AN IMMUNOGENETIC APPROACH TO LIVESTOCK RESEARCH IN TANZANIA

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SUMMARY

Five monoclonal antibodies were derived and applied to phenotype cattle according to two major histocompatibility complex (MHC) antigens. Results demonstrate significant differences in the frequency of the antigens between African and European cattle breeds. This observation is of significance in that it suggests associations of MHC antigens with economically important traits. The monoclonal antibodies are also discussed with regard to their usefulness in the serological detection of MHC and common leucocyte antigens of Tanzanian cattle. It is concluded that biotechnological applications should be enhanced for the improvement of indigenous livestock in Tanzania.

INTRODUCTION

Tanzania, like many other countries in Africa, is in a difficult situation with respect to food, especially food of animal origin. In contrast, the country is endowed with a vast pool of animal genetic resources and pasture land. Perhaps among the major problems causing food inadequacy are laxed implementation of recommended technical expertise, slow introduction of new ideas into the existing policy and to a great extent, lack of resources, infrastructure and trained manpower. These shortcomings, and possibly several others, make the current wave of biotechnological revolution a distant dream to Tanzanian applied livestock research. Nevertheless, problems of livestock production and health in Tanzania must be attended to, regardless of the existing difficulties. Partnership with developed countries and transfer of biotechnologies will therefore continue to play a vital role in the improvement of livestock in Tanzania. Four major limitations can be identified which hinder our livestock production; poor genetic composition of native livestock, inadequate nutrition, prevalence of diseases and harsh climatic and stress conditions. These limitations, notwithstanding, research need to be enhanced in areas of improved disease diagnosis and control, nutrition and genetic improvement of indigenous livestock. The present paper and a few others by Tanzanian scientists (Msolla *et al.* 1991, Lyaku *et al.* 1990, Gwakisa *et al.* a & b, 1990), validate the idea that adequate scope exists for utilization of biotechnology in Tanzania. Biotechnological applications employ new techniques for studies of biological systems, such as hybridoma (monoclonal antibody) technology, recombinant DNA technology (gene manipulation) and novel bio-processing techniques.

Monoclonal antibodies, first described by Kohler and Milstein (1975), have revolutionized the biomedical world. The principal applications for monoclonal antibodies have been in disease diagnosis, identification, localization and sorting of individual types of cells from blood and solid tissues, and the topographical analysis of antigenic determinants (gene products) of pathogenic organisms and on cells and tissues of animals (McCullough *et al.* 1987; Lalor *et al.* 1986; Milstein *et al.* 1979).

In this communication, results are given of work performed to produce monoclonal antibodies for application in phenotyping cattle for gene products controlled by alleles in the major histocompatibility complex, which in cattle is commonly referred to as the bovine lymphocyte antigen (BoLA) system. Antigens within this system are also called 'self-antigens' due to their role in the recognition of foreign antigens and involvement with immune responses and disease resistance (Warner *et al.* 1987; Lewin *et al.* 1988; Stear *et al.* 1984). Many workers have attempted to find, in a variety of species, positive associations between antigens of the MHC system and individual parameters of productivity and reproduction (Gautschi & Gaillard, 1990; Batra *et al* 1989). Identification of MHC antigens has usually relied on serological techniques employing alloantisera and monoclonal antibodies. In these techniques, cells taken from blood of the animal to be typed are mixed with different antibodies in the presence of certain blood proteins (complement) and eosin dye. If any of the antibodies recognizes and binds to an MHC glycoprotein on the cells being typed, the cells are killed. Dead cells absorb the dye in the mixture, whereas living cells do not, and cells with and without the dye can be distinguished visually with a microscope. The objectives of this study were to develop optimum conditions for derivation of monoclonal antibodies and attempt to apply them in typing cattle for MHC class I antigens. The results obtained are discussed in respect to their applicability in immunogenetic characterisation of cattle breeds.

MATERIALS AND METHODS

Animals

A total of 190 cattle were tested. Of these 100 were either Borans or their crosses with other *B.indicus* cattle, and 90 were Friesians and their crosses with other *B.taurus*. Thirty animals in either group were taken from widely separated herds in Tanzania (Sokoine University of Agriculture farms and private farms in Morogoro). The remaining 130 animals belonged to the International Laboratory for Research on Animal Diseases (ILRAD), Kenya, and were used during the characterisation and determination of specificity of the monoclonal antibodies described hereunder.

