IMMUNOLOGICAL RESPONSES OF HOLSTEIN-FRIESIAN CATTLE TO *STAPHYLOCOCCUS AUREUS IN VITRO*

By

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A THESIS SUBMITTED TO THE UNIVERSITY OF GLASGOW VETERINARY SCHOOL FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

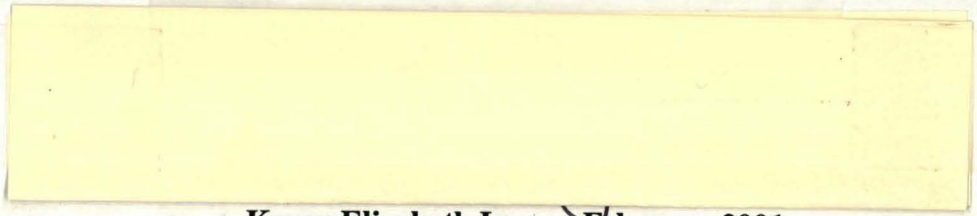
UNIVERSITY *of* GLASGOW

DEPARTMENT OF VETERINARY CLINICAL STUDIES

UNIVERSITY OF GLASGOW VETERINARY SCHOOL

FEBRUARY 2001

Apart from the help acknowledged I declare that the work described was carried out by me and is not that of any other person and, further, has not been submitted in full or in part, for consideration for any other degree or qualification



Karen Elizabeth Logan, February 2001

Acknowledgements

I would like to acknowledge the time, effort and enthusiasm that my supervisor Prof. Julie Fitzpatrick has shown throughout my time in the Department of Veterinary Clinical Studies.

I would also like to acknowledge the help of Prof. Mike Stear for statistical advice, Dr George Russell for help with the technique of PCR-RFLP and analysis of the gels, Dr Lubna Nasir for help with interpretation of the results generated by Blunt End Cloning, Dr Linda Andrew and Lesley Shields for assistance in using the Flow Cytometer.

I am for ever grateful for the help given unconditionally by the staff at Cochno farm, in particular, Alex and Iain, and at the Genus Ltd bull breeding establishments, in particular Jim Warrington and the men at Whenby Lodge. Thanks are due to the University of Cambridge Veterinary School, Liverpool School of Tropical Medicine, and the Department of Biology University of York for use of their laboratories.

I would like to thank Sonia for her continual support during my writing up process for keeping me sane when I thought the tunnel would never end. I would also like to thank Fiona for her friendship, support and company on those marathon trips to York. Thanks are also due to Steven and the VCS coffee room dwellers for their continual cheek. Finally I would like to thank Danny, my mum and dad, my nanny and the welsh one for helping me through this last year and a bit, for believing in me and encouraging me every step of the way.

This project was funded by the Milk Development Council.

Dedication

For my mum and dad

who've supported me through everything

Abstract

Mastitis is a major disease of dairy cattle worldwide. Breeding cattle for disease resistance is desirable in order to reduce the incidence of clinical and subclinical mastitis, to improve cow welfare and to reduce reliance on chemotherapeutic agents to control disease.

The aim of this study was to develop and evaluate an *in vitro* proliferative response of peripheral blood mononuclear cells (PBM) isolated from Holstein-Friesian cattle, induced by formalin-killed *Staphylococcus aureus,* a major mastitis pathogen, as a potential indicator of resistance or susceptibility to mastitis. Different strains of *S. aureus*, isolated from cows with subclinical mastitis, and identified by restriction enzyme fragmentation pattern analysis, were shown to induce different levels of proliferation of PBM *in vitro*. One particular strain (strain A), induced the strongest proliferative response compared to the other strains tested and differences in the magnitude of the proliferative response, induced by *S. aureus* strain A, was noted among cows. Phenotypic analysis of PBM of cows, by flow cytometry, showed that normal proportions of cell sub-populations were present at the start of culture and that the proliferating cell population consisted mainly of T cells expressing CD4, CD8 or γδ markers.

Proliferation of PBM of cows, induced by Staphylococcal Enterotoxin B (SEB), showed that the peak day of proliferation occurred 72 to 96 hours earlier in the presence of SEB compared to in the presence of *S. aureus* strain A antigen.

A statistically significant difference in the proliferative response induced by *S. aureus* strain A was seen in two progeny groups sired by two different commercial bulls, suggesting that genetic control of this response may be important in protection of the mammary glands against infection. The proliferative assay was, therefore, subsequently performed in bulls.

Predicted Transmitting Abilities (PTA) for somatic cell counts (SCC) estimate the effect of the sires' genes on SCC, a measure of subclinical mastitis. Two groups of

five Holstein-Friesian bulls were selected on their extreme values of PTA for SCC :bulls with a high PTA for SCC have progeny with a higher than average SCC, while bulls with a low PTA for SCC have progeny with a lower than average SCC. The proliferative response, induced by *S. aureus* strain A, of these two groups of bulls, showed a strong negative ($r=-0.7$) and statistically significant ($p<0.05$) correlation with PTA for SCC. The proliferative assay was subsequently performed on 104 bulls selected randomly from all bulls kept by a commercial breeding company, however, no significant correlation was found between the proliferative response induced by *S. aureus* strain A and PTA for SCC. These results suggest that the proliferative assay might be useful in selecting bulls that have extreme values of PTA for SCC, rather than ranking bulls in general.

Phenotypic analysis of PBM using Hotelling's T squared test, showed a significant difference ($p<0.05$) in the proportions of cell subpopulations between bulls and cows, although the biological significance of this has not been studied.

Cloning and sequencing of the Major Histocompatability Complex (MHC) class II DQB exon 2 region, was carried out. Four previously published and one novel sequence were generated from 39 clones, from three of the bulls with low PTA for SCC and from one bull with high PTA for SCC. Characterisation of DQB exon 2 by PCR-RFLP was carried out, however, the enzymatic digestion patterns generated were too ambiguous to classify.

PCR-RFLP was applied to DRB3 exon 2 of the ten bulls selected based on extreme PTA for SCC. It was found that four of the five bulls with low PTA for SCC and one of the five bulls with high PTA for SCC had RFLP patterns associated with allele DRB3.2*16 in other studies. It was also noted that all five bulls with a strong proliferative response induced by *S. aureus* strain A had RFLP patterns associated with allele DRB3.2*16, whereas all the bulls with a weak proliferative response induced by *S. aureus* strain A did not show this RFLP pattern.

Numerous published studies have associated the occurrence of allele DRB3.2*16 in dairy cows with a reduction in SCC. The occurrence of allele DRB3.2*16 in the bulls with strong proliferative responses induced by *S. aureus in vitro* supports the

hypothesis that there is potential to use the *in vitro* proliferative assay to predict subclinical mastitis caused by *S. aureus* in their progeny.

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Abbreviations

PBM	Peripheral Blood Mononuclear Cells
PTA	Predicted Transmitting Ability
SCC	Somatic Cell Count
MHC	Major Histocompatibility Complex
HLA	Human Leucocyte Antigen
SCS	Somatic Cell Score
BoLA	Bovine Leucocyte Antigen
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
REFP	Restriction Enzyme Fragmentation Pattern
RPLA	Reverse Passive Latex Agglutination
MMB	Milk Marketing Board
BMSCC	Bulk Milk Somatic Cell Count
TBC	Total Bacterial Count
IMI	Intramammary Infection
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
SE	Staphylococcal Enterotoxin
TSST-1	Toxic Shock Syndrome Toxin-1
IU	International Units
rb	Recombinant
rbBo	Recombinant Bovine
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
IFN	Interferon
IL	Interleukin
TNF	Tumour Necrosis Factor
NK	Natural Killer
Ig	Immunoglobulin
MAb	Monoclonal Antibody
LPS	Lipopolysaccharide

APC	Antigen Presenting Cell
TCR	T Cell Receptor
HIV	Human Immunodeficiency Virus
FIV	Feline Immunodeficiency Virus
HEV	High Endothelial Venule
BCR	B Cell Receptor
CTLA	Cytotoxic T Lymphocyte Antigen
ICAM	Intracellular Adhesion Molecule
LFA	Leucocyte Function Antigen
PBL	Peripheral Blood Lymphocytes
LN	Lymph Node
TAP	Transporter Associated Protein
ER	Endoplasmic Reticulum
Ii	Invarient Chain
RER	Rough Endoplasmic Reticulum
ASO	Allele Specific Oligonucleotide
DGGE	Denaturing Gradient Gel Electrophoresis
ITEM	Index of Total Economic Merit
PIN	Profit Index Number
PLI	Profitable Life Index
AI	Artificial Insemination
WCCF	White Cell Counting Fluid
PBS	Phosphate Buffered Saline
HBSS	Hanks Balanced Salt Solution
BME	Basal Medium Eagles
EDTA	Ethylenediaminetetraacetic Acid
ACD	Acid Citrate Dextrose
CMT	California Mastitis Test
BSA	Bovine Serum Albumin
FITC	Fluorescein Isothiocyanate
WC	Workshop Cluster
CD	Cluster of Differentiation
ICSCC	Individual Cow Somatic Cell Count
IQSCC	Individual Quarter Somatic Cell Count

BHI	Brain Heart Infusion
TES	
SDS	Sodium Dodecyl Sulfate
TE	Tris-EDTA
TBE	Tris-Boric Acid-EDTA
mA	milliamps
UV	Ultra Violet
3H	Methy-Tritiated Thymidine
mCi	millicuries
Co	Cobalt
CO2	Carbon Dioxide
NaCl	Sodium Chloride
HCl	Hydrochloric Acid
Na OH	Sodium Hydroxide
bp	Base Pairs
XGAL	5-Bromo-4-chloro-3-indolyl beta-D-galactopyranoside
IPTG	Isopropyl beta-D-thiogalactopyranoside
LB broth	
SI	Stimulation Index
SD	Standard Deviation
cpm	Counts Per Minute
PHA	Phytohaemagglutinin
SCMC	Spontaneous Cell Mediated Cytotoxicity
EBV	Estimated Breeding Value
BLV	Bovine Leukemia Virus
PL	Persistent Lymphocytosis
QTL	Quantitative Trait Loci
FMDV	Foot and Mouth Disease Virus
ELISA	Enzyme Linked Immunosorbent Assay
SSCP	Single Strand Conformational Polymorphism
RSCA	Reference Strand Conformation Analysis

Patent Application.

British Patent application no. 9722109.7 University of Glasgow. "Mastitis Assay". I am included within this application by my supervisor Prof. Julie Fitzpatrick along with, Prof. M.J. Stear, and Dr. D.J. Platt, October 1998. The papers based on this work were restricted from publication until the patent application was finalised. The patent application contains the data now being prepared for publication.

Conferences Attended.

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AVTRW Annual Conference (1997) Immune responses of cows to *Staphylococcus aureus* and enterotoxin B. **Logan, K.E.**, Platt, D.J., Stear, M.J., Logue, D.N., Fitzpatrick, J.L.

AVTRW Annual Conference (1998) Immunological responses of Holstein/Friesian bulls to *Staphylococcus aureus*. **Logan, K.E.**, Stear, M.J., McGuirk, B., Fitzpatrick, J.L.

AVTRW Annual Conference (1998) The effect of mycotoxins on bovine peripheral blood mononuclear cell responses in vitro. Charoenpronsook, K., Fitzpatrick, J.L., **Logan, K.E.**, Smith, J.E.

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Proliferative responses of bovine mononuclear cells to *Staphylococcus aureus* in bulls are correlated to predicted transmitting abilities for milk somatic cell count. **Logan, K.E.**, Fitzpatrick, J.L., Stear, M.J., McGuirk, B.

Proliferative responses of bovine mononuclear cells to *Staphylococcus aureus* enterotoxins. **Logan, K.E.**, Fitzpatrick, J.L., Stear, M.J., Platt, D.J.

Chapter I

Introduction

1.1 General Background

Mastitis is defined as inflammation of the mammary gland (Du Preez, 1987). Clinical mastitis can be categorised according to the severity of the infection into peracute, acute and mild cases (Schalm *et al.*, 1971). Clinical signs include abnormalities of the udder such as heat, pain, redness and swelling, and abnormalities in milk including clots, abnormal colour and odour. Mediators of the immune system are produced in response to infections such as mastitis and can result in damage to the mammary secretory tissues (Milner *et al.*, 1996). A number of these mediators can lead to a reduction in milk quality as well as reduction in milk yield (Honkanen-Buzalski *et al.*, 1981). Mastitis has been shown to increase pain perception in cows suffering from both mild and moderate cases of mastitis, compared to non-mastitic cows (Fitzpatrick *et al.*, 1998). Mastitis can also affect the cow subclinically and is much harder to diagnose than clinical disease. There may be no abnormalities of the udder or of the milk, however, an increase in the number of somatic cells present within milk, is used as an indicator of infection.

Somatic cell counts (SCC) are recorded at the bulk tank level, providing a useful method of calculating the prevalence of subclinical mastitis in UK dairy herds (Holdaway *et al.*, 1996). With the disbandment of the Milk Marketing Board (MMB) in 1994, recording of SCC at a national level has ceased. The introduction of individual cow SCC recordings by milk purchasers now means that approximately 80% of dairy cows in the UK are recorded in this way (Mrode *et al.*, 1998). Introduction of the EU hygiene directive 92/46 in January 1998, set bulk milk somatic cell counts (BMSCC) at a maximum of 400,000 cells/ml and set raw milk total bacterial count (TBC) at a maximum of 100,000 bacteria/ml (Baines, 1996). On implementation of the new legislation, problems were forecast for the 10% of dairy farmers that had BMSCC consistently over 400,000 cells/ml (Logue *et al.*, 1995).

Enumeration of somatic cells in the milk of mastitic cows may give an indication of the type of pathogen causing the elevated levels. The number of somatic cells in a healthy gland is less than 100,000 cells/ml of milk. In subclinical mastitis, this figure may rise to approximately 500,000-1,000,000 cells/ml. In chronic intramammary infection (IMI), SCC may rise to approximately 4,000,000 cells/ml of milk. A chronic IMI of this nature can be caused by *Staphylococcus aureus* infection, while an IMI due to infection with coliforms may result in SCC in excess of 20,000,000 cells/ml of milk. The large increase in SCC due to infection by coliforms can often go undetected in a single milk sample due to the transient nature of infection caused by this pathogen (Hillerton, 1996). Successful treatment of mastitis has concentrated on elimination of clinical signs rather than bacteriological clearance from the mammary gland. The lack of complete bacteriological cure allows development of chronic IMI in some cases. Identification of the factors that contribute to the incidence of mastitis helps in the development of effective control measures (Fox *et al.*, 1995).

Mastitis has severe economic and welfare implications for the dairy industry, and is noted as a major cause of mortality in adult dairy cows (Menzies *et al.*, 1995). The estimated average cost of a clinical case of mastitis is between £182-218 due to discarded milk, purchasing of antibiotics and veterinary bills (Kossaibati and Esslemont, 1997). With an average of 40 clinical cases per herd per annum, the annual bill for the United Kingdom has been estimated at approximately two hundred million pounds (Hillerton, 1998).

The introduction of the Five Point Plan to dairy farms was designed to reduce the incidence of clinical mastitis (Dodd *et al.*, 1969). The Five Point Plan suggested 1) annual checks on milking machinery, to prevent damage to the teat ends, 2) post milking teat dipping, to provide a protective barrier to the teat whilst the sphincter is still open, 3) correct use of dry cow therapy between calvings, 4) correct treatment for individual cases of mastitis and detailed recordings of cases and 5) culling of persistently infected animals that may jeopardise the health of the whole herd.

At present the breeding of dairy cattle worldwide is predominantly based on profitability, which focuses on selection based on production traits. Unfortunately the genetic correlation between production traits, for example milk yield, and subclinical mastitis is

unfavourable, indicating that as milk yield increases, so does the incidence of mastitis (Mrode and Swanson, 1996; Biochard and Rupp, 1997). Inclusion of SCC into current breeding programmes for dairy cows in addition to production traits, type traits and estimated longevity has been introduced recently (McGuirk, 1998). As a result, sires may now be selected that will result in their daughters having improved udder health through reduced SCC. The choice of SCC for inclusion into the current breeding programmes was due to SCC being reported as the most suitable single trait for the indirect selection for mastitis resistance, with a strong positive correlation (r=0.7) between mastitis and SCC (Phillpsson *et al.*, 1995; Mrode and Swanson, 1996).

1.2 Mastitis Pathogens

Repeated isolation of *Streptococcus agalactiae* and *S. aureus* from cases of mastitis made these the main bacterial pathogens responsible for IMI in the UK dairy herds during the 1940's and 50's (Booth, 1997). Since then the types of pathogen isolated from cases of mastitis has changed with a lower incidence of contagious mastitis, an increase in the incidence of environmental mastitis mostly due to coliforms and the eradication of *S. agalactiae* from well managed dairy herds (Hillerton, 1998). Mastitis in dairy herds is still attributed mostly to the pathogens *Escherichia coli, Streptococcus uberis* and *S. aureus*. Bacterial pathogens can be classified broadly into categories depending on the type of mastitis they cause, namely; environmental, summer or contagious (Blowey, 1990; Smith and Hogan, 1993).

