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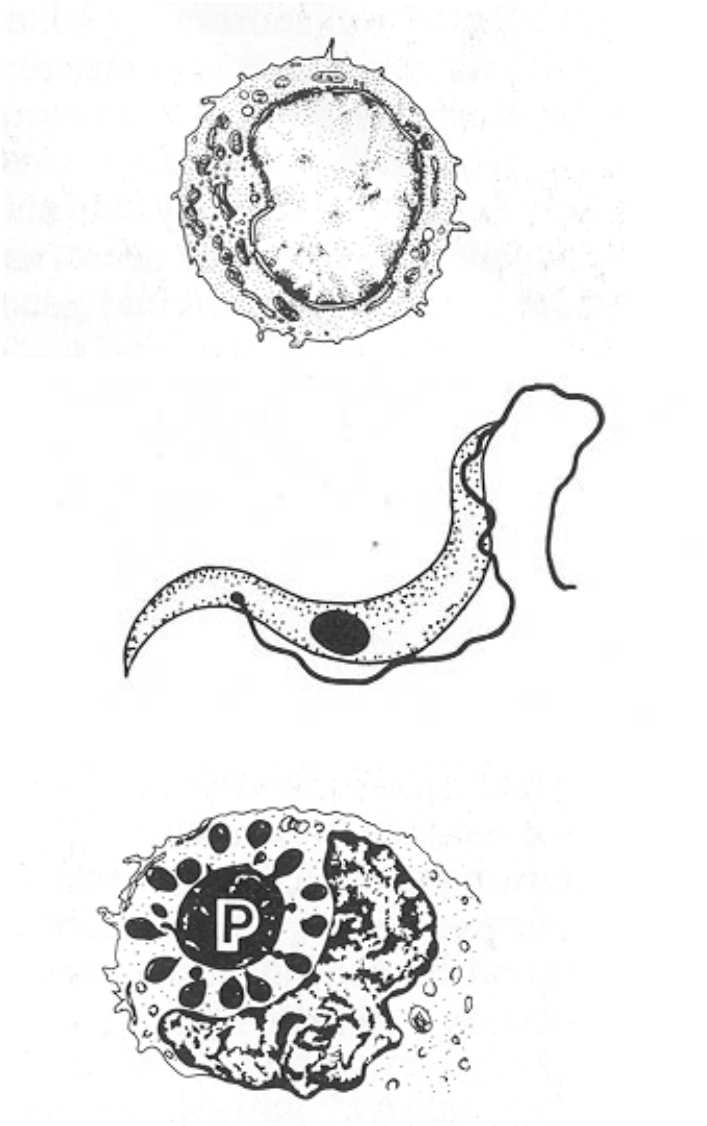
Transfecting genes

The central challenge of today's studies of disease-causing parasites is to understand the workings of the parasites and the cells of their mammalian hosts in molecular detail. Making use of technical breakthroughs in molecular biology and the natural plasticity of genes, ILRAD scientists are transferring DNA from one cell and organism to another to discover the molecules responsible for the lethal effects of parasite infection and the molecules that may be used in safe and effective vaccines against diseases.

IN THE LAST THREE DECADES, our knowledge of the biology of organisms has increased remarkably due to advances made in molecular biology. To understand how a single- or multiple-celled organism functions, scientists are studying how molecules—specifically proteins, the building blocks of all cells—function. Proteins play crucial roles in virtually all biological processes, from catalyzing chemical reactions, to transporting important molecules, regulating cell growth and differentiation, and protecting an organism from foreign pathogens. Although scientists have long been able to break up cells in ways that allow them to sort sub-cellular components, identifying the functions of these components has remained difficult, largely because the amount of a purified protein that could be obtained has been too small for analysis. Today, with technical breakthroughs in recombinant DNA technology, the genes that 'encode' the proteins are being used to produce sufficient amounts of even rare proteins so that their structures and functions can be determined for the first time.