Cells

For the purpose of production of monoclonal antibodies, peripheral blood lymphocytes (PBL), as the source of immunogen, were collected from two Boran (*Bos indicus*) heifers of known MHC class I antigens, denoted w25 and KN8 respectively, where the prefix 'w' stands for an internationally recognised MHC antigen specificity and 'KN' stands for an African antigen specificity, first defined at ILRAD, Kenya (Kemp *et al.* 1988).

Immunisation of mice and preparation of immune spleen cells

Two mice of BALB/c breed, aged 2-3 months, were immunised intravenously with the PBL taken from the two cattle of known MHC types. Approximately $2x10^7$ bovine PBL in 200 ul of phosphate buffered saline (PBS) were given to each mouse 1-4 times at 3 weekly intervals. Three days after the last booster injection, the mice were killed and their spleens removed aseptically. The spleen cells were teased into 10 ml of culture medium in a Petri dish and later transferred into a sterile tube. After clumps had settled down, the clean cell suspension was moved into another tube and centrifuged (1200rpm/10min). The cell pellet obtained was resuspended into serum-free medium and a portion of it counted in trypan blue.

Cell fusion procedure

Work was done with great care to avoid all sources of contamination. Suspensions of spleen lymphocytes from immunised mice and murine myeloma cells (X63) were mixed at a ratio of 4 lymphocytes to 1 myeloma cell in a conical bottomed tube and centrifuged gently (1000rpm/10min). Prior to cell fusion the X63 myeloma cells were routinely maintained in culture and occasionally grown in culture medium containing 8-azaguanine (complete RPMI 1640 medium with Hepes supplemented with 2 mM L-glutamine, 50 ug/ml gentamicin, 50 uM 2-mercaptoethanol and 10-20% fetal calf serum) to prevent them from 'reversion' to selection medium-resistant cells. Use of 8-azaguanine was stopped a week prior to fusion. After spinning the myeloma-spleen cell mixture, the supernatant was removed completely and 1 ml of prewarmed to 37^{0}C fusion agent (polyethylene glycol, PEG 1500) was slowly added to the cell pellet by pippeting over 1 minute. Five minutes later, 10 ml of warm serum free medium were slowly added, gently rocking the tube. The cells were gently spun and resuspended in a small volume of culture medium containing 20% fetal calf serum. The hybridised cell suspension was then topped up with selection medium (20% FCS/RPMI containing 10^{-6}M azaserine and 10^{-4}M hypoxanthine) containing mouse thymocytes as a feeder layer to bring the cell concentration to $1x10^6$ spleen cells per ml and $5x10^6$ thymocytes per ml. The cells were then plated out at 200 ul per well into 96-well cluster plates and placed in a CO_2-incubator at 37°C.

Culture of hybrids (hybridomas) after fusion

Plates were examined under inverted phase-contrast microscope daily for contamination, colony growth and pH change. After 14 days azaserine was removed from the selection medium and the wells were replenished with fresh medium containing 20% FCS and hypoxanthine alone. Screening for antibody secretion was performed only when hybridoma colonies were visible macroscopically.

Screening and selection of monoclonal antibody producing hybridomas

The microlymphocytotoxicity assay was applied essentially as described by Kemp & Teale (1987). Briefly, each supernatant antibody was dispensed under liquid paraffin into a well of a Terasaki microtiter plate. Then a lymphocyte suspension containing $3x10^3$ cells was added to the wells and the antibody-cell mixture was incubated at room temperature. Thirty minutes later, rabbit serum as a source of complement was added to the wells and the plate was incubated further. After 1 hr the reaction was terminated by adding a staining-quenching solution. Results were read 15 min later under a phase-contrast microscope using an automated assay. When applied in the Immunology Laboratory at Sokoine University of Agriculture, the assay was modified to the manual reading procedure, whereby 1% eosin and buffered formalin were used respectively, to stain and fix the cells instead of staining-quenching solution. In practice, each supernatant was screened against a panel of lymphocytes taken from the immunising animal, 2-3 siblings and 1-2 MHC-unrelated animals. Only supernatants which showed discriminate cytotoxicity on the lymphocyte panel with at least 80% killing on the targeted antigen were selected for cloning and further propagation. Positive hybridomas were cloned by limiting dilution and were cultured further as described above for primary hybrids. When the clones had grown, screening was repeated and one positive clone from the highest dilution (0.3 cell per well) was propagated further for bulk production of monoclonal antibody in cell culture supernatant. The same screening assay was followed for MHC-typing of animals.