1.2.1 Environmental mastitis

The incidence of environmental mastitis in dairy herds has increased as cases attributable to contagious pathogens has reduced (Erskine *et al.*, 1988; Schukken *et al.*, 1991). Unlike the contagious pathogens, the transmission of environmental pathogens from cow to cow during milking is rare (Eberhart *et al.*, 1979). Faecal contamination has been identified as the major reservoir of environmental pathogens (Blowey, 1990), as well as bedding and soil (Risco *et al.*, 1999).

On average, 1% of quarters of dairy cows will suffer from an IMI due to Gram negative bacteria, in comparison to approximately 35-50% of quarters due to Gram positive bacteria, (Paape *et al.*, 1986). Economically, most loss associated with environmental cases of mastitis is as a result of a reduction in milk production, with milk being discarded during infection, and a lack of response to treatment resulting in premature culling of animals (Smith and Hogan, 1993).

Treatment of environmental mastitis is complex, the short duration of the infection (Todhunter *et al.*, 1991) and the high spontaneous cure rate (Bramley and Dodd, 1984), which has been reported as high as 70% (Cravens, 1987) makes antibiotic treatment inefficient. The use of pre-milking and post-milking teat dipping for prevention of environmental cases has proved largely ineffective (Wesen and Shultz, 1970). However it has been reported that after experimental challenge with *S. uberis*, there was a 67% reduction in new infections with pre-dipping compared to dry wiping (Rasmussen *et al.*, 1991). As faecal contamination and bedding represent major reservoirs for environmental pathogens, as a preventative measure, post milking, cows should be prevented from returning directly to the stalls. At this time the teat canal is still open and is, therefore particularly vulnerable to bacterial penetration.

Escherichia coli, *S. uberis*, *Pseudomonas* species, *Bacillus* species and yeasts are known to cause environmental mastitis (Todhunter *et al.*, 1991).

1.2.1.1 *Escherichia coli*

Escherichia coli infection normally results in clinical mastitis in dairy cows. Signs associated with *E. coli* mastitis can be classified as local, systemic, or toxic. Toxic mastitis accounts for between 10-15% of IMI caused by coliforms, with a survival rate of approximately 50% irrespective of the treatment given (Green *et al.*, 1997).

Escherichia coli secretes lipopolysaccharide, an endotoxin, as a virulence factor during infection of the mammary gland (Carroll *et al.*, 1964). Lipopolysaccharide induces endotoxin shock syndrome, triggering the complement cascade, resulting in the development of a non-specific immune response (van Miert, 1991). Production of

endotoxin can result in the endogenous release of the hormone prostaglandin F2 (PGF2), (Smith, 1986). At certain levels PGF2 can result in abortion in dairy cattle through luteolysis (Giri *et al.*, 1991) and stimulation of uterine contractions.

A reduction in national BMSCC was probably mostly due to a reduction in the prevalence of contagious mastitis however these herds still have a high incidence of clinical mastitis due to environmental pathogens (Oliver and Sordillo, 1988; Schukken *et al.*, 1990; Lam *et al.*, 1997). The increase in clinical mastitis incidence was reported to relate directly to impaired defence mechanisms in the cow (Kremer *et al.*, 1993; van Werven, 1999). As somatic cells are present within the mammary gland as a defence against invading pathogens, a very low SCC may predispose a cow to a higher risk of clinical mastitis (Suriyasathaporn *et al.*, 2000). Contamination from bedding, damaged teats (Schukken *et al.*, 1991), nutritional deficiencies in vitamin E and selenium (Ndiweni and Finch, 1996) and the dry period have been implicated as causative factors in the incidence of *E. coli* mastitis (Erskine *et al.*, 1988).

The prevalence of cases of *E. coli* mastitis is lower than that associated with *S. aureus* incidence. The short duration of an *E. coli* infection results in transient increases in SCC, in comparison with the long duration of *S. aureus* infections resulting in chronic infection and persistently high SCC. An IMI caused by an environmental pathogen may be detected over a time period of between 2000-4000 milkings (Eberhart, 1977) compared to an IMI caused by a contagious pathogen which has a far greater chance of being detected by milking due to a persistently elevated SCC.

1.2.2 Summer mastitis

Relatively little is known about the transmission of pathogens and the causative factors that result in summer mastitis (Hillerton *et al.*, 1992). Cases of summer mastitis are related to the activity of the fly *Hydrotaea irritans* (Madsen *et al.*, 1991). Intramammary infections, classed as summer mastitis are prominent in dry cows and heifers during the summer months (Egan, 1990). The number of cases of summer mastitis are generally higher in younger animals (Hillerton, 1987), and have been reported to be in the range of 39-54% in England and Wales (Berry, 1998).

Characteristics of summer mastitis include acute clinical signs (Hirvonen *et al.*, 1994), with a rancid smell from the gland caused by a purulent secretion (Bouman and Booth, 1992). Signs can include toxaemia i.e. fever, depression, loss of appetite, and abortion, and in some cases, mortality (Webster, 1993). The impact of summer mastitis on the dairy herd is severe as infected quarters may be lost permanently in terms of milk production (Schalm *et al.*, 1971). Cases of summer mastitis in calving heifers is most detrimental as during the first pregnancy the mammary gland undergoes an exponential phase of growth and development that is important for milk production and subsequent lactations (Nickerson *et al.*, 1992).

Treatment of summer mastitis has focused on the correct application of dry cow therapy, which has been reported to dramatically reduce its incidence. Effective fly control, adequate treatment of injured teats, and dry cow therapy have also been recommended as preventative measures for summer mastitis (Farm Animal Welfare Council, 1997).

Actinomyces pyogenes, Peptostreptococcus indolicus and *Fusobacterium necrophorum* have been isolated from cases of summer mastitis (Pyorala *et al*, 1992). Experimental infection with these pathogens resulted in an acute response characteristic of summer mastitis (Vecht *et al.*, 1987). *Streptococcus dysgalactiae* has also been isolated from cases of summer mastitis (Egan, 1986).

1.2.3 Contagious Mastitis

Within the dairy herd, transmission of contagious pathogens occurs primarily from cow to cow during routine milking (Bramley and Dodd, 1984). Hygiene within the dairy parlour should be meticulous. Dirty cloths used to wipe the teats prior to attachment of the clusters, dirty hands of dairy workers moving among animals udders and dirty clusters are all potential routes of infection for pathogenic micro-organisms (Webster, 1993). Contagious pathogens are able to survive on the outer surface of the mammary gland and by their opportunistic nature result in an IMI (McDonald, 1984; Sutra and Poutrel, 1994).

Staphylococcus aureus, *S. agalactiae*, *S. dysgalactiae* and *Mycobacterium bovis* are classified as major contagious pathogens.

1.2.3.1 *Staphylococcus aureus*

Staphylococcus aureus has been classified as the most important of the contagious pathogens (Pederson *et al.*, 1981; Bramley and Dodd, 1984). *Staphylococcus aureus* is the predominant cause of subclinical mastitis (Wilson and Richards, 1980), although this bacterium is also frequently associated with clinical mastitis (Wilesmith *et al.*, 1986; Miltenburg *et al.*, 1996), especially acute and occasionally peracute forms of the disease (Reid and Wilson, 1959). See section 1.4.

1.2.3.2 *Streptococcus agalactiae*

Streptococcus agalactiae has been classified as a Lancefield group B streptococcus (Schalm *et al.*, 1971). Group B Streptococci are important pathogens of both humans and cattle (Facklam and Carey, 1985). *Streptococcus agalactiae* is an obligate udder pathogen and, therefore, for survival, must remain within the mammary gland environment (Schalm *et al.*, 1971; Philpot, 1975; Blowey, 1990). As with *S. aureus* IMI, *S. agalactiae* has been reported as a cause of clinical mastitis (Risco *et al.*, 1999) but has also been reported as the causative agent in cases of subclinical mastitis. *Streptococcus agalactiae* results in a low-grade persistent infection with a low self-cure rate (Farnsworth, 1987). Lactic acid produced by *S. agalactiae* results in damage to the secretory tissue within the mammary gland (MacDonald, 1984). Variation among strains of *S. agalactiae* creates different levels of virulence, attributed to the ability of the bacteria to adhere to the mammary gland epithelium (Jain, 1979).

Streptococcus agalactiae has, through successful antibiotic treatment, been eradicated from well-managed dairy herds over the past half century (Shpigel, 1994). Many penicillin-based antimicrobials are still effective in the treatment of *S. agalactiae*, for example Nafpenzal™ and Synulox™ (The Veterinary Formulary: Handbook of Medicines used in Veterinary Practice). Aminoglycosides, which inhibit protein synthesis and possess bacteriostatic properties, are also effective in the treatment of *S. agalactiae*

(Hyatt *et al.*, 1995). A comparison between the available systemic and intramammary treatments, indicated that systemic treatment was no more efficient in eliminating IMI (Tyler *et al.*, 1992). Production of a vaccine directed against *S. agalactiae* has been largely ineffective. Vaccination with formalin-fixed *S. agalactiae* provided no protection against subsequent challenge with the bacteria (Yancey, 1993: Giraudo *et al.*, 1997).

Prevention of *S. agalactiae* mastitis by using pre-milking and post-milking teat dipping has received mixed reviews. Some studies report no reduction in the number of cases of *S. agalactiae* mastitis when pre-milking and post-milking teat dipping was administered (Bartlett *et al.*, 1992). In contrast, others reported that if used correctly within well-managed herds, teat dipping has reduced but not eliminated *S. agalactiae* IMI (Sischo *et al.*, 1993).

1.2.3.3 *Streptococcus dysgalactiae*

Transmission of *S. dysgalactiae* can occur during milking, however environmental sources of infection have also been reported (Prescott and Baggott, 1988; Risco *et al.*, 1999). Intramammary infection with *S. dysgalactiae* can result in cases of clinical mastitis (Todhunter *et al.*, 1995; Waage *et al.*, 1999). *Streptococcus dysgalactiae* is most commonly isolated from cows during the dry period and from heifers (Thomas *et al.*, 1987; Bramley and Dodd, 1984). The sites of primary isolation of *S. dysgalactiae* are from skin lesions of the teat and from within the mammary gland (McDonald, 1984; Blowey, 1990). The prevalence of *S. dysgalactiae* in heifers suggests the existence of a reservoir other than the mammary gland, as these animals have yet to be exposed to the milking parlour environment, which may result in transmission of pathogens between animals (Aarestrup and Jensen, 1996).

Establishment of an IMI with *S. dysgalactiae* involves adherence of the bacteria to the mammary gland epithelium and to extracellular matrix proteins *in vitro* (Filippsen *et al.*, 1990; Calvinho *et al.*, 1996). An experimental infection in goats showed that *S. dysgalactiae* penetrated the alveolar epithelium, migrating to the interacinar tissue (Pattison and Smith, 1953), enabling the bacteria to evade the host's immune system (Calvinho and Oliver, 1998).

Effective treatment of *S. dysgalactiae* has been shown with dry cow therapy, penicillin and cephalosporins (Tyler *et al.*, 1992), however treatment with gentamicin and streptomycin has been ineffective due to antibiotic resistance (McDonald and McDonald, 1976). *Streptococcus dysgalactiae* is considered to be less contagious than *S. agalactiae.*

1.3 Genus *Staphylococcus*

Staphylococci are spherical bacteria with a diameter range of 0.5-1.5μm (Bisping and Amstberg, 1988). They are pyogenic, invasive, micro-organisms capable of causing diseases of the skin i.e. boils and impetigo; the central nervous system i.e. brain or epidural abscesses and the musculoskeletal system i.e. osteomyelitis (Schaechter, 1993). Staphylococcal species can cause mastitis in cattle, exudative epidermitis in swine and staphylococcosis in poultry (Anderson, 1983). Coagulase producing staphylococci are regarded as more pathogenic than non-coagulase producing strains and those staphylococci which produce carotenoid are regarded as more invasive than non-pigmented strains (Schaechter, 1993).

1.3.1 Staphylococcal Virulence Factors

The production of virulence factors by bacteria help to establish the infection within the host as well as improving the bacteria's chances of survival (Forsgren, 1972; Yokomzo and Igarashi, 1991). Staphylococcal virulence factors possess a variety of functions that contribute to their pathogenicity (Sutra and Poutrel, 1994), (Table 1).

VIRULENCE FACTOR	EFFECT
LEUKOCIDIN[a,b]	•destruction / inhibition of white blood cells
β-HEMOLYSINS	•destruction / inhibition of red blood cells
COAGULASE[c]	•coagulation of plasma therefore poor penetration by phagocytes
CATALASE	•reduction in killing by neutrophils
HYALURONIDASE	•destruction of hyaluronic acid between cells which results in further spreading
β-LACTAMASE	•destruction of β-lactam rings
DNAse	•destruction of DNA
LIPASES	•destruction of lipids
PROTEINASES	•destruction of proteins
PROTEIN A[d]	•activation of classical complement pathway in the absence of adherence to antibody
PEPTIDOGLYCAN[e, f]	•complement activation
TEICHOIC ACID	•complement activation •adherence to mucosal cells
α TOXIN[g, h]	•binds to cell membrane resulting in hexameric pores •emptying of cytoplasmic components •promotion of bacterial growth
β TOXIN[h, i]	•oedema and influx of neutrophils •promotion of bacterial growth

Table 1: Virulence factors associated with staphylococci: Loeffler and Norcross, 1987; b) Soboll, 1971; c) Kirby, 1944; d) Postle *et al.*, 1978; e) Kowalski and Berman, 1971; f) Adlam and Easmon, 1983; g) Bhakdi and Tranum-Jensen, 1991; h) Sutra and Poutrel, 1994; i) Ward *et al.*, 1979.

1.4 *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive, catalase producing, coccus. The production of coagulase allows identification of *S. aureus* from other staphylococci. Different biotypes of *S. aureus* have evolved and adapted for survival in different host species (Aarestrup *et al.*, 1995). Biotype A is associated with humans, B with pigs and poultry, C with cattle (Matsunga *et al.*, 1993) and D with hares. These groups are not mutually exclusive as biotype A has been reported to be able to colonise the skin of cattle (Hajek and Marsalek, 1971).

Staphylococcus aureus is the predominant cause of subclinical mastitis (Wilson and Richards, 1980), where SCC acts as an indicator of infection. Persistently elevated SCC indicates chronic infection characteristic of *S. aureus* (Holdaway *et al.*, 1996). *Staphylococcus aureus* is also frequently associated with clinical mastitis (Wilesmith *et al.*, 1986; Miltenburg *et al.*, 1996), especially peracute and acute mastitis (Reid and Wilson, 1959). Signs of peracute mastitis are characterised by systemic upset including, pyrexia, anorexia and grossly abnormal milk, which resembles bloodstained serous fluid (Webster, 1993).

1.4.1 Virulence Factors associated with *Staphylococcus aureus*

The route of infection has been reported to effect the development of an IMI. An intramammary infection caused by a strain of *S. aureus* present on the outer skin of the mammary gland was shown to require large numbers of bacteria to migrate into the mammary gland to develop an inflammatory response (Blowey *et al.*, 1992). Inoculation of bacteria directly into the gland requires far fewer numbers to develop an inflammatory response (Anderson, 1986). Production of extracellular substances such as exotoxins and enzymes, contribute to the pathogenicity of *S. aureus* (Kotzin *et al.*, 1993; Lee, 1996). Staphylococcal enterotoxins (SE) and Toxic Shock Syndrome Toxin-1 (TSST1) are extracellular metabolites produced during bacterial growth (Czop and Bergdoll, 1974) and secreted by certain strains of *S. aureus* (Yokomizo *et al.*, 1995). These SE can be subdivided into SEA, SEB, SED, SEE and SEC. SEC is further divided into subtypes SEC_1, SEC_2 and SEC_3 due to serological differences (Kenny *et al.*, 1993). Recently,

three new SE have been identified: SEG, SEH and SEI (Munson *et al.*, 1998; Li *et al.*, 1999). Amino acid homology divides the enterotoxins into two groups, SEA, SED and SEE in one with SEB and SEC_{1-3} in another (Al-Daccak *et al.*, 1998). Staphylococcal enterotoxins can cause food poisoning in humans (Miller *et al.*, 1978) and TSST1 can result in tampon related toxic shock (Todd *et al.*, 1978). Strains of *S. aureus* producing both SE and TSST1 have been identified in bovine mastitis (Yokomizo and Igarashi, 1991). The estimates of enterotoxigenic strains of *S. aureus* in ruminants has been reported previously as in the range of 4-19% of isolates (Olsen *et al.*, 1970; Harvey and Gilmour, 1985; Abbar *et al.*, 1986; Orden *et al.*, 1992). Bovine *S. aureus* isolates are reported to frequently produce more than one toxin, especially SEC, SED and TSST-1 (Ferens *et al.*, 1998). Enterotoxins are extremely heat stable, thus persisting potentially during the pasteurisation process. Milk heat treated for 15 minutes at between 77-82°C, condensed and finally spray dried showed detectable levels of enterotoxin on examination ten years later (Batish *et al.*, 1990). Others have also shown detectable amount of SEA and SED post-pasteurisation (Tatini *et al.*, 1971).