Proteins are built from a repertoire of 20 amino acids. Each gene present in a DNA molecule in the cell nucleus consists of small subunits called nucleotides. The

arrangement of nucleotides acts as a code that specifies the sequence of amino acids needed for the cell to build a particular protein. When deciphered in the mid-1960s this genetic code was found to be nearly the same for all organisms, from bacteria to mammals. This suggested that a gene coding for a particular molecule in one organism should code for the same molecule when inserted into another organism.



Molecules of three types of cells—a white blood cell (lymphocyte) of cattle (top), a trypanosome parasite (middle) and a thieileria parasite-infected bovine lymphocyte (bottom; the parasite = (P))—are being studied at ILRAD by removal and alteration of selected genes and their 'transfection' back into the original cell or into other cells.

The transfected cells will produce the proteins encoded by the foreign genes in quantities large enough to determine the functions of the molecules.

In this way, scientists will be able to discover which parasite molecules to target for attack and to determine which molecules of the bovine immune system help protect animals against disease.

DEVELOPMENT of a range of new laboratory techniques, particularly the recombinant DNA technologies which first appeared in the early 1970s, revolutionized studies in molecular biology. These techniques provide scientists with powerful means of analysing and altering genes and the proteins they encode.

Scientists are now able to clone particular genes from an organism by breaking up the

organism's DNA and inserting the fragments into plasmid DNA. The plasmids are then introduced into bacteria, where they replicate many thousands-fold. Scientists can screen the plasmid-containing bacteria to pull out a gene of interest. As predicted, genes thus isolated from one organism can be altered and either reinserted into the original cell or transferred to cells of other organisms and expressed in those cells.

This group of techniques, collectively called molecular genetics, has become indispensable in studies of many basic biological questions, such as how different proteins function and how the cell regulates protein synthesis through gene expression. (Of the thousands of genes carried in each mammalian or parasite cell, only a fraction at any one time are 'expressed', that is, transcribed into RNA and then translated into protein.)

The power and success of this new technology have greatly increased our understanding of the biology of pathogenic organisms and have raised prospects that this knowledge will benefit people in many very different ways. At ILRAD, scientists are using this technology to advance understanding of protozoan parasites that cause fatal livestock diseases. The researchers are manipulating the genetic material of these single-celled parasites—as well as the genes in cattle that control the animals' immune response to parasite infection—to discover molecules of the parasites that can be used effectively in vaccines, to discern 'weak spots' in the parasites that may be targeted for chemical or immunological attack, and to determine which components of the bovine immune system may be strengthened by administration of a vaccine. Improving the control of economically important tropical livestock diseases in such novel and cost-efficient ways will benefit both mixed farmers and nomadic pastoralists who in many parts of the world depend on their livestock for life as well as livelihood. —Ed.

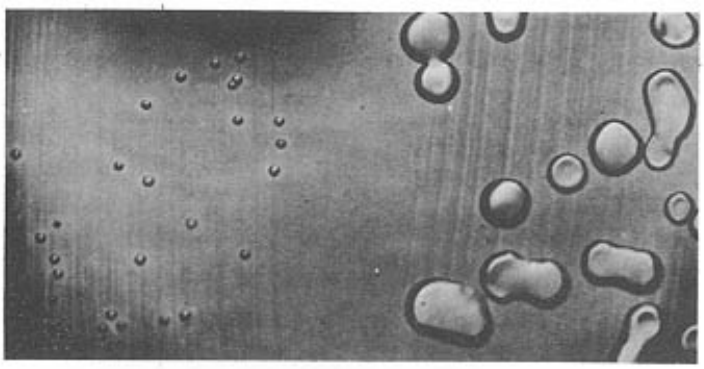
Gene transfer

The transfer of genes 'laterally', as distinct from reproductively (from one generation to the next), is not solely an invention of molecular geneticists: the phenomenon occurs naturally, for example, in bacteria, which pass a plasmid—a short, circular piece of DNA—from one individual to another. Gene transfer in bacteria was first unexpectedly observed in 1928 in a series of simple and elegant experiments conducted by the English microbiologist Frederick Griffith.

