression data showed that Toll-like receptors and mitogen-activated protein kinase pathways responded to infection, and the former

contained *TICAM1* (TIR domain-containing adapter molecule 1), which is within a trypanotolerance QTL on BTA7. Genetic analysis showed that selective sweeps had occurred at the *TICAM1* and *ARHGAP15* (Rho GTPase-activating protein 15) loci in the N'Damas, making these strong candidates for genes underlying the QTL. *TICAM1* (also known as TRIF; TIR domain-containing adapter protein inducing IFN-β) is an adapter for a few Toll-like receptor signalling cascades, while *ARHGAP15* regulates signal transduction in the immune system. These proteins are involved in inflammatory and other immune responses. Field studies using 192 cattle produced from (N'Dama × Kenya Boran) × Kenya Boran subjected to natural challenge, and using a scoring system expanded to take into account avoidance of infection and genotyping using 35 microsatellite markers spanning five bovine chromosomes that were found in the above studies to contain trypanotolerance QTLs, showed that trypanotolerance was expressed in proportion to N'Dama origin marker alleles (Orenge *et al.*, 2011) and additionally showed the importance of sex and local environment conditions in determining the response to challenge (Orenge *et al.*, 2012). Given that trypanotolerance QTLs are present on 18 chromosomes, with an allele for resistance present at nine N'Dama QTLs and five Boran QTLs, the relevant genes, or markers tightly linked to these, would need to be resolved before the QTLs can be used in marker-assisted selection. Thus, traits that distinguish between N'Dama and Boran cattle trypanotolerance in cattle proved to be regulated by multiple unlinked genes. There have been no further publications on identifying candidate genes for trypanotolerance in bovids.

Mapping of trypanotolerance genes has been an enormous undertaking and, as with so many aspects of trypanosomiasis research, it has not been rewarded with leaps in understanding. Nevertheless, as a result of these studies we are now fully aware of the genetic complexity of the trait in cattle and its distinct and lesser complexity in mice, at least with respect to survival after infection. Indeed, given that a candidate gene for *Tir1* has been proposed, it would certainly be worthwhile to determine how expression of this gene, *Pram1*, affects infection-induced mortality and changes in immune parameters that accompany this (Morrison *et al.*, 1978). With respect to determining the genetic basis for trypanotolerance in cattle, archives of genomic and cDNA have been established from the studies reported above and are available for deeper analysis as technology in this field undergoes further development to facilitate linking genes to disease-resistance traits.

Conclusions

From 1975 to 2015, scientists at ILRAD/ILCA/ILRI tried, with sustained focus and ingenuity, to identify trypanosome vaccine antigens and key aspects of trypanosomiasis pathogenesis that could be targeted immunologically or genetically to increase the productivity of cattle under tsetse/trypanosome challenge. Their research efforts, although logical, incremental and painstaking, did not achieve this goal.

Infection and treatment, a process commonly used to induce protective immunity to other pathogens, was shown to have limited success with African trypanosomes because of their huge antigenic diversity. Subsequent investigations of host-derived macromolecular nutrients that drive/sustain trypanosome cell-cycle progression were successful but did not have practical value because targeting their conserved receptors with lytic or blocking antibodies was ineffective. Although work at ILRAD identified low-, intermediate- and high-density serum lipoproteins and transferrin as required trypanosome growth factors, antisera against receptors for these molecules had little or no impact on trypanosome infectivity or growth in mammalian hosts. Despite the lack of translational impact of these investigations, the feeder layer-dependent and axenic culture systems, macronutrient bioassays and trypanosome cell-cycle analysis methodology developed at ILRAD for the above studies were forerunners of the high-throughput drug screens, genetic manipulation, and cell-cycle and differentiation pathway analyses that comprise much of the current basic research on the African trypanosomes.

Investigations at ILRAD/ILCA/ILRI to elucidate mechanisms of trypanotolerance using Cape buffalo and N'Dama and Boran cattle models were also scientifically rewarding but of little translational value. These studies showed that superior control of trypanosomiasis in Cape buffalo and, to a

lesser extent, in N'Dama cattle, was associated with the ability to suppress trypanosome parasitaemia and to sustain PCV, body weight and immune competence throughout infection, the latter evidenced by production of IgG antibodies specific for VSGs of newly arising trypanosome variable antigen types and other trypanosome antigens. However, the physiological mechanisms underlying this productive response, and its absence in trypanosomiasis-susceptible hosts, were not identified. Furthermore, analysis of the genetic basis of trypanotolerance in cattle showed this to involve 18 QTLs, all of which make relatively minor (10% or less) contributions to the trait and therefore are of little value for selective breeding to improve trypanotolerance of Borans and other livestock. While disappointing with respect to AAT control, studies of AAT pathogenesis at ILRAD/ILRI did identify the definitive question for immunological research on AAT, namely, how do trypanosomes eliminate TD antibody responses in trypanosomiasis-susceptible mammals? In addition, the work at ILRI on the genetic basis of trypanotolerance contributed a high-density single-nucleotide polymorphism (SNP) map of the bovine genome that has intrinsic value for analysis of QTLs that control other traits, including susceptibility to other diseases.

The Future

ILRI and ILRAD alumni and their global partners continue to seek solutions to AAT. Jayne Raper and colleagues are exploring genetic engineering of cattle to express a trypanosomelytic factor derived from primates that are resistant to trypanosome infection (MacMillan, 2016). S.J. Black and colleagues continue to seek a key initiator pathway of trypanosomiasis pathogenesis and have recently shown (Frenkel *et al.*, 2016) that *T. brucei*, as well as *T. congolense* (S.J. Black and D. Frenkel, unpublished data, 2019), infection co-opts host NK cells to delete splenic B2 cells and thus prevent sustained TD antibody responses against VSG and other trypanosome antigens. Strikingly, when NK cell killing is prevented, trypanosome-infected mice suppress parasitaemia, do not develop anaemia or wasting, and do not succumb to infection-induced early mortality. Thus, elucidation of the trypanosome component that activates NK cells might provide a novel target for AAT immunoprophylaxis or a probe to identify cattle that do not respond to it and hence may be trypanotolerant. Finally, other events might supersede the need to selectively control AAT. S.J. Kemp and colleagues at ILRI have initiated a multinational project in Africa to identify those rare cattle that remain healthy and productive under ambient disease and environmental stress. This will entail animal productivity reporting by legions of smallholder farmers in disease-endemic regions, SNP analyses of blood leukocytes from cattle with a high-productivity phenotype, selective breeding and validation.

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