1.4.2 Antibiotic therapy

Treatment of *S. aureus* IMI is problematic (Owens and Watts, 1987). It has been reported that despite the number of studies carried out to investigate the problems associated with antimicrobial therapy for *S. aureus* infection, little has been resolved (Ziv *et al.*, 1995). Treatment of IMI due to *S. aureus* with dry cow therapy does not always result in elimination of the pathogen from the mammary gland (Nickerson *et al.*, 1999), although some success has been reported (Clegg *et al.*, 1975; Cummins and McCaskey, 1987; Sol *et al.*, 1994). The lack of success in the treatment of IMI caused by *S. aureus* may be as a result of fibrosis, and abscessation of the infected tissue (Nickerson and Heald, 1981). The tough outer layer of the abscess acts as an impervious barrier preventing the infiltration of phagocytes and penetration of antibiotics (Sears and Heider, 1981; Makaya, 1996). The ability of *S. aureus* to persist intracellularly within macrophages, neutrophils and epithelial cells (Sandholm and Mattila, 1985) also helps to prevent destruction by the host's immune system. Production of β-lactamase by *S. aureus* can result in antibiotic resistance, thus enabling the bacteria to persist within the hosts environment (Owens, 1988; Aarestrup and Jensen, 1998). The antibiotics administered in dry cow therapy are

designed to be in contact with the bacteria for prolonged periods, possibly encouraging the development of antibiotic-resistant strains (Osteras *et al.*, 1999). Levels of antibiotic resistant *S. aureus* are increasing, with estimates suggesting that >70% of human *S. aureus* strains are resistant (Wiedemann and Kresken, 1984; Cormican and Jones, 1996), and approximately 12% of *S. aureus* strains associated with bovine mastitis showing resistance (National Veterinary Laboratories, 1996). However in the Scandinavian countries where administration of antibiotic treatment is only performed by the veterinarian, approximately 94% of *S. aureus* strains are still sensitive to penicillin *in vitro* (Nilsson *et al.*, 1997).

1.4.3 Antibiotic Residues

Antibiotic residues in milk and meat products are concerns for the respective industries. Allergic reactions to antibiotics such as penicillin can occur at very low doses (Zimmerman, 1959), which make antibiotic residues unacceptable (Ziv *et al.*, 1973). Contamination of processed dairy products with antibiotics has been reported to reduce the quality of these products (McEwan *et al.*, 1991). Milk contaminated by antibiotics (>0.005 international units, IU), results in a purchase price of 1p/l rather than the normal value of 18p/l for uncontaminated milk (Beard, personal communication). Antibiotic contamination results mainly from failure to withhold the milk of animals receiving antibiotic treatment or receiving dry cow therapy (Booth and Harding, 1986). Antibiotic failures have also been suggested as a result of natural inhibitors present in the milk i.e. colostrum and lactoferrin (VanEenennaam *et al.*, 1993) creating false positives.

1.4.4 Vaccination

Vaccine development for prevention of *S. aureus* has proved largely ineffective. Many *S. aureus* bacterins are available, however, their efficacy has not been proven by field trials or substantiated in publications. One commercial bacterin was shown to increase the number of spontaneous cures rate and significantly reduce the SCC of infected animals (Pankey *et al.*, 1985), while the incidence of new IMI and clinical mastitis remained unchanged. Vaccines based on staphylococcal toxoids and pseudocapsular materials have been shown to provide protection against clinical mastitis under experimental challenge

conditions (Watson, 1992). The reduction in clinical mastitis and new IMI following vaccination was also confirmed by others (Nickerson *et al.*, 1993; Watson *et al.*, 1996).

1.5 Anatomical Defences of the Mammary Gland

The outer epithelial layer of the teat is the mammary glands first line of defence against opportunistic pathogens. This layer of tough dead cells, comprised of stratified squamous epithelium, is impregnated with keratin, a waxy substance that possesses bacteriostatic properties. These bacteriostatic properties are esterified and non-esterified fatty acids, including palmitoleic acid and linoleic acid (Bitman *et al.*, 1988). The waxy structure of keratin prevents pathogens migrating from the outer layer of skin into the gland cistern, which may result in an IMI (Hibbitt *et al.*, 1969). If the external layer of skin is compromised i.e. chapped or cracked, colonisation of inner dermal layers by opportunistic pathogens could result in an IMI. The streak canal is also lined with squamous epithelium containing keratin. Studies have shown removal of teat canal keratin resulted in an 84% infection rate when challenged via the mammary gland with *S. agalactiae*, compared with a 27% infection rate in control quarters containing keratin (Schultze and Bramley, 1985). Cationic proteins in the streak canal disrupt the osmotic balance of invading bacteria's cell wall resulting in cell lysis. The sphincter muscle at the teat end creates an impervious barrier against potential pathogens. During milking when the sphincter muscle is relaxed to allow milk ejaculation, it is at this time that the streak canal is vulnerable to penetration by opportunistic pathogens. Implementation of pre-and post-milking teat dipping, to remove any micro-organisms prior to and after milking from the surface of the teat may help prevent colonisation of the udder by pathogens at this vulnerable time (Dodd *et al.*, 1969; Pankey *et al.*, 1983; Pankey, 1989).

1.6 Dietary defence

Basic dairy management can help protect the mammary gland if essential nutritional elements are included in the cows diet. Vitamin A and E, selenium, β-carotene, zinc and copper all contribute to the maintenance of the dairy cow's immune system.

1.6.1 Vitamin E and Selenium

Deficiencies in selenium and vitamin E reported in rats and goats have been shown to result in impairment of neutrophil bactericidal activities, and reduction in the production of chemotactic properties by macrophages (Gairola and Tai, 1985; Aziz and Klesius, 1986).

Severe vitamin E and selenium deficiencies are associated primarily with calves and borderline deficiencies occur more frequently in adult cattle. Selenium is found naturally within the soil, therefore uptake by the animal is indirect (McKenzie *et al.*, 1998). Enhancement of neutrophil migration, production of chemotactic factors and improved T cell proliferation induced by mitogenic stimuli have been reported in cows on vitamin E and selenium supplemented diets (Ndiweni and Finch, 1999). Both vitamin E and selenium have been shown to protect against hydrogen peroxide and oxygen free radicals that result in damage to healthy secretory tissue (Hogan *et al.*, 1993). A reduction in lymphocyte transformation has been observed in sheep deficient in vitamin E and selenium (Turner and Finch, 1990) and cattle (Cipriano *et al.*, 1982), as well as an increase in incidence, severity and duration of IMI (Smith *et al.*, 1984; Weiss *et al.*, 1990; Hogan *et al.*, 1993).

1.6.2 Vitamin A and Beta-carotene

Supplementation with vitamin A and a precursor, β-carotene, during the periparturient period has been shown to improve mammary gland health and is essential for the growth and maintenance of ruminant epithelial cells (Daniel *et al.*, 1991). Improvement of phagocytosis and intracellular killing as a result of vitamin A supplementation is limited (Tjoelker *et al.*, 1988a). A reduction in blastogenic T cell response has been reported in animals deficient in vitamin A, whilst B cell responses remain unaltered (Tjoelker *et al.*, 1988b).

Beta-carotene, the precursor for vitamin A, has been shown to increase T helper cell numbers by 30% whilst T cytotoxic and T suppressor levels remained unchanged

(Alexander *et al.*, 1985). Improved neutrophil function due to β-carotene has also been reported, as shown by improved bactericidal activity prior to drying off (Chew, 1993).

1.6.3 Zinc

Low levels of zinc in dietary nutrition have been shown to reduce the overall T cell numbers (Wirth *et al.*, 1984). A zinc deficit may alter keratin composition present within the teat canal, subsequently rendering the udder vulnerable to infection, as well as increasing SCC.

1.6.4 Copper

Impairment of the immune system, due to a lack of copper, results in clinical signs associated with dietary deficiency (Koller *et al.*, 1987), elevated incidences of IMI, due to a reduction in response to acute inflammation (Harmon *et al*, 1994) and a reduction in intracellular killing (Babu and Failla, 1990).

1.7 Humoral Immunity – non specific

1.7.1 Lactoferrin

Lactoferrin is an iron binding protein found in milk (Gordon *et al.*, 1962) and in the granules of neutrophils (Masson and Heremans, 1966). This chelating protein starves bacteria such as staphylococci and coliforms of iron, an essential element for bacterial growth (Bishop *et al.*, 1976). Lactoferrin has also been shown to work in conjunction with IgG_1 in the inhibition of *E. coli* and *Klebsiella pneumoniae* (Oliver and Bushe, 1987). In a normal, healthy, gland lactoferrin concentrations are relatively low, recorded as between 0.1-0.3mg/ml of milk (Oliver and Smith, 1982). Levels are seen to rise at drying off and reach 20-30mg/ml of milk between three to 30 days involution (Harmon *et al.*, 1975). During an IMI levels of lactoferrin in milk are elevated, suggesting a possible role in the defence of the udder, which may arise through modulation and control of lymphocytes, macrophages and neutrophil function (Sanchez and Watts, 1999).

1.7.2 Complement

Complement is a set of approximately 30 heat labile proteins capable of lysing micro-organisms and cells (Klein and Horejsi, 1999), with receptors found on the surfaces of neutrophils, eosinophils, macrophages, monocytes and gastrointestinal epithelial cells. In a normal healthy gland, complement levels are relatively low, with higher levels reported in colostrum. Levels of complement have been reported as significantly increased during and IMI with the production of Protein A by *S. aureus* shown to activate the classical complement cascade. The antibacterial properties of complement are thought to have a minimal role within the udder (Reiter *et al.*, 1983).

1.7.3 Lysozyme

The enzyme, lysozyme, can cleave peptidoglycan from the cell wall of Gram positive and Gram negative bacteria (Reiter, 1978). Digestion of the peptidoglycan may occur immediately, resulting in destruction of the micro-organism, or alternatively, additional enzymes maybe required for the destruction of the outer cell wall to expose the underlying peptidoglycan. It has been suggested that lysozyme may enhance lactoferrin binding, contributing to the defence of the mammary gland against pathogenic organisms. The concentration of lysozyme in the mammary gland of cows has been reported as 300 times lower than those of human breast milk (Chandran *et al.*, 1964), which may suggest that cattle have an ineffective concentration of lysozyme to deal with invading micro-organisms.

1.7.4 Lactoperoxidase thiocyanate hydrogen peroxide system

The lactoperoxidase thiocyanate hydrogen peroxide system has been shown to elicit bacterostatic effects on Gram positive (*S. aureus*) and bactericidal effects on Gram negative bacteria (coliforms), (Outteridge and Lees, 1988). Neutrophils produce large amounts of hydrogen peroxide during the respiratory burst due to alterations in the plasma membrane (Paape *et al.*, 1991). In a healthy animal, levels of lactoperoxidase are low within the udder, and if the animal is suffering from nutritional stress, a further reduction has been observed in the levels of this enzyme (Sordillo *et al.*, 1997).

1.7.5 Cytokines

Cytokines are important in the specific and non-specific humoral immune system in cattle. These proteins have a potent biological effect at picomolar to nanomolar levels, and are produced by a variety of immune and non-immune cells. The primary role of human peripheral blood monocytes involves the regulation of the synthesis and secretion of biologically active molecules, such as cytokines (Alderson *et al.*, 1991). Their interactions as individual cytokines and in combination with others can be synergistic, additive or antagonistic, spanning multiple cell targets (Lawman *et al.*, 1987), with certain cytokines stimulating cells directly (Tosato and Jones, 1990; Cheung *et al.*, 1991). There have been greater than 30 bovine cytokines identified by their regulatory activities, many of which affect the cells present within the mammary gland (Table 2), (Sordillo *et al.*, 1997). To date there have been more than 80 cytokines characterised in humans (Oppenheim, 1994).

CYTOKINE	EFFECT
G-CSF[a]	•increase in milk somatic cell counts
GM-CSF[b]	•enhanced neutrophil activity
IFN-γ[c]	•enhanced neutrophil phagocytosis and bacteriostatic/cidal activity
IL-1[d,e]	•increase in neutrophil numbers •no alteration in mononuclear cell proliferation
IL-2[f, g]	•increase in plasma cell numbers •enhanced lymphocyte cytotoxicity and bacteriostatic/cidal activity

Table 2: Cytokines and their various effects on the immune system; a) Kehrli *et al.*, 1991; b) Sordillo *et al.*, 1992; c) Sordillo *et al.*, 1991; d) Daley *et al.*, 1991; e) Torre *et al.*, 1992; f) Nickerson *et al.*, 1989; g) Sordillo *et al.*, 1991

1.7.5.1 Interleukin-1

Interleukin-1 (IL-1) is an inflammatory cytokine, that has been reported to contribute to the amplification of the host's inflammatory response and to aid in the restoration of damaged tissue (Neta and Oppenheim, 1988). Interleukin-1 is secreted by a variety of nucleated cells and along with IL-6, tumour necrosis factor (TNF)-α, and TNF-β, is highly pleiotropic (Dinarello, 1989; Beutler and Cerami, 1989; Hirano *et al.*, 1990). Intramammary infusion with recombinant (rb) bovine (Bo) IL-1β was reported to increase SCC and enhance the production of superoxide by milk neutrophils (Daley *et al.*, 1993), although an improvement in phagocytosis was not evident. Elevated levels of IL-1 have been reported in response to intramammary infusion with endotoxin (Shuster *et al.*, 1993), suggesting that the production of this cytokine precedes, or is concurrent with, leucocytic influx and systemic response.

1.7.5.2 Interleukin-2

Interleukin-2 (IL-2) is the best characterised of the bovine cytokines. Primarily produced by T helper cells, IL-2 effector function can lead to clonal expansion of T cells, growth and development of the B cell population, activation of natural killer (NK) cells (Swain, 1991) and induction of T cytotoxic activity (Caligiuri *et al.*, 1990). Interleukin 2 can initiate an increase in thymocyte proliferation (Lawman *et al.*, 1987) as well as the enhancement of bactericidal activity, MHC II expression and neutrophil infiltration (Sordillo *et al.*, 1991). Colostrum collected from the mammary gland during the final week of gestation indicated very low levels of IL-2 (Sordillo *et al.*, 1992), correlating with the increased susceptibility of the mammary gland to infection during this time. Initially difficulty in purifying large quantities of bovine IL-2 hindered studies looking at the potential use of this cytokine in treating mastitis, however the production of rbIL-2 has since removed these constrictions (Cerretti *et al.*, 1986; Price *et al.*, 1987). Research has shown that treatment of milk mononuclear cells with rbIL-2 enhanced the proliferative response induced by mitogenic stimulus at a concentration that when challenged previously in the absence of rbIL-2 was not sufficient to induce a proliferative response (Torre *et al.*, 1992). *In vivo* studies administering rbIL-2 by the intramammary route via surgically implanted mini osmotic pumps resulted in an increase in the total

number of lymphocytes, macrophages and IgG bearing plasma cells (Nickerson *et al.*, 1989). The data from the previously published studies suggests the potential of using rbIL-2 as a therapeutic agent in the prevention or reduction of IMI (Torre *et al.*, 1992).

1.7.5.3 Granulocyte–Colony Stimulating Factor

Granulocyte–Colony Stimulating Factor (G-CSF) is one of a group of colony stimulating factors, required for development, differentiation and survival of phagocytes and granulocytes (Sordillo *et al.*, 1997). The primary secretory cells of G-CSF include monocytes, fibroblasts and endothelial cells (Fukunaga *et al.*, 1990). Administration of rbG-CSF subcutaneously to cows led to a two to five fold augmentation in the number of peripheral blood neutrophils (Kehrli *et al.*, 1991).

1.7.5.4 Granulocyte Macrophage–Colony Stimulating Factor

Granulocyte Macrophage–Colony Stimulating Factor (GM-CSF) has been reported to induce the development of granulocytes and macrophages from hematopoietic stem cells (Metcalf *et al.*, 1985). The primary secretory cells of GM-CSF include macrophages, T lymphocytes, fibroblasts and endothelial cells (Gearing *et al.*, 1989). Intramammary infusion with rbGM-CSF did not result in an influx of cells into the mammary gland, however it was noted that neutrophils were stimulated to produce superoxide via the respiratory burst (Daley *et al.*, 1993; Tao *et al.*, 1993). Administration of rbGM-CSF to cattle has been shown to significantly enhance the bactericidal and chemotactic properties of neutrophils in peripheral blood and the mammary gland (Sordillo *et al.*, 1992). Recombinant GM-CSF has also been reported previously to prevent apoptosis of bovine peripheral blood neutrophils during *in vitro* culture (Colotta *et al.*, 1992). The ability of rbGM-CSF to stimulate peripheral blood and mammary gland neutrophils and enhancement of the bactericidal and chemotactic properties suggests treatment with this cytokine may induce a more efficient immune response towards invading pathogens (Sordillo *et al.*, 1992).

1.7.5.5 Interferon-γ

Interferons (IFN) were discovered in the 1950s, and are characterised by the ability to induce an antiviral status *in vitro*. Interferon-γ belongs to the second class of IFN, and is derived from T lymphocytes and NK cells (Aguet *et al.*, 1988). This cytokine has many immunomodulatory properties and has been shown to improve antibody dependent cell cytotoxicity, NK activity and T lymphocyte cytotoxic ability. A reduction in the severity of clinical signs and levels of mortality were reported in cattle challenged with Bovine Herpes Virus and subsequently treated with IFN-γ (Babuik *et al.*, 1987). Treatment of immunosuppressed cattle with IFN-γ improved neutrophil function even compared to normal non-immunosuppressed cattle (Roth and Frank, 1989). Interferon-γ has been reported to be involved in the regulation of MHC II expression on cell surfaces, as well as the regulation of complement receptors on monocytes and macrophages (Bielefeldt Ohmann *et al.*, 1986). A reversal of the suppressive effects of milk on mammary gland neutrophils was reported *in vitro* following treatment with IFN-γ (Sordillo *et al.*, 1991). The significant enhancement of neutrophil function as a result of treatment with IFN-γ may improve mammary gland defences against pathogens such as *S. aureus*.