The form of the pneumococcus bacterium that causes pneumonia in animals is surrounded by a capsule responsible for the bacterium's pathogenicity. There also exists a mutant form of the bacterium that, lacking this capsule, does not cause disease.

It was known that mice infected with the live virulent form of the bacterium died of septicaemia, whereas neither the virulent form when killed by heat nor the avirulent mutant live form were lethal to mice. Griffith injected mice with a mixture of live avirulent and heat-killed virulent pneumococci. Two striking results followed: the mice died and the blood of the dead mice was found to contain *live virulent* pneumococci. The heat-killed virulent bacteria had thus somehow 'transformed' the live avirulent form into live virulent pneumococci. The change, moreover, was permanent: the transformed bacteria yielded pathogenic progeny of the virulent form. This suggested that the heat that killed the pathogenic cells did not damage those cells' genetic components, which, liberated somehow from their original cells, passed through the cell wall of the living recipient cells and subsequently underwent genetic recombination with the recipients' genetic apparatus.

Griffith named the factor responsible for the transformation from avirulent to virulent strain the 'transforming' principle. The significance of this discovery was not fully understood until 1944, when Oswald Avery, Colin MacLeod and Maclyn McCarty at the Rockefeller Institute published their landmark discovery that the active principle effecting this transformation is deoxyribonucleic acid (DNA).



Transformation of nonpathogenic (small colonies) to pathogenic pneumococci (large) by DNA from heat-killed pathogenic pneumococci. (From Avery, MacLeod, McCarty, Journal of Experimental Medicine 79,1944, p. 158.)

Transfecting modified trypanosome genes into trypanosomes

The above account illustrates how studies of a mutant form of an organism can produce important information about biological processes in the normal form. In a similar way, studies of mutant genes often yield insights into the functions of normal genes. ILRAD scientists working on improved control methods for the animal disease trypanosomiasis are developing 'transfection' systems for trypanosomes, the causative parasites. (The term 'transfection' is used to describe the artificial transfer of DNA from one organism to another.)

After extracting a gene of potential interest from trypanosomes, the scientists modify the gene using molecular biology techniques and then reintroduce the gene into the parasite to examine the effects of its alteration. Use of reliable transfection systems will help the researchers locate important receptor molecules on the parasite surface.

ILRAD scientists have isolated several genes in two species, *Trypanosoma brucei* and *Trypanosoma congolense,* that are expressed, or 'turned on', in particular rather than all stages of the parasites' life cycle. This selective gene expression suggests that the proteins encoded by these genes play important roles in the development and growth of the parasites. The protein products of these genes are thus potential targets for chemotherapeutic or immunological interventions aimed at stopping the parasites from proliferating in their animal hosts. ILRAD scientists are using transfection systems to discover the functions of these developmentally regulated proteins.

Transfection technology is also being used to discover the mechanisms responsible for development of parasite resistance to commonly used trypanocides. ILRAD scientists recently began to use transfection techniques to identify the genes responsible for the resistance phenotype and to establish the role these genes play in conferring resistance. Results of these studies will enable them to identify the targets of the drugs and should help disease control workers make more effective use of drugs.

In collaboration with scientists from the Netherlands Cancer Institute (Amsterdam), ILRAD workers are developing systems for the permanent (stable) transfection of trypanosomes. Vectors are constructed by inserting a selectable marker—a gene encoding resistance to an antibiotic—into a cloned parasite gene. The vector is introduced into live trypanosomes by a method known as electroporation. High-voltage electric currents are passed through the parasite medium, causing a temporary disruption of parasite cell membranes, through which the vectors enter the cells. The parasites that are resistant to the antibiotic are then selected.

These experiments have shown that the transfected trypanosomes contain the antibiotic resistance gene inserted at the genomic location of the cloned parasite gene. Using this

'targeted insertion', the ILRAD scientists are able to alter cloned trypanosome genes, target them back to their original genomic locations and examine the effects of the alteration. This technique is being used to examine the effects of altering genes identified as playing important roles in trypanosome cell division.