Preparation of ascitic fluid in mice

To obtain large amounts of monoclonal antibodies, 10^6 hybridoma cells were injected i.p. into BALB/c mice which had been injected i.p. 7-60 days previously with pristane. When tumours had grown (7-15 days later), the mice were killed, the ascitic fluid taken and cleaned by spinning at 2000 rpm for 30 min. Clean ascitic fluid was titrated using the screening procedure described above and kept either at -20°C or at 4°C with sodium azide until use.

RESULTS

Development of monoclonal antibody technology

By following the procedures described above, at 10-15 days post - fusion most culture wells contained colonies of growing hybrids. Most wells produced antibody, but only a few of them secreted antibody of desired specificity. Results of four independent fusions are shown in Table 1. It is apparent that multiple intravenous injections of antigen resulted in a much higher frequency of hybridomas secreting specific antibodies when compared with one single injection performed in early experiments. Large numbers of wells showing growth of hybrids 7 days after fusion were observed when the fusion agent (PEG 1500) was warmed to 37° prior to its addditon to the myeloma-spleen cell pellet. Moreover, the number of wells with growing hybridomas was high in all fusions when selection medium was supplemented with 20% fetal calf serum, rather than when a lower serum concentration was used. Likewise, more hybridomas secreting specific antibody were generated when selection medium was supplemented with hypoxanthine and azaserine rather than with hypoxanthine, aminopterin and thymidine. Prior to use in MHC typing the monoclonal antibodies were titrated using the microlymphocytotoxicity assay on PBL of several MHC-matched animals. Thus, the established titre was maintained in all subsequent studies.

Table 1. Summary of four fusions

Fusion number	Target antigen	No. of injections	Wells showing hybrid growth	Wells secreting specific antibody
1	w25	1	200/960	3
2	KN8	2	432/960	8
3	w25	3	900/960	56
4	KN8	4	850/960	60

It is interesting to note that wells to which thymocytes were added showed very rapid colony growth. No adverse effects on colony growth were observed when the density of feeder layer cells (thymocytes) was increased.

Addition of thymocytes as a feeder layer to the wells containing fused cells was found to be crucial.

Selection of monoclonal antibodies for MHC typing

Selection of monoclonal antibodies prior to their application for typing cattle according to bovine lymphocyte antigens was based on results from family and population studies, immunochemical and biochemical assays described in detail elsewhere (Gwakisa *et al*, 1990). Five of the derived antibodies were confirmed to be specifically directed against MHC class I antigens. Two of these Code-named IL-A89 and IL-A94 were reactive on cells expressing a w25 antigen. Three others, IL-A91, IL-A92 and IL-A93 were seen to be reactive upon a *Bos indicus* antigen, KN8.

Genetic studies

In an attempt to compare antigen frequencies of the two MHC antigens (w25 and KN8) in cattle populations of African breeds (Boran)

and taurine breeds (Friesians and crosses), the defining monoclonal antibodies were included in a panel of reagents, known to define class I MHC antigens. The panel comprised of 45 reagents, of which 30 were monoclonal antibodies, the rest being alloantisera. Some of the reagents employed in the BoLA typing panel are shown in Table 2.

Most of the reagents in the panel were raised against
Bos indicus tissue in ILRAD (Kemp *et al.* 1988, Gwakisa *et al.* 1990a, Teale & Kemp unpublished data). MHC typing on Tanzanian *Bos indicus* cattle and exotic breed crosses revealed interesting results. Thus, animals of either breed expressed the same antigens. However, marked differences in antigen frequencies were seen between *Bos indicus* and *Bos taurus* breeds.

Table 2. Part of the BoLA typing panel

Antigen	Reagents	References
w25	IL-A89	Gwakisa *et al.* 1990
"	IL-A94	"
"	KA017	Kemp *et al.* 1988
"	KMA031	"
KN8	IL-A91	Gwakisa *et al.* 1990
"	IL-A92	"
"	IL-A93	"
"	KKA005	Kemp *et al.* 1988

The frequency of the KN8 antigen was elevated in African cattle, whereas the antigen w25 was relatively rare in African cattle compared with *Bos taurus* animals. Table 3 presents separately the gene frequencies of the two antigens in Tanzanian Boran and Friesians or their crosses

Common leucocyte antigens

Two monoclonal antibodies, IL-A37 and IL-A39 (a kind gift from Dr. Teale & Dr. Kemp of ILRAD) were included in the panel for definition of common leucocyte antigens (CLA), IL-37 and IL-39.

Table 3. Gene frequency (%) of the lymphocyte antigens in different breeds.