1.8 Humoral immunity – specific

Immunoglobulins (Ig) are involved in humoral immunity and the acquired immune system. Secretion of Ig occurs primarily from B-lymphocytes that have been stimulated and have matured into antibody secreting cells. There are five isotypes of Ig namely; IgM, IgA, IgD, IgG and IgE, with IgG further subdivided into IgG_1 and IgG_2. Immunoglobulin G is the predominant isotype in the serum of cattle compared to humans where IgA predominates (Lascelles, 1979).

Ruminant neonates are born devoid of Ig acquiring the required Ig from their mother's colostrum (Howe, 1921; Aschaffenberg, 1949). The transfer of Ig from the mother's colostrum to the calf is different to humans, where Ig absorption takes place *in utero* via the placenta (Butler, 1974; Brambell, 1970). Colostral Ig is a concentrated source of serum antibodies (Dixon *et al.*, 1961), which is transferred from the maternal serum to the

colostrum, which the calves suckle (Sasaki *et al.*, 1976; Watson and Lascelles, 1973). Colostral IgM and IgA are both derived from maternal serum and synthesised locally within the mammary gland of cattle (Newby and Bourne, 1977). In humans and mice most synthesis of Ig occurs locally rather than from transportation from serum (Drife *et al.*, 1976).

The transfer of Ig from serum to the mammary gland was suggested to arise from leakage/transudation, a mechanism which would be viable during severe cases of mastitis or during the dry period, but would not be functional in a normal healthy gland (Linzell and Peaker, 1974). The single layer of cells at the periphery of the mammary gland consists of milk producing secretory cells, mainly alveoli and ducts (Mackenzie and Lascelles, 1968), which is responsible for the specific transportation of Ig to the mammary gland (Brandon *et al.*, 1971)

Levels of Ig are not constant in the mammary gland and have been reported to increase during colostrogenesis and infection (Musoke *et al.*, 1987), with concentrations in the mammary gland during parturition far exceeding those in serum (Concha *et al.*, 1980). For neonate absorption of Ig from colostrum the first 24 to 30 hours postpartum are the most critical (Deutsch and Smith, 1957). A delay in suckling can result in a reduction in Ig absorption and an increased susceptibility to infection (Gay, 1965).

The four main isotypes thought to be associated with defence against mastitis are IgG_1, IgG_2, IgA and IgM (Butler, 1974; Spiegelberg, 1974). Both IgD and IgE are present in small quantities within the mammary secretions but are not associated with protection. Immunoglobulin G_1, IgG_2 and IgM act as bacterial opsonins, improving phagocytosis by macrophages and neutrophils by direct binding of bacteria to the cell surface or via the C3b component of complement (Smits *et al.*, 1996). Immunoglobulin A provides a better defence as it has enhanced resistance to proteolytic enzymatic degradation that may enhance its survival in the mammary gland (Underdown and Dorrington, 1974). Immunoglobulin A's enhanced resistance to proteolytic enzymatic degradation may also prevent bacterial colonisation through agglutination and toxin neutralisation (Musoke *et al.*, 1987).

1.9 Cell mediated immunity

Somatic cells present in milk represent immune cells in the mammary gland and comprise neutrophils, macrophages, lymphocytes and epithelial cells. The percentages of each cell type present are dependant on a number of factors, including, stage of lactation, whether the mammary gland is healthy or infected, and if infected what degree of inflammation is present. Macrophages are the predominant cell type in the healthy mammary gland, followed by neutrophils, lymphocytes and epithelial cells (Miller *et al.*, 1991). During early and late lactation, neutrophils increase in number and the lymphocyte population decreases (McDonald and Anderson, 1981). Cell numbers present within the gland increase rapidly within hours of an IMI, rising from $<10^5$ to $>10^6$ cells per ml (Paape *et al.*, 1981). The initial response mounted by the immune system to an IMI should be quick and efficient to minimise damage and prevent establishment of a chronic infection.

1.9.1 Neutrophils

Neutrophils are part of the immune system's first line of defence against infection (Paape *et al.*, 1991), and have been shown to play an important role in cattle in the defence against *E. coli* (Hill, 1981) and *S. aureus* mastitis (Schalm, 1976). Activation of neutrophils leads to diapedesis of cells to the site of inflammation, and subsequently the destruction of pathogens via phagocytosis, enzymatic degradation and oxidative burst metabolism (Spitznagel, 1983). The ability to respond to invading pathogens varies between neutrophils present in blood and those in milk, with peripheral blood neutrophils being more effective phagocytes than mammary gland neutrophils (Kent and Newbould, 1969). The presence of cytokines at the site of inflammation has been shown to enhance neutrophil function (Steinbeck *et al.*, 1989) whereas stress (Kelley, 1980) and viral pathogens (Abramson and Mills, 1988) have been reported to impair the phagocytic properties of neutrophils. During late pregnancy, calving and early lactation cows have been shown to be affected by physical and metabolic stress (Kehrli *et al.*, 1989a). The rate of IMI increases during this period and coincides with reported immunosuppression and a reduction in bactericidal activity of neutrophils (Gilbert *et al.*, 1993). Neutrophil function is known to effect the incidence of mastitis, but has also been reported to effect the severity of the IMI (Craven *et al.*, 1985).

Temporary disruption of the epithelial cells, followed by resealing of the tight junctions, permits neutrophils to migrate from peripheral blood into the mammary gland (Lin *et al.*, 1995). Epithelial cells and tight junctions are reported to restrict neutrophil diapedesis from blood to the mammary gland (Evans *et al.*, 1983). Migration of neutrophils to the site of infection is dependant on energy resources, which are required for effective phagocytosis (Newbould, 1973). The mammary gland energy sources required for the effective phagocytosis are at their lowest when the cow is most susceptible to an IMI. A reduction in neutrophil function has also been reported due to the ingestion of fat and casein present in the milk (Paape and Guildry, 1977). To overcome the problems associated with the mammary gland a large number of neutrophils are required to tackle an IMI (Schultze and Paape, 1984). Neutrophils have been reported as contributing 90% of total leukocytes during an IMI (Sordillo *et al.*, 1987). Ideally neutrophil migration to the site of inflammation should be quick, as extended periods of diapedesis can result in damage of the mammary parenchyma tissue, which contribute to a reduction in milk yield (Sordillo and Nickerson, 1988).

1.9.2 Dendritic cells

Dendritic cells are the most potent stimulators of T cell proliferation (Steinman and Witmer, 1978). Although present in very low numbers they form an extensive network that pervades the entire body. During circulation, they acquire the capacity to process and present antigen, express MHC class II and MHC class I and co-stimulatory molecules (Engering *et al.*, 1998; Sunshine *et al.*, 1983; Hayashi *et al.*, 2000). They are morphologically distinct from other cells and lack the classical markers associated with T and B cells (Steinman and Cohn, 1973). The MAb, CC21, has been used to identify a subpopulation of PBM expressing the B cell marker and has been shown to stain cells around the follicles of the lymphoid tissue of cattle with dendritic cell morphology (Naessens and Howard, 1991). It is not known whether these dendritic cells express antigen passively or acquired from other cells as hypothesised in humans. Bacterial products i.e. LPS and inflammatory stimuli e.g. TNF induce the maturation of dendritic cells (Watts 1997b; Cella *et al.*, 1999). Maturation of the dendritic cells alters the functional state and within 10-20 hours biosynthesis of MHC class II is upregulated and

maintains a high endocytic capacity (Pieters, 2000). This ensures that dendritic cells retain a high capacity for peptide loading (Pierre *et al.*, 1997). Production of LPS and TNF also induce migration of dendritic cells towards lymphoid tissue (Adema *et al.*, 1997). Down-regulation of endocytosis and MHC class II recycling during migration ensures the peptides derived from the pathogen are not replaced as well as stabilising the half life of the dendritic cell (Cella *et al.*, 1997). This pathway of events results in potent antigen presenting cell (APC) in the lymphoid tissue where immunologically naïve T cells can be stimulated (Banchereau and Steinman, 1998).

1.10 Lymphocytes

There are two main sets of lymphocytes, namely T and B lymphocytes, with many subsets, sub-subsets and clones identified by expressed cell surface markers. These different sets and subsets enable the immune system to process different antigens from various sources (Grey and Chesnut, 1985; Cooper, 1987; von Boehmer, 1988). B-lymphocytes respond to externally presented antigen, for once the antigen becomes internalised, the antibodies produced by the B lymphocytes are largely ineffective. T lymphocytes respond to internalised antigenic peptides presented on the surface of APC, in association with MHC molecules (Swain, 1983). For an optimal immune response, both B and T lymphocytes are required (Beckmann and Levitt, 1984).

1.10.1 T lymphocytes

Lymphocytes originate from pluripotent stem cells within bone marrow. These pluripotent stem cells differentiate into lymphoid stem cells and further develop into pre-T and pre-B-lymphocytes. After differentiation from lymphoid stem cells, T cells are educated as to the differences between self and non-self antigen within the thymus, an absolute requirement for differentiation (Bill and Palmer, 1989; Shortman *et al.*, 1991; Marrack and Kappler, 1997). Children suffering from DiGeorge syndrome, lack mature T cells as a result of an athymic condition, and fail to induce a proliferative response when stimulated by mitogens (Markert *et al.*, 1998). The survival of naïve T cells present in the thymus without antigenic stimulus is dependant on the expression of MHC molecules (Sprent *et al.*, 1990; Tough and Sprent, 1994; Takeda *et al.*, 1996; Rooke *et al.*, 1997).

The MHC molecules and self peptide present in the thymus in combination result in either survival or elimination of the immature T cell (Jameson *et al.*, 1995). In mammals, puberty triggers a reduction in size of the thymus known as thymic involution (Steinmann and Mullerhermelink, 1984), which scales down the requirement for T cell education as the residual population mature and develop into memory cells. The thymus was believed to be largely ineffective by the onset of puberty, however further studies have now shown that the thymus continues to participate in lymphocyte development throughout adult life (Bertho *et al.*, 1997).

T lymphocytes can be characterised by immunological markers present on their cell surface. The T cell receptor (TCR), is a cell surface marker which has been studied extensively in mice and humans since the molecular structure was unveiled (Yanagi *et al.*, 1984; Hendrick *et al.*, 1985). The TCR is a transmembrane molecule composed of two chains linked via a disulphide bond that can be combined as αβ or γδ (Pereira *et al.*, 1997). Many similarities exist between αβ and γδ chains in function, structure and association with CD3 (Bank *et al.*, 1986; Borst *et al.*, 1987; Van Neerven *et al.*, 1990). Each T cell clone has a unique TCR that is non-covalently associated on the surface of the T cell with a complex of transmembrane polypeptides, CD3. The CD3 complex comprises three molecules γ, δ, and ε and is required for T cell development (Tanaka *et al.*, 1995), as well as assembly and efficient surface expression of the TCR (Exley *et al.*, 1995). The CD3 complex and TCR are tightly associated with zeta (ζ), a molecule composed of two identical chains. The CD3 complex is T cell specific unlike ζ, however neither bind antigen directly but rather act as signal transducers initiating signal relay to the T cell molecule once the antigen has bound to the TCR (Wegener *et al.*, 1992).

1.10.1.1 T lymphocytes expressing the αβ TCR

Once the lineage of the TCR has been established, αβ T cells develop into T helper ($CD4^+$ T cells), (Baldwin *et al.*, 1986), T cytotoxic or T suppressor cells ($CD8^+$ T cells), (Chan *et al.*, 1998), each with different regulatory immunological functions (Ellis *et al.*, 1986). Expression of CD4 and CD8 on the T cell surface strengthens the bond that exists between the T cell and the APC. This bond is important due to the low affinity of MHC-

antigenic peptide complex binding to the TCR. The $CD4^+$ T cell binds to the invariant portion of the MHC class II molecule (Glass *et al.*, 1991), and the $CD8^+$ T cell bonds to the invariant portion of the MHC class I molecule (Sordillo *et al.*, 1997). The affinity of $CD4^+$ T cells for MHC class II and $CD8^+$ T cells for MHC class I is known as MHC restriction (Zinkernagel and Doherty, 1979).

T cells expressing the CD8 cell surface marker have been identified in large numbers within the paracortex of lymph nodes, discrete Peyers Patches and periarteriolar T-dependant areas of the tonsils (Ellis *et al.*, 1986; Morrison *et al.*, 1988). $CD8^+$ T cells have also been identified in the red pulp of the spleen of young calves and in the epithelium of the gut mucosa of cattle (Parsons *et al.*, 1989; Naggi and Babiuk, 1989). Phenotypic identification of $CD8^+$ T cells can be achieved by staining with monoclonal antibodies as described in humans (Johnson, 1989) and cattle (Ellis *et al.*, 1986).

$CD8^+$ molecules have been associated with two types of regulatory T cell; T suppressor and T cytotoxic cells (VonBoehmer, 1988). T suppressor cells generate a negative feedback loop for the immune system, downregulating the responses of both T and B cells, ensuring the immune response is self-limiting and not damaging (Chin and Hay, 1980; Callard *et al.*, 1982; Cooper, 1987). T suppressor cells secrete IL-4, a cytokine associated with a Th2-type immune response, which has been shown to shift the immune response induced from a Th1-type response to a Th2-type response (Hsieh *et al.*, 1992; Mocci and Coffman, 1995). The shift in the type of Th response is carried out by differential expression of chemokine receptors on the cell surface of T suppressor cells (Kim *et al.*, 1998; Sallusto *et al.*, 1998). T cytotoxic cells have been reported to initiate the destruction of infected or altered host cells mediated by cell lysis through the release of cytotoxic granules that contain perforin monomers (Fong and Mosmann, 1990). The granules create homopolymeric pores resulting in rapid osmotic lysis of the infected cell (Liu *et al.*, 1995). T cytotoxic cells also function as effective scavengers, removing old or damaged secretory cells that may impair immune function (Taylor *et al.*, 1994).

T cells expressing the CD4 cell surface marker have been identified in the organised lymphoid tissue of the paracortex of the lymph nodes, the periarteriolar region of the spleen, and the interfollicular area of the discrete Peyers Patches and tonsils (Parsons *et*

al., 1989). $CD4^+$ T cells are also located in the non organised lymphoid tissue of the gut mucosa in the lamina propria, but unlike $CD8^+$ T cells have not been identified in the epithelium (Parsons *et al.*, 1989). $CD4^+$ T cells are classified as regulatory T cells (Fong and Mossman, 1990). Once stimulated by antigen, $CD4^+$ T cells can induce responsiveness in both T and B-lymphocytes (Sattentau and Weiss, 1988; Mazerolles *et al.*, 1991; Gaubin *et al.*, 1996). The induction of a response may result in the maturation of B cells into antibody producing plasma cells (Semple *et al.*, 1993). $CD4^+$ T cells have been reported as essential in responding to helminth infection in humans, and in directing the host's immune response towards the infectious agent (Romagnani, 1994). $CD4^+$ T cells are important in the development of a fully functional immune response, as differentiation of B cells into memory cells is usually T helper cell dependant (Rouse *et al.*, 1982).

In human and murine immune systems, T helper cells are divided into further subsets. T helper cells originate as Th0-type cells then differentiate into Th1- or Th2-type cells (Mosmann *et al.*, 1986; Cherwinski *et al.*, 1987). Characterisation of Th1-type and Th2-type cells can be achieved by their cytokine profiles (Abbas *et al.*, 1996; Seder and Paul, 1994; Mosmann *et al.*, 1986). Production of IL-12 by monocytes and macrophages, and production of INF-γ by natural killer cells, trigger differentiation of the precursor Th0-type cell to the Th1-type cell (Tam *et al.*, 1999). Interleukin-4 production from unconventional natural killer cells as well as basophils, eosinophils and mast cells, triggers differentiation of the precursor Th0-type cell to the Th2-type cell (Powrie and Coffman, 1993).

Prior to differentiation, the precursor Th0-type cell secretes IL-2, IL-3, IL-4, IFN-γ and TNF-β (Gurish *et al.*, 1992). Classically Th1-type cells produce both IL-2 and IFN-γ, which are reported to promote cellular responses induced by intracellular pathogens, in particular mycobacterial infections (Cher and Mosmann, 1987; Fong and Mosmann, 1989). This cellular response requires activation of cell mediated defence mechanisms and the induction of specific immunity. The presence of Th1-type cells has also been associated with the development of immediate hypersensitivity and allergic responses. Th2-type cells are characterised by the production of IL-4, IL-5 and IL-10 and have been

associated with the regulation of Ig isotype produced by B cells (Coffman and Carty, 1986).