Transfecting cattle genes into mouse cells

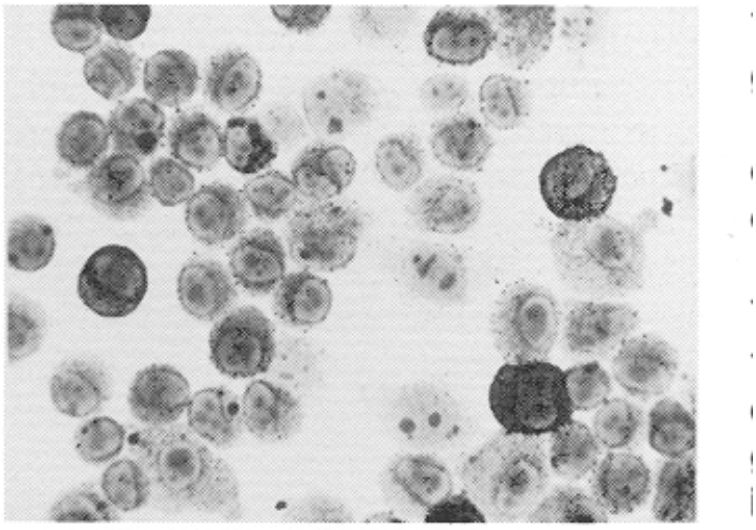
Foreign DNA can be introduced into mammalian cells using one of several methods. The choice of method depends largely on the cell type to be transfected and whether or not the gene of interest has been isolated. The following discussion illustrates how some of these methods have been employed at ILRAD for use in studies of important molecular functions in the immune responses of cattle to infection with *Theileria parva,* a protozoan parasite that causes East Coast fever.

East Coast fever is a virulent form of theileriosis. The disease is endemic in 11 countries of Africa where it is a severe constraint not only to the production of milk and beef, but also to mixed farming: keeping at least one or two cattle is essential for the survival of many of Africa's small mixed crop–livestock farms.

Confirmation of a second MHC class I locus in cattle

Some cattle naturally survive an infection with *T. parva* and these animals are thereafter immune to East Coast fever. Evidence suggests that this immunity is mediated by a subpopulation of bovine white blood cells known as cytotoxic T lymphocytes (CTLs). These cells bind to and kill host cells that are infected with *T. parva* by recognizing foreign (parasite) proteins that appear on the surface of the infected cells.

Research on other disease systems has shown that CTLs recognize foreign proteins on host cells only when these appear in close association with a particular type of molecule produced by the host cell and known as an MHC class I molecule. These molecules are encoded by genes located in an area of the mammalian genome designated the major histocompatibility complex (MHC). With a couple of exceptions, each mammalian species has a pool of many MHC class I molecules. Each individual within a species inherits a very small proportion of the available MHC class I pool. Thus, there can be significant differences in the composition of expressed MHC class I molecules from one individual to another.



The darkly stained cells in this group of mouse cells have been 'transfected' at ILRAD by

insertion of a cattle gene that encodes a class I MHC molecule.

The great advantage of using transfection technology is that it allows scientists to study a single gene and its protein product in isolation.

These MHC differences among individuals may help to prevent the eradication of an entire species by a lethal pathogen. Today, these variations must be considered when interpreting results of immunization trials and when conducting experiments aimed at identifying parasite molecules that may be used in vaccines. To gain a better understanding of the complexity of the bovine MHC class I gene system, ILRAD scientists wanted to know how many types of MHC class I molecules are expressed on the surface of a single bovine cell.

Past results obtained at ILRAD and other laboratories suggested that cattle have more than one genetic locus expressing MHC class I molecules. To obtain clear evidence of this, ILRAD scientists transfected total (genomic) DNA from an animal into mouse fibroblast cells using a calcium-phosphate-mediated transfection system.

Calcium-phospate-mediated gene transfer is one of the most widely used transfection procedures. In essence, the DNA to be transfected is precipitated by the addition of calcium phosphate and added to the recipient cells. The precipitated DNA particles are taken up by the cells and the DNA is permanently incorporated into the chromosomes of the transfected cells. The unique advantage of this technique is that it can be used to transfect genes that have not been isolated but are known to be present in the genomic DNA from a particular cell type.

