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The mechanisms by which cattle acquire immunity to trypanosomiasis

To find out why the disease African trypanosomiasis develops in some animals infected with trypanosomes but not in others, scientists at ILRAD are comparing the immune responses of two breeds of experimentally-infected cattle: one that is resistant to trypanosomiasis and one that is susceptible. The aim of these studies is to determine whether the immune response of resistant animals helps to control the disease, and if so, the mechanisms that effect this. Once this is understood, it may be possible to develop methods that mimic the natural disease control mechanisms and use the methods to improve immune responses of susceptible animals, which greatly outnumber trypanotolerant animals on the continent. A better understanding of the control mechanisms will also facilitate the search for genetic markers that one day may be used to identify highly resistant cattle for use in livestock breeding programs.

The trypanosome is a protozoan parasite transmitted by the bite of a tsetse fly to people and to wild and domestic animals, in which it causes trypanosomiasis (the disease is called sleeping sickness in people and *nagana* in cattle). Infection in domestic livestock impairs the animal's immune system and causes anaemia, weight loss and reproductive disorders; infected animals may die if not treated. While pathological effects such as these have been well documented for much of this century, we know surprisingly little about what causes the pathology.

A few breeds of livestock that are indigenous to Africa, such as the N'Dama cattle (*Bos taurus)* of West Africa, are able to tolerate trypanosomes well and in many cases appear to suffer no ill effects from infection. This ability, which is shared by many of Africa's wild ruminants, is known as 'trypanotolerance'. The more common and more recently arrived livestock breeds in Africa, in which trypanosomiasis readily develops, such as the Zebu (*Bos indicus)* and imported European cattle breeds, are referred to as 'trypanosusceptible'.

Understanding the basis of resistance to trypanosomiasis is fundamental to research aimed at curing or preventing the disease. Because of its ability to control the harmful effects of trypanosome infections, the N'Dama breed was chosen by ILRAD scientists as a model for studies comparing the

immune responses of trypanotolerant and trypanosusceptible animals to infection. In 1983, they successfully implanted ten N'Dama embryos obtained from The Gambia into foster mothers of an East African Zebu breed called the Boran. These embryo transfers gave rise to 5 male and 5 female calves, which became the basis of a breeding herd. ILRAD now has a total of 45 N'Dama (Figure 1), which are being used for genetic and immunological research into trypanotolerance.

FIGURE 1. *N'Dama cattle produced at ILRAD using embryo transfer techniques for the purpose of studying the immune responses of cattle that are resistant to trypanosomiasis.*

In a series of experiments begun in 1985, N'Dama and Boran cattle were experimentally infected with *Trypanosoma congolense.* The N'Dama demonstrated a strong ability to control 'parasitaemia', the numbers of parasites in the blood. In the beginning of the infection, the N'Dama and Boran showed similar levels of parasitaemia. But as the infection progressed, high numbers of parasites persisted in the Boran until about 6 weeks into the infection, when the cattle became severely anaemic and had to be treated with a trypanocidal drug to prevent possible death. In contrast, the parasitaemia in the N'Dama decreased with time. After about 20 weeks, the N'Dama were parasitaemic only periodically. These results clearly showed that the trypanotolerant N'Dama can control parasitaemia, but the mechanism(s) responsible for this control remained obscure.

Antigenic variation

ILRAD is committed to developing immunological methods to control its two target livestock diseases, trypanosomiasis and theileriosis (East Coast fever). Several non-protozoan livestock diseases, such as rinderpest, are already controlled through vaccination, which commonly is not only the cheapest control method available but also the most environmentally sound.

Vaccines exploit the ability of animals to control disease-causing organisms by mounting an immune response. When an animal is infected with a pathogen, the host's immune system recognizes molecules exposed on the parasite and targets an immune response to those molecules, which are called antigens (*antibody generators).* This leads to the destruction and removal of the pathogen by 'effector' components of the immune system.

Most vaccines contain one or more of these accessible molecules purified from the parasite. These stimulate an immune response without having to expose the host to infection with the whole organism. The immune system remembers these antigens and when the host is exposed to the pathogen, the cells of the immune system are primed and ready to control the infection. This response is faster and qualitatively superior to the response elicited following primary exposure to the antigen. In this way, vaccinated animals are able to eliminate parasites quickly and efficiently and no disease develops.

Results of cell and molecular biology studies of trypanosomes have established that these parasites have evolved a remarkable mechanism that enables them to evade attack by the immune system. This defense mechanism is known as 'antigenic variation'.