There is no absolute restriction to which Th-type of response develops, however, detrimental shifts from Th1-type to a Th2-type have been reported with respect to some viral infections and allergic conditions (Mosmann and Sad, 1996; Carter and Dutton, 1996). It has been noted in patients infected with HIV, a shift from Th1-type to Th2-type has been correlated with disease progression (Clerici and Shearer, 1994). The shift from Th1-type to Th2-type has also been reported to be detrimental to those suffering from atopic disorders such as asthma (Gelfand *et al.*, 1996; Leung, 1997). Staphylococcal superantigens (SAg), which are known cytokine stimulators, have also been implicated in detrimental shifts in Th-type (Campbell *et al.*, 1999). The expression of Th1 cytokines has been reported to enhance anti-viral immunity against Hepatitis B and anti-tumour immunity against human melanoma (Van Elsas *et al.*, 1997; Chow *et al.*, 1998). Promotion of a Th1-type immune response has been suggested as beneficial in enhancement of the immune response induced by certain bacterial infections (O'Sullivan *et al.*, 1995; Greenberger *et al.*, 1996; O'Suilleanbhain *et al.*, 1996). Conversion of Th1-type cells to Th2-type cells may elicit a protective role in the down-regulation of TNF-α, preventing its overproduction and subsequent tissue damage. Interleukin-4 and IL-10, both Th2-type cytokines, are potent anti-inflammatories that can suppress the production of pro-inflammatory cytokines, therefore, reducing the risk of possible tissue damage (Zhou *et al.*, 1994; Clarke *et al.*, 1998). Production of IL-4 has been reported to modulate the balance between an inflammatory and an immune response induced by bacterial infection in pigs (Zhou *et al.*, 1994). Not all cytokine production can be classified as either Th1-type or Th2-type as production of IL-3 and GM-CSF is common to both Th-types (Gurish *et al.*, 1992).

1.10.1.2 T lymphocytes expressing the $\gamma\delta$ TCR

Expression of the cell surface markers CD4 and CD8, which act as co-receptors promoting signal transduction, do vary between $\gamma\delta$ and $\alpha\beta$ T cells (Gay *et al.*, 1987; Rudd *et al.*, 1988; Veillette *et al.*, 1989; Newell *et al.*, 1990). Expression of the cell surface markers CD4 and CD8 on $\gamma\delta$ T cells is not usual (Haas *et al.*, 1993; Zorbas and Scollay,

1993). Expression of MHC molecules is not an absolute requirement for the development of γδ T cells, and has no effect on the number of γδ T cells (Correa *et al.*, 1992; Bigby *et al.*, 1993), unlike T cells bearing the αβ TCR, as reported in mice. Although cells expressing the αβ TCR and the γδ TCR differ in the chains of their TCR they do share the same composition of cluster of molecules known as CD3. The CD3 is responsible for signal transduction (Bank *et al.*, 1986; Borst *et al.*, 1987; Van Neerven *et al.*, 1990), as well as function of the T lymphocytes (Hanby-Flarida *et al.*, 1996). Large numbers of γδ T cells have been identified in peripheral blood, in the gut, and at the epithelial surfaces, with lower numbers reported in the lymph node, thymus and spleen (Mackay *et al.*, 1988). The lower numbers of γδ T cells reported in the lymph node, thymus and spleen suggests that this population does not readily re-circulate. Expression of L-selectin on the cell surface of γδ T cells suggests that they are able to migrate from peripheral blood into the lymph node via high endothelial venules (HEV), (Mackay *et al.*, 1988; Howard *et al.*, 1992). Sequentially, the afferent lymph, peripheral blood and the efferent lymph contribute the largest number of γδ T cells present in the lymph node in sheep (Mackay *et al.*, 1990). The location of the γδ T cells suggests that those present in the lymph nodes were extracted by endothelium in peripheral tissue as opposed to the HEV of lymph nodes. Examination of lymph node sections showed that the entry of γδ T cells was via the afferent lymph (Mackay *et al.*, 1988).

When γδ T cells are induced by antigenic stimuli the response is similar to that of a T cytotoxic cell. Cytokine production by activated γδ T cells is dependent on the type of antigenic stimulus. Stimulation of murine peritoneal γδ T cells with *Listeria monocytogenes,* showed a pattern of cytokine production that was characteristic of a Th1 type response (Ferrick *et al.*, 1995). However on stimulation of the same cells with a different antigen, *Nippostrongylus brasiliensis,* the pattern of cytokine production was characteristic of a Th2 type response (Ferrick *et al.*, 1995). The *in vitro* culture of γδ T cells with IL-2 has been shown to induce the destruction of malignant breast carcinoma cells (Miescher *et al.*, 1990). γδ T cells have also been reported to play an important role against challenge from bacterial pathogens (Mackay and Hein, 1991).

The addition of γδ T cells to a mixed lymphocyte reaction resulted in a disproportionate proliferation (Clevers *et al.*, 1990). When γδ T cells were depleted *in vivo*, the antibody response generated towards intravenously administered human type O erythrocytes increased (Howard *et al.*, 1989). The combination of these effector functions has resulted in speculation that γδ T cells are associated with the regulation of response induced by antigen in other subsets of lymphocytes. It has also been suggested that γδ T cells may possess cytolytic activity but this has still to be established (Mackay, 1988; O'Reilly and Splitter, 1991).

T cells expressing the γδ TCR are a major subpopulation in cattle (Wyatt *et al.*, 1996). Levels of γδ T cells expressing the WC1 cell surface marker are considerably lower within secondary lymphoid tissue and mammary gland secretions (3-8%), (Park *et al.*, 1992; Wyatt *et al.*, 1994), but higher in the tissue of the gut and the skin (McClure *et al.*, 1989; Howard *et al.*, 1992). Levels of γδ T cells lacking expression of the WC1 cell surface marker represent a small percentage of the circulating lymphocyte population, between 2-5%. A large proportion of those cells lacking the expression of the WC1 cell surface marker are present in the spleen, mammary gland and its secretions, as well as the gut epithelium (Wyatt *et al.*, 1994 and 1995; MacHugh *et al.*, 1997). These two disparate subpopulations have been reported in cattle and pigs (Binns, 1994; Licence *et al.*, 1995). The first subset express cell surface markers similar to those of γδ T cells in other species namely WC1, CD2, CD5, CD6 and they may, or may not, express CD8 (Davis *et al.*, 1996). Expression of CD6 has been reported to enhance T cell responsiveness (Yang *et al.*, 1996). The second subset lacks expression of WC1, CD2, CD6 and CD8 but does express the CD5 cell surface marker (Clevers *et al.*, 1990). Expression of CD5 on both subsets of bovine γδ T cells is unlike humans where CD5 is expressed on only one of the subsets (Spour *et al.*, 1990). The proportion of γδ T cells in the peripheral blood of adolescent ruminants has been reported as approximately 40% in three week old lambs (Washington *et al.*, 1992) and 27% in three week old calves (Clevers *et al.*, 1990). Levels are shown to decrease too less than 15% in adults (Tizard, 1992). The reduction in γδ T cell numbers due to age is in direct contrast to the pattern observed with $CD4^+$ T cells (Mackay *et al.*, 1990; Howard *et al.*, 1991; Mason and Powrie, 1990).

About 60% of the lymphocytes present in the skin of cattle have been identified as expressing the γδ cell surface marker (Mackay and Hein, 1991), as well as having been identified in the epithelial tissues of cattle (Wijngaard *et al.*, 1992). As has been suggested in other species (Janeway *et al.*, 1988), the function of γδ T cells identified in the skin of ruminants may be the protection of the epithelial surfaces (Hein and Mackay, 1991; Morrow-Tesch *et al.*, 1996).

1.10.1.3 Expression of naïve/memory phenotype on T lymphocytes

Expression of a cell surface marker, CD45, was first identified through the production of a mouse anti-human monoclonal antibody, which was able to identify $CD4^+$ T cells that could respond to mitogenic stimulus but not soluble antigen and vice versa (Morimoto *et al.*, 1985). These T cells were named CD45RA and further studies identified a lower molecular weight (MW) isoform of the CD45 cell surface marker, named CD45RO (Smith *et al.*, 1986).

CD45 is a membrane protein tyrosine phosphatase (Tonks *et al.*, 1988) expressed on all nucleated haematopoetic cells in various isoforms (Kong *et al.*, 1995a). The requirement for CD45 has been reported for T and B cell activation after antigenic stimulation (Pingel and Thomas, 1989; Chan *et al.*, 1994; Justement *et al.*, 1994). This result was confirmed by the addition of anti-CD45 MAb, which resulted in the loss of ability by B cells to respond to stimuli, which initiates cross-linkage of the BCR (Gruber *et al.*, 1989).

B cells lacking the expression of CD45 can produce normal levels of antibody after antigenic challenge and maintain the ability to switch Ig class with $CD4^+$ T cells assistance (Kong *et al.*, 1995a). This suggests that expression of CD45 is not an absolute requirement for B cell development or signal transduction involving surface Ig. However mice lacking CD45 expression do exhibit impaired T cell development and function (Kishihara *et al.*, 1993; Kong *et al.*, 1995b).

In mammals expression of the higher MW isoform of CD45 can result in unresponsiveness or low levels of response when challenged after vaccination with a specific antigen. The isoforms associated with this unresponsiveness were identified

using MAb in humans and rodents and classified as CD45RA/RB/RC (Streuli *et al.*, 1987; Streuli *et al.*, 1988; Johnson *et al.*, 1989). A similar state of unresponsiveness is reflected in cattle and sheep in association with the high MW CD45, where high MW CD45 are mainly CD45RB/RA respectively (Mackay *et al.*, 1990; Howard *et al.*, 1991).

From studies carried out in humans, it was suggested that T cells expressing the high MW isoform of CD45 were naïve in terms of antigenic exposure (RA) and those T cells expressing the low MW isoform of CD45 were memory T cells (RO), (Terry *et al.*, 1988). Expression of the isoforms is not mutually exclusive, and the presence of both CD45RA and RO has been observed on a small subset of T cells in humans (Mason and Powrie, 1990). Dual expression may suggest that this subset of T cells has a function that is distinct from that of other $CD45RO^+$ T cells, or that it may be an intermediate phenotype, which develops during alteration in activation status (Bell and Sparshott, 1990; Sparshott *et al.*, 1991; Michie *et al.*, 1992). Persistent exposure to antigenic stimulus has also been suggested as a potential explanation for dual expression of CD45RA and RO (Salmon *et al.*, 1994).

Approximately 10% of bovine T cells have been shown to be double positive for CD45RA/RO, which is consistent with the results published in humans (Bembridge *et al.*, 1995). Bovine $CD4^+$ T cells have been reported to express a higher percentage of CD45RO molecules than $CD8^+$ T cells (Akbar *et al.*, 1993). Expression of the CD45RO isoform is positively correlated with age, for as the animal matures the level of $CD45RO^+$ T cells increases (Sanders *et al.*, 1988; Beverly, 1990; Hannet *et al.*, 1992). This is not unexpected for as the cattle mature, their exposure to antigenic stimuli will increase.

Expression of CD45RO is not restricted to $CD4^+$ and $CD8^+$ T cells. $\gamma\delta$ T cells in mice have been reported to uniformly express the CD45RO isoform parallel with CD25 (Capone *et al.*, 1998), the receptor ligand for IL-2. The expression of these two cell surface molecules suggests an activated phenotype.

1.10.2 B Lymphocytes

B and T lymphocytes are derived from the same cell lineage, originating from pluripotent stem cells. These pluripotent stem cells differentiate into lymphoid stem cells and further develop into pre-T and pre-B lymphocytes. The maturation of immature B cells creates plasma cells, which are capable of producing antibody. Antibody producing B cells are educated within the bone marrow and Peyers patches and B cells have also been identified in the cortex of the lymph nodes and the marginal zone of the spleen (VonBoehmer and Kisielow, 1990). B cells respond to external antigen through the production of antigen specific antibody (Morafo *et al.*, 1999).

Generation of an antibody response is a two-stage process. Initial cell to cell contact between the B cell and the Th cell in addition to co-stimulation by adhesion molecules is required, followed by stimulation of the B cell by cytokines secreted from the Th cell population (Hermann *et al.*, 1995). Promotion of B cell proliferation and polyclonal Ig secretion is associated with a Th1-type response, although this does not result in antigen specific antibody formation (Mosmann *et al.*, 1986; Cherwinski *et al.*, 1987; Reiner and Seder, 1995). A Th2 type response results in up-regulation of IgG_1, IgA and IgE (Tam *et al.*, 1999). The final stage in the maturation of the B cell into an antibody secreting plasma cell takes place after re-circulation (Pierce *et al.*, 1979; Husband, 1982).

B cells possess a B cell receptor (BCR), which is comprised of different glycoproteins and functions much like the TCR. The BCR initiates antigen specific signals from the B cells and also processes and presents antigen through phagocytosis and proteolysis similar to conventional APC (Butler, 1998). A unique feature of the BCR is that, unlike the TCR, they can bind antigen, which is not present on the surface of an APC, after internal processing (Klein and Horejsi, 1999).

Interaction between the BCR and antigenic peptide must be accompanied by co-stimulation to trigger the immune response and result in the production of antibody. Co-stimulation between the B cell surface involves CD40 (van Kooten and Banchereau, 1997) and its ligand, CD40L on the surface of activated T cells, which are predominately $CD4^+$ (Roy *et al.*, 1993; Banchereau *et al.*, 1994; Foy *et al.*, 1996). The interaction

between CD40 and CD40L enhances B cell proliferation, antibody production and Ig isotype switching and is essential for the development of an antibody mediated response towards T cell antigens (Durie *et al.*, 1994). Studies carried out *in vivo* reported that the interaction between CD40 and CD40L was able to initiate the development of a humoral response (Allen *et al.*, 1990) as well as the transient activation of macrophages (Stout *et al.*, 1996). The interaction between CD40 and CD40L has also been shown to be essential for the production of *bcl*-2 a molecule known to promote B cell survival by preventing apoptosis (Liu *et al.*, 1991). The expression of *bcl*-2 is restricted to memory B cells, with only 5% of maturing B cells developing into plasma cells and the rest being deleted by apoptosis (Parry *et al.*, 1994).

It has been suggested that a reduction in the expression of CD40L may affect the efficiency of T and B cell responses. In neonates whose T cells expressed low levels of CD40L (Nonoyama *et al.*, 1995; Durandy *et al.*, 1995; Flamand *et al.*, 1998) the development of a specific antibody response was slower. Cytokine expression in these neonates also preferentially promoted a Th2-type response (Barrios *et al.*, 1996; Kovarik and Siegrist, 1998). The expression of CD40L is regulated via transcriptional and post-translational mechanisms, resulting in limited expression time on activated T cells (Graf *et al.*, 1995). The level of expression of CD40L on T cells has been reported to limit the rate and magnitude of the specific antibody response (Perez-Melgosa *et al.*, 1999). Various studies have been carried out to remove CD40L as the limiting factor in the process of antigen presentation and recognition. Addition of an anti-CD40 antibody to the culture, which mimics CD40L, resulted in successful presentation and recognition of antigen (Flamand *et al.*, 1998). Other studies have altered the type of cell presenting the antigen to dendritic cells thus eliminating the requirement for CD40L in the presentation of antigen (Ridge *et al.*, 1998).

1.10.3 Co-stimulatory and Adhesion Molecules

The expression of CD4 and CD8 on the cell surface of αβ T cells aids the adhesion of the T cell to the APC during presentation of the antigenic peptide. Other co-stimulatory and adhesion molecules are expressed on the surface of T cells, which participate in the process of antigen presentation and recognition.

1.10.3.1 CD28 and B7 Interaction

Expression of the CD28 molecule is essential for T cell activation and induction of an immune response (Allison, 1994; DeBenedette *et al.*, 1997). The expression of the ligand B7 binds the CD28 molecule to the surface of the APC. B7 exists as two molecules; B7.1 (CD80), (Freedman *et al.*, 1987; Freeman *et al.*, 1991; Croft *et al.*, 1994) and B7.2 (CD86), (Azuma *et al.*, 1993; Freeman *et al.*, 1993). Cloning of B7.1 and B7.2 has been successful and the existence of another molecule, B7.3, has also been suggested (Boussiotis *et al.*, 1996). The interaction between CD28 and B7 sends a signal to the T cell, which amplifies the signal transmitted via the MHC-peptide-TCR complex interaction. The interaction between CD28 and B7 is essential for the production of IL-2 from unprimed T cells (Lindstein *et al.*, 1989; Linsley *et al.*, 1991; Harding *et al.*, 1992; Norton *et al.*, 1992; Harding and Allison, 1993; Linsley and Ledbetter, 1993). Production of IL-2 has been reported to activate macrophages and enhance their microbicidal activity (O'Suilleabhain *et al.*, 1996). Regulation of the co-stimulation between B.7 and CD28 has also been reported to be mediated by IL-10 (Tam *et al.*, 1999). Production of IL-10 results in the up-regulation of B7.1 in monocytes, paralleled by a down regulation of B7.2 in monocytes, Langerhans and dendritic cells (Buelens *et al.*, 1995; Kawamura and Furue, 1995; Creery *et al.*, 1996). Whether B7.1 and B7.2 induce identical, or different, gene effector programmes involved is unclear (Freeman *et al.*, 1995).

1.10.3.2 CTLA-4 and B7 Interaction

CTLA-4 is a co-stimulatory molecule expressed on the cell surface of T cells. This co-stimulatory molecule has affinity for the B7 ligand expressed on the cell surface of the APC (Linsley *et al.*, 1990; Linsley *et al.*, 1991). The interaction between CTLA-4 and B7 may result in a down-regulation of the signal sent from the T cell to the MHC-peptide-TCR complex, resulting in a reduction in the production of IL-2 (Krummel and Allison, 1995; Krummel and Allison, 1996; Chambers *et al.*, 1996; Boussiotis *et al.*, 1996; Sperling and Bluestone, 1996; Waterhouse *et al.*, 1996).