Cells transfected with the genomic DNA may express one or more of the foreign genes. Those transfected cells that express the gene of interest can be identified and isolated. This is particularly straightforward if the product of the gene of interest is an antigenic molecule located on the surface of the original cell. Using monoclonal anti-bodies that bind to this surface antigen and a technique known as fluorescence-activated cell sorting, scientists can isolate the transfected cell population. The disadvantages of this technique are that it is successful only for certain cell types, and that it does not allow easy recovery of the gene of interest.

In the ILRAD experiment, DNA was obtained from an animal known to have inherited identical MHC regions from each of its parents. (Such an individual is said to be homozygous at the MHC class I region.) When genomic DNA from this animal was transfected into mouse fibroblast cells, two distinct transfected cell lines were produced. Each line expressed a different MHC molecule as shown by the reactivities of the cells with monoclonal antibodies. This result was confirmed using genomic DNA from a second homozygous animal to produce another two transfected mouse cell lines expressing different MHC molecules.

These transfection experiments thus established that cells of each animal from which the DNA was obtained were expressing at least two types of MHC class I molecules. This means that each of the two MHC regions the animals inherited from their parents had two different class I genes.

Most animals are heterozygous rather than homozygous for MHC class I genes. The ILRAD results thus indicate that cattle can express at least four different MHC class I molecules on each cell. Parasite proteins could be presented to CTLs of the bovine immune system by any or all of these molecules.

Use of transfected cells to characterize monoclonal antibodies

Monoclonal antibodies that recognize and bind to surface antigens of lymphoid cells are essential reagents for the dissection of any immune response. For example, probing bovine cells with monoclonal antibodies allows scientists to identify subpopulations of

immune cells, and thus to determine the contribution a particular subpopulation makes to an immune response. In this way, it was discovered that cytotoxic T cells play an important role in the protective immune response cattle mount against East Coast fever.

Monoclonal antibodies have traditionally been obtained by inoculating mice with cells expressing the appropriate antigen. Monoclonal antibodies derived from these mice are characterized by establishing their reactivities with subsets of cells or tissue sections, and by noting the size of antigens immunoprecipitated by the monoclonal antibodies. This procedure is often complicated by the fact that the expression of the antigen may not be clearly restricted to defined cell populations and that not all monoclonal antibodies will immunoprecipitate their respective antigens.

An alternative approach is available if the gene encoding the relevant antigen is isolated and transfected into cells that normally do not express the gene. The specificity of candidate monoclonal antibodies can be easily established by their reactivity with the transfected cell line. This approach was used at ILRAD to identify monoclonal antibodies specific for the bovine interleukin 2 (IL-2) receptor. IL-2 acts as a molecular messenger among lymphoid cells. Those lymphoid cells that are actively proliferating in response to an infection express elevated levels of the IL-2 receptor.

The gene encoding the IL-2 receptor had been isolated by workers elsewhere using the human IL-2 receptor gene as a probe. A mouse cell line permanently transfected with the bovine gene was produced at ILRAD and used to establish that several candidate monoclonal antibodies recognize the IL-2 receptor. These antibodies are now being used to obtain a clearer understanding of the roles that IL-2 and the IL-2 receptor play in the bovine immune response to trypanosomiasis.

Isolating genes that encode parasite antigens recognized by monoclonal antibodies

The traditional way to isolate a gene that encodes an antigen is to construct gene 'library' in which all the DNA expressed by an organism is cloned into plasmid DNA, which is then introduced into bacteria for expression of the gene product. Scientists can identify which bacteria contain the gene of interest by probing the colonies with monoclonal antibodies that specifically recognize the protein product of the gene.

In a recently developed variation of this procedure, the gene library is transfected directly into mammalian cells. This modified approach is particularly useful to geneticists attempting to isolate a mammalian gene. Bacteria and mammalian cells process proteins differently. Thus, a mammalian gene expressed in a transfected mammalian cell may produce a protein that resembles the native gene product more closely than the same gene expressed in a transfected bacterium.