In the course of a trypanosome infection, the number of parasites in the blood of the mammalian host fluctuates from low to high in a series of waves. Each wave consists of a population of parasites most of which display one kind of glycoprotein on their surface. After a few days, in which the immune system of the host first recognizes the dominant glycoprotein and then builds up a response against it, most of the parasites in such a population are killed.

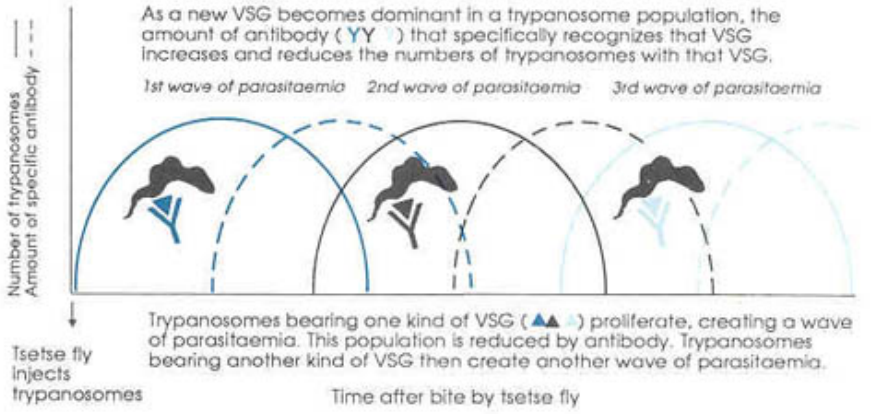

FIGURE 2. *Diagram illustrating the process of antigenic variation, which enables trypanosomes to survive attack by the host's immune system,*

However, a few of the parasites in the first wave are not destroyed because their surface coat is made up of a different glycoprotein. It takes time for the host to mount an immune response to this new glycoprotein. Meanwhile, the parasites expressing this glycoprotein continue to multiply and in a few days produce another full-scale parasitaemia. This process, as illustrated in Figure 2, continues in successive waves of parasitaemia, with the parasites staying one step ahead of the immune response.

Trypanosomes are able to express many kinds of glycoproteins, designated 'variable surface glycoproteins' (VSGs). The VSGs are a major cell product of the trypanosome, accounting for 10% of all the proteins synthesized by the parasite. (It is estimated that between 300 and 1,000 trypanosome genes code for VSGs.) Each of the many different strains of trypanosomes displays a particular set of VSGs. Further complicating the matter, genetic recombination among trypanosome populations may occur in the field, resulting in an enormous potential for antigenic diversity. Clearly, a conventional type of vaccine, which primes an animal's immune system against only one or a few antigens, will not be broadly effective against trypanosomiasis. With this in mind, ILRAD scientists are researching other vaccine-based methods of controlling the disease. In the following approach, they are attempting to determine the mechanisms that enable trypanotolerant livestock to resist trypanosomiasis, and thus to survive in tsetse-infested areas where more susceptible cattle quickly succumb and die.

Antibody responses to VSG antigens

Experiments conducted at ILRAD in the late 1970s and early 1980s showed that antibodies of trypanosome-infected Boran cattle generated against a VSG eliminated parasites bearing that particular VSG. This antibody response was observed to be the primary mechanism by which the host's immune system controlled parasite numbers. During prolonged infections, a particular VSG occasionally appeared more than once. When this happened, the immune system was able to rapidly clear trypanosomes from the blood by producing antibodies against the VSG. This indicates that the immune system remembered the VSG from its first appearance and was thus able, on its reappearance, to quickly act against it.

Other evidence that antibody plays a role in controlling parasitaemia was obtained by infecting cattle with a population of trypanosomes known to cause only mild disease. The animals controlled the infection and were resistant to reinfection with the same parasites. This host resistance was associated with the presence of anti-VSG antibodies.

In other experiments, trypanosomes were heavily irradiated so they could no longer multiply. The irradiated parasites were inoculated into cattle, which developed antibodies to the dominant VSG expressed in that population and were thereafter resistant to infection with non-irradiated trypanosomes expressing the same VSG. However, although the antibodies prevented the population of trypanosomes with that VSG from growing, the cattle became infected with parasites

bearing a different VSG, which multiplied normally.

These results, which indicated that antibody played a major role in controlling trypanosome parasitaemia, led ILRAD scientists to question if the trypanotolerant N'Dama cattle produced a more efficient antibody response than trypanosusceptible cattle to the many different VSGs arising throughout a trypanosome infection. A better antibody response would account for the demonstrable ability of N'Dama to control trypanosome infections better than Boran.