1.10.3.3 CD2 and Lymphocyte Function associated Antigen Interaction

The adhesion marker, CD2, is a pan T cell marker expressed on surface of T cells. CD2 has affinity for its ligand, Lymphocyte Function associated Antigen (LFA), which is expressed on many different cell types (Dustin *et al.*, 1987). The interaction between CD2 and LFA contributes to the overall binding affinity of T cell to APC (Arnauot, 1990). The LFA ligand has also been reported to take part in the trans-endothelial migration of T cells (Oppenheimer-Marks, 1991), and the multi-step extravasation of lymphocytes.

1.10.3.4 IntraCellular Adhesion Molecule-1

Intracellular Adhesion Molecule-1 (ICAM-1), is an Ig receptor and adhesion molecule, which mediates firm adhesion and transmigration of neutrophils (Sakamoto *et al.*, 1997; Morland *et al.*, 1997), and T cells to hepatic endothelial cells *in vitro* (Yoong *et al.*, 1998). Expression of ICAM-1 has been noted on macrophages and cortical epithelial cells (Dustin *et al.*, 1986; Singer *et al.*, 1990). The presence of ICAM-1 has been shown to enhance antigen recognition of T cells through the interaction of T cell and APC (Dustin *et al.*, 1986; Dustin and Springer, 1991).

1.10.4 Proportion of Lymphocytes present in peripheral blood and in the mammary gland of cattle

Percentages of lymphocytes in the peripheral system have been shown to vary within different species depending on the subject's age. In humans, children up to 16 years of age have approximately 34-57% circulating T cells and 15-42% B cells. As humans mature the percentage of lymphocytes fluctuate, and in adults approximately 56-83% are T cells and 8-21% are B cells (Bhat *et al.*, 1992).

In adult cows it has been reported that 46-75% of T cells were $CD2^+$ (Davis *et al.*, 1988; Baldwin *et al.*, 1988), and that approximately 23-35% of those were $CD4^+$ (Baldwin *et al.*, 1986), and 9-26% were $CD8^+$ (Ellis *et al.*, 1986; MacHugh and Sopp, 1991). Expression of the CD5 cell surface marker has been reported on approximately 70% of T

cells (Rabinovsky and Yang, 1986) and 16-35% on B cells (Yang *et al.*, 1997). The high levels of $CD5^+$ T cells in adult cows is not unexpected as CD5 is a mature cell surface marker reported on $CD2^+$, $CD4^+$, $CD8^+$ and γδ T cells (Yang *et al.*, 1995). Expression of MHC class II molecules that are involved in presentation of antigenic peptide to $CD4^+$ T cells, has been reported as 19-28% of peripheral blood lymphocytes in cattle (Taylor *et al.*, 1993), and also as 30-50% of peripheral blood lymphocytes in cattle (Tizard, 1992). Variation in CD4/CD8 T cell ratios in peripheral blood has been noted among species during lactation, $CD4^+$ T cells predominate in goats (Ismail *et al.*, 1996a) where as in lactating pigs (Chabaudie *et al.*, 1993), and mice (Ismail *et al.*, 1996b) $CD8^+$ T cells predominate.

Approximately 5% of peripheral blood lymphocytes in adult cattle are γδ T cells. In young ruminants, γδ T cell expression is much greater with levels reported as approximately 26% of the peripheral blood lymphocyte population in calves aged three weeks, and 40% in lambs aged four weeks (Clevers *et al.*, 1990). Work carried out by Ayoub and Yang (1996) reported a rapid increase in the number of $CD4^+$, $CD8^+$ and γδ T cells in newborn calves, which peaked at six to eight months of age. As the calves aged, the levels of $CD4^+$ and $CD8^+$ T cells decreased to a point then remained unchanged. The γδ T cell population however continued to decrease as the calves aged, results which are supported by work previously published in sheep (Hein, 1994). As well as γδ T cell numbers altering with age, alteration in lymphocyte numbers can also be attributed to disease status. A two-fold increase in B cell numbers was reported in cows suffering from lymphocytosis (Muscoplat *et al.*, 1974).

In cattle, it has also been reported that during lactation, $CD8^+$ T cells are predominant in milk, a reversal of the situation reported in the peripheral system (Park *et al.*, 1992). As the days in lactation increased an increase in the proportion of $CD4^+$ T cells in the mammary gland was reported (Taylor *et al.*, 1994). Lymphocyte numbers have been shown to be highest during late lactation, in particular T cells (Park *et al.*, 1992). Levels of $CD8^+$ T cells remain fairly constant throughout lactation but numbers of $CD4^+$ T cells start very low and increase throughout lactation (Asai *et al.*, 1998).

The type of milk, as well as the stage of lactation may also affect the types of cells present. Work carried out by Concha *et al.* (1996), showed that stripping and residual milk did not differ significantly in the proportion of CD2, CD4 and CD8 expressing T cells, but both did differ significantly from mid lactation milk in CD2 and CD4 T cell numbers. A reduction in T cells was observed during the periparturient period compared with during lactation. Numbers increased during the dry period (Asai *et al.*, 1998). During the dry period the population dynamics of cells within the mammary gland alters considerably (Lee *et al.*, 1980; Jensen and Eberhart, 1981), with the most dramatic fluctuations seen in macrophages and neutrophils (Wilson *et al.*, 1986).

Induction of a proliferative response induced by antigen *in vitro* is representative of cellular immunocompetence (Kristensson *et al.*, 1994). Bovine mammary gland lymphocytes isolated from foremilk are hyporesponsive when cultured *in vitro* (Smith and Schultz, 1977; Nonnecke and Kehrli, 1985; Park *et al.*, 1993). The state of hyporesponsiveness exhibited by mammary gland lymphocytes has been suggested as an effect of increased numbers of macrophages (Collins and Oldham, 1986). Others have reported no improvement in proliferative response when macrophage numbers were reduced (Concha *et al.*, 1996). Although a reduction in responsiveness has been noted between mammary gland lymphocytes and PBL, mammary gland lymphocytes still possess the ability to respond to mitogenic stimuli (Nonnecke and Kehrli, 1985), allogenic histocompatibility antigens (Parmely *et al.*, 1976), and virus infected cells (Kohl *et al.*, 1981).

Parturition and lactation have been reported to effect the type of cells present in the mammary gland and their function. The postpartum period in cattle is associated with higher incidence of mastitis and a reduction in immunological response (Oliver and Sordillo, 1988; Sordillo *et al.*, 1997). The reduction in immunological response has been reported to affect phagocytosis, cellular proliferation, antibody production and secretion of inflammatory mediators such as cytokines (Harp and Nonnecke, 1986; Nagahata *et al.*, 1992; Ishikawa *et al.*, 1994; Detilleux *et al.*, 1995).

Development of an inflammatory response is a main requirement for elimination of a bacterial pathogen (Hill, 1981). Neutrophils act as the host's primary line of defence

against invading pathogens. Impaired neutrophil function in cattle during early lactation can result in an increased susceptibility to disease (Guildry *et al.*, 1975; Kehrli *et al.*, 1989a) and has been reported as essential in the prevention of staphylococcal infections (Lee, 1996).

During the postpartum period a reduction in the proportion of lymphocytes has been reported in peripheral blood, mammary secretions and mammary parenchyma (Shafer-Weaver *et al.*, 1996; Van Kampen and Mallard, 1997; Yang *et al.*, 1997). A reduction in the total number of lymphocytes and a reduction in the proportion of $CD4^+$ T cells has been used to indicate a compromised immune function in humans (Fahey *et al.*, 1984; Kalish and Schlossman, 1985). The number of $CD4^+$ T cells has been reported to differ significantly in cows before and after parturition and in cows in mid-lactation (Harp *et al.*, 1991). During the postpartum period, T cells expressing CD4 secrete low levels of IL-2 and IFN-γ, both Th1-type cytokines. Both of these cytokines are reported to stimulate up-regulation of MHC class II molecules on the surface of mammary epithelial cells (Fitzpatrick *et al.*, 1992) and to activate and enhance the microbicidal activity of macrophages (Paul and Seder, 1994). T cells expressing CD4 during the postpartum period have been identified as expressing mRNA transcripts for Th2 type cytokines (Shafer-Weaver *et al.*, 1999). During the postpartum-period the reduction in both IL-2 and IFN-γ was positively correlated with an increased incidence of mastitis in dairy cows (Shafer-Weaver *et al.*, 1999). Neutrophil and lymphocyte function, phagocytosis, cellular proliferation and antibody production are all reduced in the mammary gland during the postpartum period (Harp and Nonnecke, 1986; Kehrli *et al.*, 1989b; Detilleux *et al.*, 1995). The presence of hormones such as oestrogen and progesterone, has been suggested as a possible mechanism of immunosuppression in the mammary gland during the postpartum period (Roth *et al.*, 1982; Blalock, 1994). The elevated levels of these hormones present during the postpartum period may alter the type of cytokines secreted from Th1-type to Th2-type (Rook *et al.*, 1994). The imbalance between Th1-type and Th2-type cells and the cytokines they produce has been linked to disease susceptibility (Clerici and Shearer, 1994; Romagnani, 1994; Carter *et al.*, 1996). The expression of T suppressor cells dominates that of T cytotoxic cells during the postpartum period compared to the proportions present mid to late lactation (Shafer Weaver *et al.*, 1997). The presence of T suppressor cells during the postpartum period has been reported to

reduce the responsiveness of $CD4^+$ T cells present in the mammary gland (Shafer Weaver and Sordillo, 1997). The presence of T suppressor cells in peripheral blood and the mammary gland during the postpartum period has resulted in a reduction of IL-2 and IFN-γ (Park *et al.*, 1993; Sordillo *et al.*, 1991).

The immunosuppression reported in the mammary gland during the postpartum period was reversed during mid to late lactation. During mid to late lactation an increase in the levels of IL-2 and IFN-γ in the mammary gland and in peripheral blood, due to the large proportion of Th1-type $CD4^+$ T cells was reported (Ishikawa *et al.*, 1994; Shafer-Weaver *et al.*, 1996; Sordillo *et al.*, 1991).

Monocytes present in peripheral blood are the precursors for macrophages, which are responsible for non-specific cellular immunity (Durum *et al.*, 1984). Macrophages in cattle are known to reside as Kupffer cells in the lining of the sinusoids of the liver, microglia in the brain, alveolar macrophages in the alveoli of the lung and those present in lymphoid and mammary gland tissue (Bielefeldt Ohmann *et al.*, 1986; Bryan *et al.*, 1988). Pulmonary intravascular macrophages account for a large number of macrophages in cattle and other species and are involved in the clearance of blood borne bacterial pathogens and particulate debris (Winker *et al.*, 1988).

Mammary gland macrophages ingest fat and casein indiscriminately from the milk. This results in fat globules within the cytoplasm of macrophages, which allows distinction between those present in the peripheral system and those in the mammary gland, as their cytoplasm appears foamy (Jenson and Eberhart, 1975). Non-specific ingestion of fat by macrophages has been shown to result in a reduction in phagocytic capability of mammary gland macrophages, and is particularly compromised prior to calving. Macrophages contain phagocytic and antibacterial properties (Higginbotham and Pruett, 1994), which are functional whilst they are resting but once activated become more aggressive, capable of ingesting bacteria, cellular debris and milk components (Sordillo and Nickerson, 1988). An important product of macrophage activation is the prompt release of IL-1, IL-6 and TNF-α, which can trigger acute phase and inflammatory responses (Politis *et al.*, 1991). Macrophages contribute to cell mediated immunity, acting as APC involved in processing and presenting antigen to lymphocytes (Nickerson,

1985) and have been reported to be the predominant cell type in a healthy lactating mammary gland (Lee *et al.*, 1980; Jensen and Eberhart, 1981; Concha and Holmberg, 1990). Presentation of antigen by peripheral blood macrophages occurs in association with MHC class II expressed on their cell surface (Weaver and Unanue, 1990; Sordillo *et al.*, 1997), and has also been reported in macrophages within the mammary gland (Fitzpatrick, 1992). Macrophages also express ligands for co-stimulation with T cell markers (Ding and Shevach, 1996). These ligands can be expressed (Oyaizu *et al.*, 1997) or secreted (Kiener *et al.*, 1997). The macrophage population, like lymphocytes, divides into two subsets effected by the presence of various cytokines. The two subsets differ phenotypically and have been reported to develop as a result of antagonistic induction pathways (Orlikowsky *et al.*, 1996; Orlikowsky *et al.*, 1997+
; Kummerle-Deschner *et al.*, 1998).

1.11 Initiation of an Immune Response

A dual signal model was first suggested to try and explain why lymphocytes once they have encountered an antigenic stimulus, either induce an immune response or remain unresponsive (Bretsher and Cohn, 1970). The first signal was described as being induced by the coupling of the antigen receptor to antigen, which if not backed up by a second signal would result in unresponsiveness. The primary signal is now recognised as the engagement of the TCR with the MHC-antigenic peptide complex. Various suggestions have been made as to the nature of the second signal. Some of these include the interaction previously described between B7.1 (Freeman *et al.*, 1989; 1991), or B7.2 (Azuma *et al.*, 1993; Freeman *et al.*, 1993; Hathcock *et al.*, 1993; Ranger *et al.*, 1996). The interaction between CD28 (June *et al.*, 1990), and CTLA-4 (Linsley *et al.*, 1991b); the interaction between CD28 and CD2 (Meuer *et al.*, 1984; Kato *et al.*, 1992; Holter *et al.*, 1996); the interaction between CD28 and ICAM-1 (Kuhlman *et al.*, 1991; Dubey *et al.*, 1995; Cai *et al.*, 1996; Deeths and Mescher, 1997); and the interaction between CD28 and LFA-3 (Parra *et al.*, 1997). Cytokines, for example IL-1 (Williams *et al.*, 1985; Houssiau *et al.*, 1988; Houssiau *et al.*, 1989; Vink *et al.*, 1990; Holsti *et al.*, 1994), IL-2, IL-6 (Houssiau *et al.*, 1988; Houssiau *et al.*, 1989; Vink *et al.*, 1990; Holsti *et al.*, 1994; Joseph *et al.*, 1998) and TNF-α (Vella *et al.*, 1997; Joseph *et al.*, 1998), have also been suggested as candidates for initiation of the second signal. Recent studies have reported

that if the primary signal relayed to the T cell is exceptionally strong, the requirement for the second signal to initiate the immune response may be obsolete (Goldstein *et al.*, 1998; Luxembourg *et al.*, 1998). Others have dismissed this theory and reported that $CD8^+$ T cells, whose response is based upon a single signal, die rapidly if no secondary signal follows (Sepulveda *et al.*, 1999). Work showing that the interaction between CD28 and B7, as well as providing a secondary stimulatory signal for induction of an immune response, also provided a survival signal for those $CD8^+$ T cells, supports the theory for a dual signal (Sperling *et al.*, 1996). The age and maturity of the cell being induced must be taken into account as it has been suggested that fewer TCR-ligand interactions and co-stimulatory molecules are required by mature effector cells when compared to immature effector cells (Dubey *et al.*, 1996).

1.11.1 Cell cycle events

The recognition of antigenic peptide by lymphocytes by virtue of cell surface markers results in proliferation and differentiation of cells. Differentiation produces clones of cells with corresponding receptors to the antigenic determinant or epitope (Benjamini *et al.*, 1996). This mechanism is more commonly described as clonal selection (Klein and Horejski, 1999).

Resting lymphocytes appear as a homogenous population with a low RNA and DNA content and are referred to as G_0 cells (Kristensen *et al.*, 1982). On stimulation the G_0 cells transform into the G_1 phase of their cell cycle, which is characterised by an increase in RNA synthesis (Darzynkiewicz *et al.*, 1976; Stadler *et al.*, 1980). Once an increase in DNA synthesis is detectable the lymphocytes have entered into the S phase of their cell cycle. On completion of DNA-synthesis the lymphocytes progress to an interphase known as the G_2 phase (Klein and Horejski, 1999). From the G_2 phase the lymphocytes undergo mitosis (M phase) and subsequently return to the G_0 phase (Kristensen *et al.*, 1982). It has also been suggested that lymphocytes undergoing mitosis go directly to the G_1 phase of their cell cycle during the logarithmic growth phase. It has been demonstrated previously in mice and humans that an IL-2 signal in addition to the activation signal is required for T cell proliferation (Baker *et al.*, 1980; Gillis *et al.*, 1980). The IL-2 is responsible for completion of the cycle involving RNA synthesis and

proliferation (Kristnesen *et al.*, 1981). T cells have been reported previously to secrete IL-2 after binding mitogen or antigen to the cell membrane and after receiving a signal from IL-1 secreted from adherent cells (Smith *et al.*, 1980).