Antigen (BoLA/CLA)	Boran crosses n=30	European n=30
w25	6	20
KN8	25	0.5
IL-37	10	45
IL-39	2	20

Results obtained showed differences in the frequency of the two antigens within and among different cattle populations (Table 3). The antigen IL-39 was apparently less frequent than IL-37 in Boran cattle as well as in European crosses. The frequency of both leucocyte antigens was on the whole lower in Tanzanian Borans than in exotic cattle or their crosses.

DISCUSSION

As results presented here indicate, the monoclonal antibodies derived in this study have proved to be invaluable tools for the definition of lymphocyte antigens of Tanzanian cattle. Besides other advantages, monoclonal antibodies are easily produced in unlimited quantity and permit repeatability of results, thus allowing inter-laboratory exchange of standard reagents as shown in this work. The studied MHC antigens occurred with varying frequencies in different breeds, and one of

them, the KN8 antigen, was unique to *Bos indicus* cattle. Although the gene frequencies presented in this study may not necessarily be representative of the breeds as a whole, these data support previous observations by other reseachers, that differences exist between African and European cattle breeds for MHC antigens (Maillard *et al.* 1989, Kemp *et al.* 1988). The differences in the frequency of MHC antigens between cattle populations are not unexpected. One of the explanations for the differences is assumed to be inheritance and geographical background. Similar differences have also been reported for human races (Dick, 1978). In cattle, differences among breeds in the frequency of MHC class I antigens have been reported by Spooner *et al.* (1987) for 14 antigens in three breeds of African cattle, by Oliver *et al.* (1981) for 14 antigens in six breeds of British cattle, by Stear *et al.* (1987, 1988) for 29 antigens in six breeds of Australian cattle and for 37 antigens in seven breeds of American cattle respectively, by Caldwell *et al.* (1979) for six antigens in six breeds of North American cattle, and by Amorena & Stone (1980) for 11 antigens in six breeds of North American cattle. The same findings were observed when the animals were phenotyped according to the polymorphic system of common leucocyte antigens. Although the significance of these differences was not studied in this work, they may reflect responses to differing environmental pressure, stress and disease challenges. It is known that African cattle face intense challenge by a number of pathogenic organisms and ectoparasites and survive under conditions (high temperature, low availability of water and intense sunlight) which frequently prove fatal to European breeds. Moreover, European breeds do not receive constant prophylaxis and protection from the environment. These facts serve as the impetus for searching for genetic markers which distinguish African and European cattle breeds. If significant differences in the frequency of polymorphic markers can be established, this might suggest which alleles should be studied further with the aim of identifying associations between such markers and productivity or disease resistance. Clearly, from this work and earlier findings by Trail *et al.*(1989), Maillard *et al.* (1989), and many others, MHC antigens have been shown to be important markers of cattle breeds.

The differences in the frequency of inherited blood protein systems among cattle populations are important to establish 'marker-trait' associations. In *Bos taurus* cattle, evidence has been given of association between presence of MHC antigen type in animals and fitness or immune responsiveness. MHC antigen w16 has been linked with high immune response to serum albumin (Lie *et al.* 1986). The specificity w16 has also been linked to the M blood group antigen (Leveziel, 1983) and an association has been suggested between expression of the M antigen and susceptibility to mastitis (Larsen *et al.* 1985) and trypanosomiasis (Carr *et al.* 1974). Stear and co-workers (1991) have shown that increased antibody-dependent neutrophil cytotoxicity and decreased susceptibility to subclinical mastitis segregate with MHC antigens w14/w8. Therefore, study of MHC antigens, such as this one, can provide useful information for defining genetic markers and subsequently apply such markers in association studies with economically important traits of production and health. The role of MHC antigens in the control of immune responses and disease resistance has been reviewed in detail elsewhere (Gwakisa, 1989).

In summary, this work has led to two achievements. First, monoclonal antibodies which phenotypically distinguish cattle according to genetically controlled lymphocyte antigens have been produced. Secondly, application of the described antibodies together with several other reagents has enabled comparison of Tanzanian Borans with exotic cattle breeds for frequencies of

lymphocyte/leucocyte antigens. In this communication emphasis has been put on an immunogenetic approach to animal improvement; indeed, production of monoclonal antibodies is among the biotechnologies which should find even wider application in various aspects of livestock improvement, such as immunodiagnosis. As more biotechnological products (monoclonal antibodies, DNA probes, sequenced DNA fragments of genes responsible for particular traits, transfected cells, etc.) are generated, the scope for biotechnological application will broaden for the strengthening of research into the biological systems which play key roles in immunological (disease resistance) and physiological (production) interactions in our indigenous cattle.

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