To answer this question, a study was set up to analyse antibody responses to trypanosome VSGs. In this experiment, 4 N'Dama and 4 Boran animals were infected with *T. congolense.* Serum was collected from each animal at weekly intervals for antibody analysis. In addition, parasites were collected from the cattle every 4 days and stored, frozen but viable, in liquid nitrogen. Soluble non-VSG trypanosome proteins, purified VSG and whole trypanosomes were used as targets in the antibody assays. The amounts of antibody circulating in the blood were also measured. The results of this experiment showed that levels of serum antibody increased in both the N'Dama and the Boran following infection. No difference in the magnitude of the increase was detected between the two breeds.

Two types of tests were used to detect antibody generated against the surface of the living trypanosome. In one, known as the complement lysis assay, trypanosomes that had been taken from the infected cattle at selected times and stored were thawed, allowed to multiply and then mixed with sera from the infected cattle. Serum proteins, collectively called complement, were then added. Complement is activated in the host when antibody binds to the trypanosomes; complement then acts in several ways to complement and amplify the action of antibody, eventually causing the lysis (rupture) of the parasites. Quantifying the number of trypanosomes killed by complement gives a measure of the amount of anti-trypanosome antibodies generated. By comparing the ability of sera taken at different times following infection to lyse trypanosomes in the complement lysis assay, researchers determined that higher levels of specific antibodies had been produced in the N'Dama than in the Boran (Figure *3 left).*

A second test was conducted to determine to which class the anti-trypanosome antibodies belonged. Five classes of antibodies, collectively called immunoglobulins [abbreviated as Ig], are found in higher vertebrates. Each class is determined by the kind of heavy polypeptide chains that make up the molecule. IgM antibodies, for example, have μ. (mu) chains and IgG antibodies have (gamma) chains. Each of the five classes of immunoglobulins mediates a different biological response following the binding of antibody to antigen. Using an enzyme-linked immunosorbent assay (ELISA) in the second test, ILRAD scientists found that the levels of antibody in the N'Dama were again higher than those in the Boran. In both breeds the antibodies generated were of the IgM class (Figure 3 *right).*