1.11.2 Re-circulation

Large proportions of lymphocytes, which reside in the central lymphoid organs namely bone marrow and thymus, and in the secondary lymphoid organs of the lymph nodes (LN) and spleen remain within these sites. Approximately 10% of lymphocytes in adult animals are able to migrate from peripheral blood to tissue and then into the lymph nodes (Trnka and Cahill, 1980). This mechanism is known as re-circulation (Gowans and Knight, 1964). Re-circulation, prior to elucidation of the mechanism involved, was believed to occur randomly. A model in sheep depicted T cells homing in tissue specific streams towards the gut (Cahill *et al.*, 1976). The ability of lymphocytes to re-circulate is an inherent trait important in foetal development for long term development of immunity (Pearson *et al.*, 1970). The thymus contributes to the determination of tissue-specific T cell re-circulation, as well as contact with antigen in foetal development (Washington *et al.*, 1994; Kimpton *et al.*, 1995).

Initial attachment of lymphocytes to vascular endothelial cells is an absolute requirement for cells that leave the periphery and enter the surrounding tissue (Janeway and Golstein, 1992; Springer, 1994). The attachment of lymphocytes to endothelial cells is regulated by cell surface molecules expressed on lymphocytes and on the endothelium of the target tissue. Adhesion molecules are essential for the re-circulation of lymphocytes to the lymphatic organs or effector sites (Springer, 1994; Picker, 1994; Springer, 1995). After adhesion to the vascular endothelium, rolling of the leucocyte on endothelial cells in post capillary venules or HEV of peripheral lymph nodes or mucosal tissue of the gut is mediated by expression of selectin or integrin (Ley and Tedder, 1995; Bargatze *et al.*, 1995). The majority of lymphocytes and HEV interactions have been studied in humans and mice, using the methodology associated with the binding of MAb to tissue sections or monocultures to provide enlightenment as to the mechanisms involved (Kishimoto *et al.*, 1989; Dustin and Springer, 1991; Picker and Butcher, 1992). The production of MAb for

ruminant species now makes it possible to study lymphocyte re-circulation and homing in these animals (Trnka and Cahill, 1980; Miyasaka and Trnka, 1986; Pearson *et al.*, 1976).

Lymphocyte entry into the LN from peripheral blood is predominately via post capillary venules (PCV), (Harp *et al.*, 1990; Kraal and Mebius, 1997), which are distinguished morphologically from HEV but function in a similar manner. Approximately 10% of the total lymphocyte population is transported to the LN via the afferent lymph, and transported from the LN via the efferent lymph (Binns, 1988).

Endothelial cells express adhesion molecules with complementary ligands expressed on the surface of the circulating T cells (Jutila *et al.*, 1989). One such ligand is CD62, known as L-selectin and regarded as a homing receptor. L-selectin has been well conserved throughout mammalian evolution with expression reported in sheep (Mackay *et al.*, 1992), and cattle (Howard *et al.*, 1984). L-selectin promotes binding of lymphocytes to the HEV on endothelial surfaces and permits diapedesis of lymphocytes into the LN. Lymphocytes deficient in L-selectin have been shown to be unable to migrate to the peripheral nodes (Arbones *et al.*, 1994; Steeber *et al.*, 1996; Tang *et al.*, 1998). Another such adhesion molecule is ICAM-1, which has been shown to mediate firm adhesion and transmigration of neutrophils (Sakamoto *et al.*, 1997; Morland *et al.*, 1997) and T cells to hepatic endothelial cells *in vitro* (Yoong *et al.*, 1998). Lymphocyte Function associated Antigen has been reported to aid in the trans-endothelial migration of T cells (Oppenheimer-Marks *et al.*, 1991), and in the multi-step extravasation of lymphocytes (Picker *et al.*, 1990).

The uterus of the sheep provides an environment where foreign antigen and memory T cells are prevented from entering the tissues and, therefore, the homing patterns of T cells can be followed and distinguished between naïve and memory T cells (Pearson *et al.*, 1976; Cahill and Trnka, 1980). From studies using the ovine uterus, lymphocytes have been reported to begin re-circulating 75 days into the gestation period, migrating from peripheral blood and entering the tissue, during which time their growth is exponential (Cole and Morris, 1973).

The immunological development that takes place in the thymus, spleen, lymph node and Peyers patches in human foetal development is very similar to that reported in sheep during that period. By way of comparison, at the time of birth, rats and mice have inadequately developed lymphoid organs. The similarity between human and sheep development makes the sheep model useful for determining different stages of human immunological development (Kimpton *et al.*, 1995). At approximately day 80 in the gestation period, the foetal sheep thymus is fully developed as identified by immunohistological and cellular appearance (Mackay *et al.*, 1986; Maddox *et al.*, 1987; Kimpton *et al.*, 1995).

Re-circulation of lymphocytes in the sheep foetus is comparable with numbers of lymphocytes present in the postnatal immune system (Kimpton *et al.*, 1989; Kimpton *et al.*, 1990; Kimpton *et al.*, 1995). Transportation of cells from the thymus in the postnatal animal approximates to 1% of thymocytes per day and has been reported in mice (Scollay *et al.*, 1980), pigs (Binns *et al.*, 1988) and humans (Miyasaka *et al.*, 1990). Re-circulation in humans of lymphocytes approximates too roughly that reported for other species under non-inflammatory conditions. Of these lymphocytes, $CD4^+$ T cells more actively re-circulate than $CD8^+$ T cells (Lemaire *et al.*, 1998).

1.11.3 Lymphocyte Homing

T cells expressing a memory phenotype preferentially re-circulate to the site of initial antigenic stimulus; termed lymphocyte homing (Mackay *et al.*, 1992; Picker and Butcher, 1992). It has been reported that T cells expressing the memory phenotype re-circulate more actively in the gut when compared to other non-lymphoid sites (Lemaire *et al.*, 1998). Non-lymphoid sites that have shown T memory cell re-circulation included intestinal mucosa, skin, pulmonary tissue and joints (Mackay *et al.*, 1992; Picker and Butcher, 1992; Butcher and Picker, 1996). The migratory pathways associated with naïve and memory T cells are distinct. Memory T cells migrate preferentially through non-lymphoid tissue, whereas naïve T cells are transported via HEV in the LN. The development of the distinct pathways for memory and naïve T cells is regulated by exposure to antigen (Butcher and Picker, 1996; Mackay, 1993).

As a result of antigenic challenge, a dramatic reduction in migration of cells, termed shutdown, from within the LN during the initial hours post challenge has been reported (Hall and Morris, 1967). The reduction in the migration of cells coincides with an influx of lymphocytes into the LN, mediated by an increase in blood flow to the stimulated node (Cahill *et al.*, 1976). The lymphocytes that migrate from within the LN during antigenic challenge, amplify and disseminate the immune response to the whole body (Hall *et al.*, 1967), highlighting the necessity for an efficient lymphatic system to circulate memory and effector cells.

The existence of a common mucosal immune system has been demonstrated in monogastric species such as pigs (Harp and Moon, 1988). Priming of immune cells with antigen via the gastrointestinal route lead to antigen-specific immune cells being present in the mammary gland (Goldblum *et al.*, 1975; Parmely and Beer, 1977; KortbeekJacobs and Van der Donk, 1981). The existence of a common mucosal immune system, known as the entero-mammary link, is less functional in ruminants (Chang *et al.*, 1981; Moon and MacDonald, 1983). The less functional entero-mammary link was highlighted in cattle when radiolabeled mammary LN lymphocytes localised in the node of origin (mammary LN) and a peripheral node (prescapular LN) (Harp *et al.*, 1988). However, very few of the radiolabeled cells localised in the intestinal mesenteric node, as previously reported in sheep (Harp and Moon, 1987).

1.12 Major Histocompatibility Complex

The murine MHC, named H-2 (Snell, 1958) was discovered by the recognition of molecules present on lymphocytes which resembled a blood group antigen. Twenty years later, the chicken B complex (Briles *et al.*, 1950), followed by the human leucocyte antigen system (HLA; Klein, 1986) were reported. The MHC was recognised as the major locus controlling the rejection of foreign transplantation tissue in chickens (Schierman and Nordskog, 1961) and humans (Tiercy *et al.*, 1991) as well as influencing susceptibility to autoimmune diseases (Gregersen *et al.*, 1987; Thomson, 1988). The MHC has now been described in mammalian, avian, amphibian, domestic species (Klein and Figueroa, 1986), pigs (Vailman *et al.*, 1970), and cattle, where it is termed the Bovine Leucocyte Antigen System (BoLA), (Amorena and Stone, 1978). Definition of BoLA

was carried out using antisera that had been raised from recipients of skin grafts and multiparous cows (Spooner *et al.*, 1978; Spooner *et al.*, 1979).

The location of the MHC varies among species, the murine H-2 is located on chromosome 17, HLA on chromosome 6 of man (Korman *et al.*, 1985), and BoLA on the short arm of chromosome 23, identified by use of *in situ* hybridisation. Within the MHC there are three different regions, class I, class II, and class III. In both the HLA and BoLA system, the chromosomal organisation is in the order of centromere: class II genes first, separated from class I genes by class III in the middle (Peelman *et al.*, 1996).

The region between class II and class III appears conserved in humans, pigs and mice (Lunney, 1994), unlike the region between class I and class III, which appears more variable (Bahram *et al.*, 1994). The class III genes are less variable and show conservation in mammalian species. Information on MHC class III genes in horses, cattle and goats is limited, with the organisation suggested as similar to that in humans (Cameron *et al.*, 1990). The location of structural genes required for serum complement components have been reported in the MHC class III region (Hood *et al.*, 1983), with their function reported as unrelated to that of class I and class II genes (Klein *et al.*, 1986)

It was first observed in the 1960s that mice and guinea pigs could be categorised as high or low immune responders based on antibody production induced by a synthetic polypeptide (Paul *et al.*, 1968; McDevitt and Benacerraf, 1968), and that the ability to respond to antigenic stimulus was possibly due to the presence of immune response genes (McDevitt *et al.*, 1972). These immune response genes were categorised as MHC class II, and the MHC molecules identified by classic serology were defined as MHC class I.

The MHC is regarded as a major gene in immune responsiveness (Falconer, 1989). MHC genes encode highly polymorphic cell surface molecules, which present antigenic peptides to T lymphocytes (Allen *et al.*, 1987). High and low antibody responder lines of mice were created by selective breeding to antigen of different specificity's (Biozzi *et al.*, 1979). Immune responsiveness was shown to be under polygenic control, with MHC-linked genes playing a partial and irregular role (Biozzi *et al.*, 1979). After seven generations of selective breeding the differences between the high and low antibody titre

lines was calculated to be the effect of alleles at four independent loci (Cabrera *et al.*, 1982). One line of responder mice in particular with a high antibody titre carry a MHC class II with a specific β-chain sequence, which renders those mice susceptible to chronic relapsing experimental allergic encephalomyelitis (Liu *et al.*, 1993). In humans the alteration of the HLA-DQ β-chain sequence is associated with insulin-dependant diabetes mellitus (Tait and Harrison, 1991). Greater than 10% of differences in immune responsiveness towards various antigens can be attributed to MHC in mice (Biozzi *et al.*, 1974), and cattle (Mallard *et al.*, 1989).

It is well established that cellular and humoral immune responses are influenced through the MHC, in presentation of simple antigens (Benacerraf and Germain, 1978; Klein, 1986) and in disease susceptibility. Presentation of antigenic peptides to lymphocytes usually takes place in the presence of a MHC molecule for the induction of an immune response. Antigenic peptides are joined to a respective class of MHC molecule depending on the responding lymphocyte type. Further work revealed the crystallised complex of the TCR coupled to peptide embedded in the MHC molecule (Garcia *et al.*, 1996). The ability of lymphocytes to recognise non-self antigen in conjunction with self-MHC molecules is known as MHC restriction (Zinkernagel and Doherty, 1974). Major Histocompatibility Complex restriction was first described with regards to MHC class II in the guinea pig (Shvach and Rosenthal, 1973) and has been defined in mice (Shreffler and David, 1975) and humans (Bergholtz and Thorsby, 1978). In general, MHC class I molecules present antigenic peptides to T cytotoxic lymphocytes, expressing the CD8 cell surface molecule and are expressed on the surface of all nucleated cells. In contrast MHC class II molecules present antigenic peptides to T helper lymphocytes, expressing the CD4 cell surface molecule (Batra *et al.*, 1989; Parham and Ohta, 1996) and are expressed on the cell surface of macrophages, dendritic cells, B lymphocytes and some epithelial cells (Wiman *et al.*, 1976). Expression of MHC class II molecules have also been identified on the surface of activated lymphocytes (Seeg *et al.*, 1982). During the latter stage of the immune response, T cytotoxic cells have been identified as expressing MHC class II molecules (York and Rock, 1996). This may result in a down-regulation of the immune response resulting in the prevention of antigen presentation to T helper lymphocytes (Braakman *et al.*, 1987).

The manner in which antigen is processed is dependant on which class of MHC molecule is expressed. Endogenous antigen is processed by cells expressing MHC class I (Moore *et al.*, 1988) and exogenous antigen is processed by cells expressing MHC class II (Lamb *et al.*, 1982; Hanke *et al.*, 1985).

1.12.1 Major Histocompatibility Complex class I

Major Histocompatibility Complex class I molecules are integral cell surface glycoproteins. Expression of MHC class I has been identified on all nucleated somatic cells (York and Rock, 1996). The heavy chains of the MHC class I molecules are bound non-covalently to β_2-microglobulin (Madden, 1995). Beta 2-micoglobulin although not encoded for by the MHC, is thought to provide structural stability for the MHC class I molecule (Townsend *et al.*, 1990; Elliott *et al.*, 1991). The MHC class I genes are receptors for endogenous antigen to be processed and presented to the immune system (Moore *et al.*, 1988).

Ubiquitin, a small stable protein, attaches the target pathogen to a proteosome, a complex of different proteases (Englehart, 1994), with different peptidase activity. The proteosome unfolds the target pathogen and during this process releases the ubiquitin. Generation of antigenic peptides from the whole target pathogen transpires at the centre of the proteosome. The antigenic peptides generated are attached to transporter proteins, Transporter for Antigen Processing-1 (TAP1) and TAP2, which prevent further degradation by the proteosome. Antigenic peptides are delivered via the TAP molecule across the cytoplasm and into the lumen of the endoplasmic reticulum (ER). In TAP deficient cell lines accumulation of MHC class I molecules within the ER can result in unstable heterodimeric complexes (Tan *et al.*, 1997) effecting the presentation of antigenic peptides to cytotoxic T lymphocytes (Powis *et al.*, 1991; Spies and DeMars, 1991). In the lumen of the ER peptide fragments are coupled to MHC class I molecules, which are bound non-covalently to β2-microglobulin (Heemels and Ploegh, 1995). The peptides are loaded into the MHC binding groove, to form a peptide-MHC complex and transported from the ER to the cell surface for recognition by T cytotoxic lymphocytes.

Studies characterising the crystallised structure of the HLA class I molecule reported antigenic peptides presented in a groove formed by the α1 and α2 domain (Bjorkman *et al.*, 1987). The structure of the groove was reported as an α1α2 unit, supported by a β pleated sheet floor containing eight strands bounded by two α helices, one from α1, and one from α2 (Brown *et al.*, 1993; Stern *et al.*, 1994). Once the structure had been detailed, the groove was renamed as the peptide binding groove. More detailed examination of the peptide binding groove revealed the presence of pockets (Garrett *et al.*, 1989), reported to house anchor residues involved in peptide recognition (Saper *et al.*, 1991). It was reported that polymorphic amino acid residues present in the HLA heavy chain created the pocket structure (Madden *et al.*, 1992). The class I peptide binding groove is restricted to binding peptides of eight to nine amino acids in length (Rotzschke *et al.*, 1990; van Bleek and Nathenson, 1990; Falk *et al.*, 1991). This is due to the peptide binding grooves structure being closed at both ends (van Bleek and Natheson, 1990; Schumacher *et al.*, 1991; Falk *et al.*, 1991; Madden *et al.*, 1991). Following expression on the cell surface a portion of the MHC class I molecules are endocytosed, either being recycled back to the plasma membrane or retained intracellularly and degraded (Reid and Watts, 1990). This level of regulation of MHC class I ensures a constant level of expression is maintained.

1.12.2 Major Histocompatibility Complex class II

Major Histocompatibility Complex class II genes reside in the D locus of BoLA (Groenen *et al.*, 1990). Restriction Enzyme Fragmentation Pattern (REFP) analysis (Andersson, 1988) and characterisation of cloned MHC class II genes (Groenen *et al.*, 1990) resulted in mapping studies, which reported that the order of genes in the BoLA system was similar to those in the HLA system (Bensaid *et al.*, 1991).

Major Histocompatibility Complex class II molecules, like MHC class I molecules, are cell surface glycoproteins (Klein, 1986), and exist as dimers composed of an α and β-chain. Expression of BoLA-MHC class II molecules has been reported on classical APC such as B cells (Lewin *et al.*, 1985), monocytes (Taylor *et al.*, 1993), and alveolar macrophages (Ohmann *et al.*, 1986). Expression has also been noted on other cell types

including, activated T lymphocytes (Taylor *et al.*, 1993), mammary epithelial (Fitzpatrick *et al.*, 1992) and bronchial epithelial cells (Spurzem *et al.*, 1992).