This approach has been used successfully in the transient expression COS cell system. COS cells are derivatives of an African green monkey kidney cell line (CV-1) infected with a replication-defective mutant of the simian virus 40 (SV40). These cells produce large amounts of the large tumour antigen of SV40. When specially designed plasmids are introduced into COS cells, the SV40 large tumour antigen present in the cells causes the plasmids to replicate as independent extrachromosomal elements to high copy number. Thus, any gene also inserted into the plasmid is expressed at very high levels if transfected into COS cells.

Scientists at the University Hospital in Utrecht, the Netherlands, working in collaboration with ILRAD, recently employed this procedure to isolate the gene that encodes an antigen produced by *T.parva* schizonts, the form of the parasite present in infected bovine lymphocytes. A library of genes expressed by schizonts was constructed in a plasmid specifically designed for expression of foreign genes in COS cells.

COS cells were transfected with the gene library and probed with anti bodies specific for a schizont antigen. Using an enzyme-based-detection system, scientists identified the COS cells containing the relevant plasmids. The recombinant plasmids were recovered from the COS cell and the gene encoding the schizont antigen was isolated This gene will now be used to characterize the antigen. The scientists particularly want to know if the antigen is recognized by CTLs as well as antibodies from immune cattle (see below).

Identification of antigens recognized by CTLs

As mentioned earlier, CTLs are believed to be important in protecting cattle against East Coast fever. A major objective of ILRAD's theileriosis research is to identify the parasite antigens recognized by CTLs obtained from cattle that are immune to East Coast fever; such antigens will form the basis of an effective vaccine against the disease.

One way to determine if a particular parasite antigen is recognized by CTLs is to transfect the gene encoding the antigen into a cell line expressing the appropriate bovine MHC class I molecule, such as the mouse fibroblasts transfected with bovine MHC class I molecules, as described above. These cells can then be used as target cells in a cytotoxicity assay to determine whether the gene encodes an antigen recognized by CTLs from an immune animal. In this way, it may be possible to identify from an array of candidate antigens those that could induce a protective CTL response. These antigens would form the basis of an effective vaccine against East Coast fever.

This article is based on reports of ILRAD studies of gene transfection written by Noel Murphy (trypanosomes) and Philip Toye (mammalian cells). Other ILRAD scientists involved in the mammalian cell transfection studies were Niall MacHugh, Ivan Morrison (now at the Compton Laboratory, UK), Jan Naessens and Alan Teale. Collaborators in this work include Piet Borst, of the Netherlands Cancer Institute (Amsterdam), and Hans Clevers and colleagues at the University Hospital, Utrecht, the Netherlands. The following article (page 5) is based on a report by former staff member Vinand Nantulya.

CORRECTION
The wording of a paragraph (page 2, last paragraph) in the recently published *ILRAD 1991 Highlights* is misleading. The paragraph should read as follows:

During the year, ILRAD scientists also identified a 24-kDa molecule present in infected bovine lymphocytes. The purified molecule was recognized *in vitro* by clones of helper T cells derived from immune cattle. The role of the 24-kDa antigen in inducing protective responses to *T. parva* is being investigated

New tools for better diagnosis of African trypanosomiasis

DIAGNOSIS is a critical element in the management of disease, both at the level of the individual animal when the decision to treat or not to treat has to be taken and for evaluating the success of disease control programs. The diagnostic tests used should be simple, rapid, specific and highly sensitive. They should also be able to differentiate between closely related parasite species if the disease syndromes they cause require different management approaches. Ideal tests should be suitable for field application and the cost should be within the means of the communities affected by the disease. ILRAD has developed simple tests for diagnosis of African trypanosomiasis that promise to fulfil many of these requirements.

Trypanosomiasis is characterized by severe anaemia, weight loss, reduced productivity, infertility and abortion, with death occurring in some animals during the acute phase of the disease. Animals that survive often remain infected with trypanosomes for several months or years and exhibit a low level of fluctuating parasitaemia. During this phase, the animals may manifest no overt clinical signs.