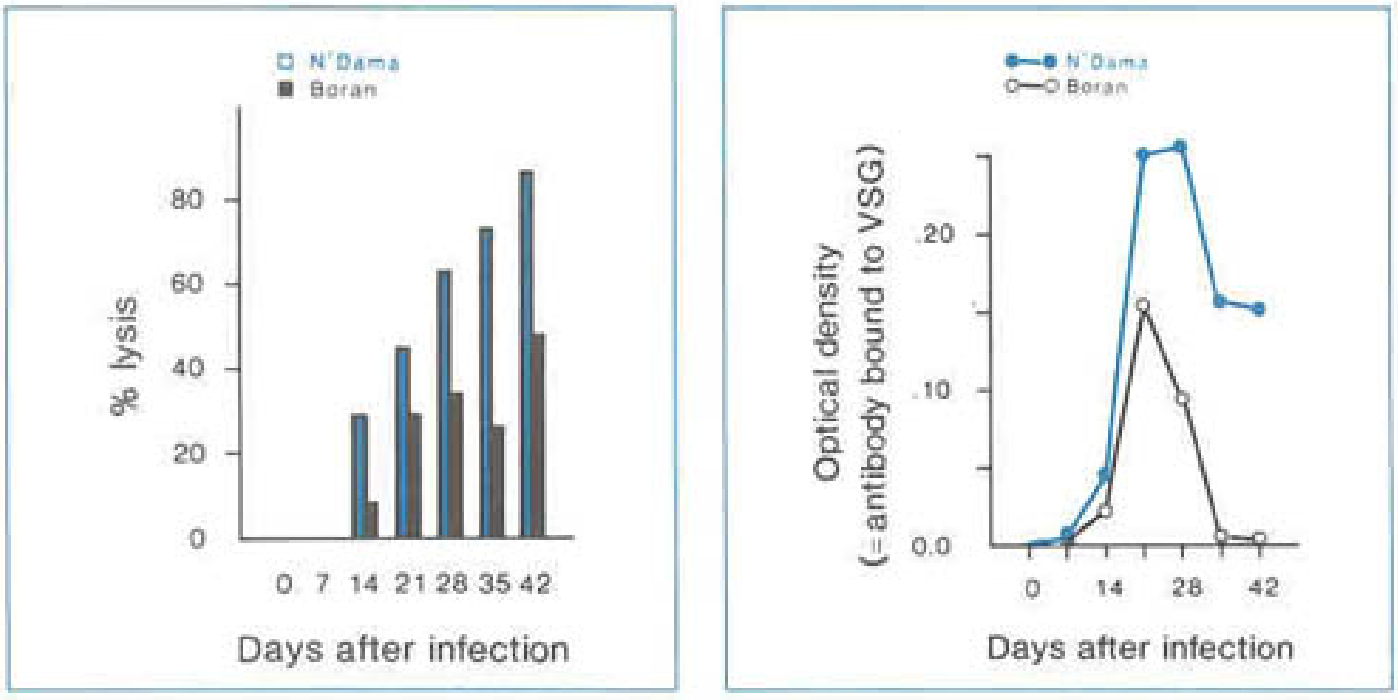

FIGURE 3. *(*left*) Results of a complement lysis assay demonstrated that when infected with trypanosomes, the trypanotolerant N'Dama cattle produce higher levels of specific antibodies to*

*trypanosome VSG antigens than the trypanosusceptible Boran. (*right*) Results of an ELISA demonstrated that both cattle breeds generate the IgM class of antibodies to trypanosome VSGs, but that the N' Dama produce higher levels of this antibody than the Boran.*

Both the complement lysis assay and the ELISA detected antibodies that were bound to those parts of the VSG molecule that in living trypanosomes are exposed on the parasite surface. Another assay was carried out in which VSG was purified from the trypanosome surface and used to detect antibody. In this test, antibodies of both IgM and IgG classes were detected in sera from both breeds of infected cattle, but the N'Dama produced predominantly IgG antibodies while the Boran produced mainly IgM antibodies.

The findings contrasted in an interesting way with the results of the complement lysis assay and ELISA experiments. The work on purified VSG suggested that only part of the VSG molecule is exposed on the surface of living trypanosomes. Antibodies apparently are first generated to those exposed parts of the molecule. This antibody binding to the epitopes (the regions on an antigen that bind with antibody) subsequently brings about the destruction of the parasites. After a trypanosome is killed, however, and its VSG is released into the circulation, different antibodies are generated to newly exposed parts of the VSG molecule that were hidden on the living trypanosome. In this experiment, the Boran produced IgM antibodies against the previously hidden epitopes while the N'Dama produced IgG antibodies. It is known that a change with time from an IgM-dominated immune response to an IgG-dominated response indicates development from an early primary immune response to a mature response. Scientists therefore suspect that the immune response in the susceptible Boran is in some way prevented from maturing as rapidly as the response in the N'Dama.

Antibody responses to invariant antigens

In addition to comparing antibody responses in N'Dama and Boran cattle to the *variable* surface antigens of trypanosomes, ILRAD scientists compared and characterized antibody responses in the two breeds to trypanosome *invariant* antigens. These consist of all the molecules of the parasite other than the surface glycoproteins. Results of this work showed that N'Dama cattle produced antibodies to more invariant antigens than the Boran.

Using the Western blotting technique to analyse responses to individual trypanosome antigens, scientists identified two proteins that were recognized in different ways by the immune systems of the two breeds. The first protein has a relative molecular mass of 69 kiloDaltons (kDa). Antibodies to this molecule were detected in sera from both N'Dama and Boran cattle, and the molecule appeared to be the main antigen of *T. congolense* that induces an immune response (such an antigen is said to be 'immunodominant'). Most of the specific IgM produced in both breeds was directed against this protein, but the IgM levels in the Boran were significantly higher than those in the N'Dama. However, against the 69kDa protein, the N'Dama also produced antibodies of the IgG_1 class (a subclass of IgG), whereas the Boran produced only very low levels of this type of antibody (Figure 4a).

Further studies showed that the 69kDa protein is present in *T. brucei* and *T. vivax* as well as in *T. congolense* and that it is present in all stages of the parasite's life cycle. It has since been shown that the 69-kDa molecule is similar in several ways to a group of molecules called heat shock proteins. The precise function of heat shock proteins is still unknown, but they are thought to act as molecular chaperones, facilitating the correct folding of newly synthesized proteins. As such, they may have a vital role to play in the synthesis of VSG molecules. If this is the case, immune responses directed against the 69-kDa protein may interfere with the ability of the trypanosome to maintain an intact surface coat. In addition, the identification of this protein as an immunodominant antigen following trypanosome infection has two major practical applications: the protein may prove useful for diagnosing trypanosome infections and it will serve as a tool for further analysis of the differential immune responses in the two breeds to determine why Boran cattle fail to switch from the production of IgM antibodies to that of IgG_1.

Antibody recognition of the second trypanosome protein identified at ILRAD appeared to correlate with resistance to trypanosomiasis. This protein has a molecular mass of 33 kDa. All the N'Dama produced IgG_1 antibodies against it. In addition, antibodies in sera obtained from three trypanosome-infected Cape buffalo—which, like the N'Dama, are resistant to trypanosomiasis—also recognized the 33-kDa antigen. In contrast, the Boran animals produced no detectable antibodies to this protein during the primary infection (Figure 4b). Some Boran that were repeatedly infected and treated eventually produced detectable levels of antibodies against the 33-kDa antigen. When these responder Boran were rechallenged with a repeat infection, they developed a less severe anaemia than that following the primary infection and they did not require drug treatment, although they

remained parasitaemic. On the other hand, when Boran cattle that had no detectable anti-33-kDa antibodies in their sera were rechallenged, they again became highly parasitaemic and anaemic and required drug treatment to terminate the infection. The presence of detectable levels of antibodies against the 33-kDa protein thus appears to be associated with resistance to trypanosomiasis.

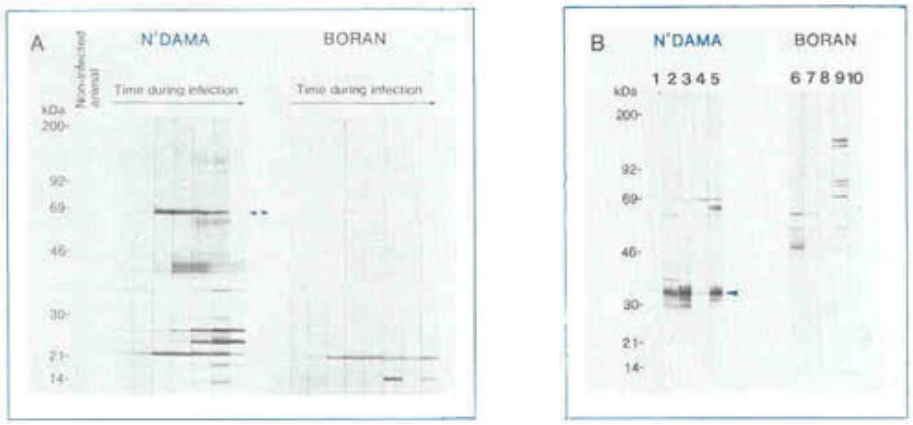

FIGURE 4. *(a) A Western blot showing the pattern of antibody recognition of T. congolense antigens during infection in an N'Dama and a Boran. The trypanotolerant N'Dama recognizes more invariant antigens than the Boran and also produces more antibodies of the IgG_1 class to a 69-kDa antigen (arrow). (b) A Western blot showing that N'Dama cattle (animals numbered 1–5) produce antibodies against a 33-kDa invariant antigen of T. congolense (arrow), whereas Boran cattle (6–10) don't produce antibodies against this molecule during a primary infection. The presence of detectable levels of antibodies against the 33-kDa protein appears to be associated with resistance to trypanosomiasis.*

Characterization of the 33-kDa protein has shown that it is a cysteine protease (an enzyme that helps break down protein) normally found in the endosomal and lysosomal compartments of *T. congolense.* Proteases have been implicated in the pathogenesis of several parasitic diseases and may be involved in the breakdown of host molecules such as immunoglobulins and complement, or they may activate host systems to release large quantities of substances such as tumor necrosis factor, gamma interferon and interleukins, which can produce the symptoms of trypanosomiasis. Work is in progress to determine whether antibodies to the cysteine protease ameliorate its possible pathogenic effects.

Conclusions

Studies carried out at ILRAD on antibody responses in trypanotolerant and trypanosusceptible cattle following *T. congolense* infection show that the two breeds differ in the quantity, quality and specificity of antibodies they generate. The trypanotolerant N'Dama cattle produce higher levels of anti-VSG specific antibodies than the Boran; they produce more IgG_1 antibodies to invariant trypanosome antigens, and they produce a greater response to two trypanosome-derived molecules, one of which may be useful in diagnosis while the other may have important implications in the control of the disease.

Much more work is required to determine the relevance of these superior antibody responses to trypanotolerance. However, this research, together with studies being carried out on other aspects of the bovine immune response to trypanosome infection, will determine whether the immune system plays a primary role in controlling trypanosomiasis in trypanotolerant livestock.

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Theileria Annulata Workshop at ILRAD, 17–19 September 1990

In September 1990, ILRAD invited a group of experts on *Theileria annulata*, a protozoan parasite that causes tropical theileriosis, to participate in a workshop to review recent developments in