There are 12 loci in the class II region of BoLA, with two sub-regions in the MHC class II; class IIa and class IIb. These two sub-regions are approximately 15 centimorgans apart, identified previously by genetic mapping (Andersson, 1988; van Eijk *et al.*, 1992). The DQ and DR genes present in sub-region IIa of BoLA are in tight linkage disequilibrium, where there are only a few exceptions of DQA alleles that are not associated with the same DQB alleles (Sigurdardottir *et al.*, 1988). Linkage disequilibrium generates haplotypes or combinations of allelic variants of genes located on one chromosome. The gap between the subregions of class IIa and class IIb has created a hot spot for intra-MHC recombination, which alter haplotypes normally inherited as a complete unit (Andersson, 1988). The genes residing in the MHC class IIa region are DRA, DRB1, DRB2, DRB3, DQA, and DQB. In this sub-region, the function of these genes has been well studied (Davies *et al.*, 1994b). The genes residing in the MHC class IIb region are DYA, DYB, DIB, DOB and DNA. Much less is known of the function of these genes within this group (Andersson and Rask, 1988).

Cellular internalisation of exogenous antigen is contained within endosomal or lysosomal vesicles (Werdelin, 1987; Wubbolts *et al.*, 1997). These vesicles contain proteases and peptidases, which provide an extremely acidic environment (pH~4) for degradation of the antigen into peptides of various lengths (Allen, 1987). Synthesis of the MHC class II dimer molecule takes place in the ribosomes of the rough endoplasmic reticulum (RER), where association with an invariant (Ii) chain prevents peptide binding at this stage. The MHC class II molecule and Ii complex are transported via the Golgi and trans-Golgi network to the cell surface. En route to the cell surface the vesicles containing the MHC class II molecules intercept the acid vesicles containing the peptide. When the MHC class II molecules and peptide fuse the Ii chain degrades and the MHC and peptide complex is transported to the cell surface for presentation to $CD4^+$ T lymphocytes. The loading of MHC class II molecule with peptide occurs via the endocytic pathway.

The antigen binding groove of MHC class II molecules comprises eight strands of anti-parallel β sheets for the floor and two anti-parallel helical regions create the sides (Brown

et al., 1993; Stern *et al.*, 1994). The antigen binding groove of MHC class II molecule is open ended (Rudensky *et al.*, 1991) and can accommodate peptides up to 34 amino acids in length (Chicz *et al.*, 1993). The MHC class II peptide binding groove is restricted also as peptides with a minimum of eight or nine amino acids are required (Sinigaglia and Hammer, 1994). The MHC class II binding groove lacks the amino acid chains, which act as a lid on the class I groove (Chicz *et al.*, 1993). Attachment of peptide in the MHC class II binding groove occurs preferentially in the middle of the cleft allowing longer peptides to bind (Engelhart, 1994).

1.12.2.1 Major Histocompatibility Complex class IIa DQB

A high degree of polymorphism in the MHC class IIa loci was identified by the use of human probes and RFLP (Andersson, 1986; Sigurdardottir *et al.*, 1988). Polymorphisms differ between the MHC and proteins as the number of alleles and number of amino acid replacements at a single locus is often very high in the MHC, with amino acid substitutions reported to be in excess of 10% (Schenning *et al.*, 1984; Trowsdale and Kelly, 1985). Both DQA and DQB, especially in exon 2 (Ballingall *et al.*, 1997) loci have been shown to be highly polymorphic with 31 DQA (Davies *et al.*, 1994) and 37 DQB alleles (Davies *et al.*, 1997) identified by RFLP. The DRA locus however does not exhibit the same level of polymorphisms and has been reported as monomorphic (Aida *et al.*, 1995). Recent studies have reported the possible identification of another DQA locus (Ballingall *et al.*, 1997). Complexity of bound MHC repertoire of peptides is still largely unknown. It has been estimated that in excess of 2000 peptides can bind to the murine MHC class II-IA^d region, which is the equivalent to the DQ region in humans (Englehart, 1994). A study carried out by Chicz *et al.* (1993) reported greater than 200 peptides presented by each of the HLA-DR alleles they investigated. One hypothesis for the existence of polymorphisms at the MHC locus is that some alleles may be favourable for protection to one disease whilst other alleles may be favourable for resistance to another disease (Biozzi *et al.*, 1979).

1.12.2.2 Major Histocompatibility Complex class IIa DRB

Three DRB loci have been reported, DRB1 has been reported as an unexpressed pseudogene, DRB2 is poorly expressed (Burke *et al.*, 1991; Russell *et al.*, 1994) but does express polymorphic properties (Mugglicockett and Stone, 1991). The DRB3 locus is expressed and is highly polymorphic (Sigurdardottir *et al.*, 1988). The DRB3 locus has also been reported to possess functional restriction elements (Burke *et al.*, 1991; Fraser *et al.*, 1996). Numerous haplotypes have been associated with the HLA-DRB locus, whereas BoLA-DRB3 has been reported as well-conserved (Sigurdardottir *et al.*, 1991), adding to the possible functional importance of this locus in cattle. Genetic polymorphisms are predominately within exon 2 of DRB3, which has been reported to encode the antigen-binding site (Ellegren *et al.*, 1993). DRB3, in particular alleles from exon 2, have been associated with traits involved in immunity, somatic cell score and mastitis incidence (Dietz *et al.*, 1997b).

1.12.3 Major Histocompatibility Complex and disease

Resistance to infection can be partially determined by genetics (Gavora and Spencer, 1983). Farmers select animals based on production traits: however higher yielding animals are associated with increased health costs (Jones *et al.*, 1994). Selection of animals for improved health traits can be problematic due to low heritability of some traits and limited availability of records of disease incidence (Starkenburg *et al.*, 1997).

The association between the MHC and infectious disease has been best defined by Marek's disease in chickens (Briles *et al.*, 1977; Hepkema *et al.*, 1993). Many B haplotypes have been associated with specificity of immune response and resistance to specific diseases (Kean *et al.*, 1994). The index used to gauge immune responsiveness combines humoral, cellular and reticuloendothelial aspects of the immune system (Kean *et al.*, 1994). In chickens, the B haplotype, B^{21}, has been shown to improve resistance to Marek's disease, as were the B^{2} and B^{6} haplotypes (Briles *et al.*, 1980). The B^{21} haplotype, as well as improved resistance, has been shown to promote increased antibody production (Martin *et al.*, 1990). The B^{19} haplotype has been reported to increase susceptibility to the disease as did the haplotypes B^{3}, B^{5}, B^{13} and B^{15} (Briles *et al.*, 1980).

Association between the MHC and subclinical bovine leukaemia virus infections (Lewin, 1988) and equine sarcoids (Lazary *et al.*, 1985; Meredith *et al.*, 1986) have also been reported.

Associations among production traits, health traits, and MHC class I alleles in cattle have been reported. These include percentage milk fat (Hines *et al.*, 1986), protein yield (Batra *et al.*, 1989), ketosis (Mejdell *et al.*, 1994), a reduction in the number of cases of mastitis and health costs attributed to increased milk yield and high fat content (Weigel *et al.*, 1990). Parasitic infection, determined by faecal worm egg counts (Stear *et al.*, 1988), and tick infestation (Stear *et al.*, 1989) have also been associated with MHC class I alleles. MHC class I genes have also been shown to have an association with the incidence of clinical mastitis (Oddgeirsson *et al.*, 1988; Mejdell *et al.*, 1994; Schukken *et al.*, 1994) and the susceptibility or resistance to mastitis (Solbu, 1983; Spooner *et al* 1988). The identification of allele BoLA-A14 has been linked to low SCC (Weigel *et al.*, 1990), to resistance to enzootic bovine leucosis (Lewin and Bernoco, 1986) and late seroconversion in those cattle infected with enzootic leucosis (Lewin and Bernoco, 1986; Palmer *et al.*, 1987). The effect elicited by this one allele highlights the fact that selection for a single trait may affect many other traits.

At least 170 BoLA-A-DRB3 haplotypes have been identified within the major breeds associated with both dairy and beef production (Lewin, 1996). Variation in haplotype among breeds was noted, however, detection of ancestral haplotypes was also observed. These ancestral haplotypes permit elucidation of the evolutionary relationship among breeds of cattle and relates them to the migratory patterns of humans over the years (MacHugh *et al.*, 1994).

Associations between BoLA MHC class II alleles and bull breeding values have been shown with respect to clinical mastitis, ketosis, retention of the placenta and milk fever (Lunden *et al.*, 1991). In one study in cattle, it was reported that allele DRB3.2*16 increased the susceptibility to mastitis as estimated by an increase in SCC (Dietz *et al.*, 1997a). In the same study, alleles DRB3.2*11, 12, 23 were suggested to improve resistance to clinical mastitis. Other studies have contradicted this previous work by reporting an association between allele DRB3.2*16 and a reduction in SCS in Holstein

cows in their first lactation (Sharif *et al.*, 1998a) and second lactation (Starkenburg *et al.*, 1997). Allele DRB3.2*23 reported previously to reduce the incidence of clinical mastitis was reported by others to increase the severity of the incidence of coliform mastitis (Sharif *et al.*, 1998a).

Evidence of heritability associated with immune response and the MHC has been reported previously in pigs (Mallard *et al.*, 1992) and in cattle (Detilleux *et al.*, 1994). The association in cattle has been reported with respect to BoLA class I alleles and measurable parameters of non-specific immunity (Weigel *et al.*, 1991). A significant association was also reported previously between immune parameters of BoLA MHC class II DRB alleles (Dietz *et al.*, 1997b). The suggestion from the study carried out by Dietz *et al.* (1997b) was that further studies were required to determine the interaction between the MHC class II alleles and the traits of innate and adaptive immunity.

Mastitis is a multi-factorial disease with complex aetiology (Anderson, 1983). Variation in susceptibility due to causative agent, environmental factors, genetics and the interaction of all of the above will contribute to the risk of an individual animal suffering from an IMI. Resistance or susceptibility to clinical mastitis by BoLA has been shown to vary among breeds. In Norwegian Red cattle, BoLA-A11 has been linked to susceptibility to clinical mastitis, whereas in Holstein and Danish Black Pied cattle, it has been linked to resistance to clinical mastitis (Weigel *et al.*, 1990), and low SCC, respectively (Aarestrup *et al.*, 1995). Levels of BoLA-MHC class II expression present on PBM has been shown to vary depending on the reproductive cycle. Levels of MHC class II on the monocytes of cows were shown to be highest prior to parturition and after parturition, with levels falling after calving (Van Kampen and Mallard, 1997). Selection of haplotypes for resistance to a particular disease, making the animals homozygous at the MHC loci would create holes in the peptide-binding repertoire (Lewin *et al.*, 1999). The implementation of haplocide, homozygosity at the MHC loci, may result in animals resistant to one pathogen and susceptible to another (Lewin, 1989). The complex aetiology of mastitis makes it very difficult to achieve absolute resistance to any infectious agent in an outbred species even in the well characterised MHC linked resistant loci in Marek's disease (Briles *et al.*, 1977).

1.12.4 Methods of Detection of Major Histocompatibility Complex Polymorphisms

Various methods for characterisation of polymorphisms in BoLA MHC class II genes and products have been described. For detection of protein polymorphisms, serology (Emery *et al.*, 1987; Williams *et al.*, 1991) and one-dimensional isoelectric focusing have been used (Watkins *et al.*, 1989; Glass *et al.*, 1992). To elucidate the genetic organisation of the class II genes RFLP (Sigurdardottir *et al.*, 1988), DNA sequence analysis (Groenen *et al.*, 1990; Bernoco *et al.*, 1991; Sigurdardottir *et al.*, 1991; Davis *et al.*, 1997), and allele specific oligonucleotide (ASO) hybridisation have been used (Sitte *et al.*, 1995). Availability of HLA sequence data on genes within the MHC allowed the development of a PCR based technique leading to sequence based HLA typing methods (Saiki *et al.*, 1988). These techniques used single stranded oligonucleotides and restriction endonucleases (PCR-RFLP), (Maeda *et al.*, 1989; Dekker and Easteal, 1990; Uryu *et al.*, 1990) for identification of polymorphisms in PCR amplified products from the HLA-MHC class II.

1.12.4.1 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

The combination of PCR and RFLP techniques saw the development of an extremely powerful tool for the determination of genetic polymorphisms in the functional domain of BoLA-DRB3 (van Eijk *et al.*, 1992; Russell *et al.*, 1997), combining simplicity and speed (Mallard *et al.*, 1999). The use of PCR-RFLP permitted analysis of multiple samples and animals identifying important allelic patterns in the β1 domain by enzymatic degradation (Maeda *et al.*, 1989; Dekker and Easteal, 1990; Uryu *et al.*, 1990). A possible drawback of PCR-RFLP when looking at heterozygotes is preferential allele amplification, which results from mismatching in the regions where the primers used for amplification anneal. Unwanted products of PCR which form when complementary strands of DNA pair up are known as heteroduplexes, and out-compete the oligonucleotides for hybridisation with their template strand. The interference of heteroduplexes is restricted to those that form during the final annealing step within the PCR, as others formed prior to this will dissociate. Work carried out by Van Eijk *et al.* (1992) used PCR-RFLP to successfully characterise 14 alleles which had been previously identified by DNA sequencing (Sigurdardottir *et al.*, 1991).

1.12.4.2 Advances in Methodology for the Detection of MHC Polymorphisms

The development of microsatellite haplotyping (Ellegren *et al.*, 1993) has reported a technique that is as powerful as PCR-RFLP, and extremely useful in large scale MHC disease association studies related to outbred populations as well as paternity testing (van Haeringen *et al.*, 1999). Denaturing Gradient Gel Electrophoresis (DGGE) has been reported as having an increased sensitivity and specificity for identification of new BoLA alleles (Aldridge *et al.*, 1998). The technique is rapid and allows sequencing without the requirement for cloning, and is applicable to other gene loci (Myers *et al.*, 1988; Weber, 1991; Lessa, 1993).

1.13 Dairy Cattle Breeding

Improvement in management practices has resulted in a significant reduction in the incidence of clinical and subclinical mastitis in the UK (Booth, 1988). Increasing costs associated with treatment of cases of mastitis and implementation of milk quality penalties has resulted in a need to breed cows that have a lower incidence of mastitis and lower SCC. It has been calculated that an increase of 100,000 cells/ml milk over an average BMSCC of 200,000 cells/ml milk results in a 2.5% reduction in milk yield (Edmondson, 1995). As well as the production benefits associated with breeding for lower SCC, inclusion of SCC in breeding programmes has the potential for improved control of subclinical and clinical mastitis (Coffey *et al.*, 1986).

Presently, selection of dairy cattle for breeding worldwide is based mainly on profitability. The selection of dairy cattle in the UK utilises two indices; Profit Index Number (PIN) and Index of Total Economic Merit (ITEM). The PIN is a genetic index with economic weighting, which incorporates production traits including milk yield, and percentage of milk solids (Animal Data Centre, Fox Talbot House, Chippenham, Wiltshire). The PIN reflects the expected rise in revenue for each daughter's lactation, compared to an animal with a PIN value of zero. These calculations are based on expected future prices for milk, quota, transportation and feed requirements (Holstein-Friesian Society, www.hfs-gbi.org).

The ITEM, introduced for dairy cows and bulls in the UK in 1995 (Veerkamp *et al.*, 1995), includes production traits as in the PIN, and the main type traits associated with longevity, namely udder depth, teat length, angularity and hoof angle (Brotherstone and Hill, 1991). Clearance of the udder from the ground and tighter fore udder attachment have been correlated to a reduction in log SCC, somatic cell score (SCS), and subsequently a reduction in the incidence of clinical mastitis (Rogers *et al.*, 1991; Lund and Jensen, 1996; Boettcher *et al.*, 1998). The traits included in the ITEM have also been associated with lameness in cattle as well as contributing to economic profit and longevity (Rogers, 1993). Estimation of type traits is carried out using multi-trait linear models (Brotherstone and Hill, 1991; Jairath *et al.*, 1998), or alternatively the threshold model (Gianola, 1982) for higher estimates of heritability than the linear model (Weller and Ron, 1992).

More recently, the Profitable Life Index (PLI) has been published. This index assesses longevity through direct measurements utilising those type traits, which directly affect the life span of dairy cows (Brotherstone *et al.*, 1997). Low milk production, poor reproduction and a high incidence of mastitis have been cited as the top three reasons for premature culling from the dairy herd (Allaire *et al.*, 1977). Enhanced longevity or herd life of dairy cattle is of economic importance (Rendel and Robertson, 1950), as it is associated with a reduction in culling (van Arendonk, 1985) and reduces the costs associated with purchasing and raising replacement animals (Boettcher *et al.*, 1999). It has been estimated that longevity accounts for 25-70% of the economic value attributed to milk production (Allaire and Gibson, 1992; Weller and Ron, 1992).

Selection of dairy cattle based on production traits has been extremely successful, resulting on average in an improved annual yield of approximately 2-3% (Honkenboken, 1987). However selection for improved udder health has been sacrificed for improvement in production traits (Rogers *et al.*, 1998). Studies have shown that selection for increased milk yield may result in tissue damage in the mammary gland resulting in a reduction in milk yield (Mrode *et al.*, 1998). Recent studies have reported a reduction in milk yield of 1.29kg/day in young cows and 2.04kg/day in older cows with each $\log_{10}$ increase in SCC (Koldeweij *et al.*, 1999). The damage sustained by mammary tissue may

lead to an increase in susceptibility to subclinical mastitis (Arriens *et al.*, 1994). An unfavourable genetic correlation has been reported between protein yield and udder health in Danish and Swedish Holstein cattle (Emanuelson *et al.*, 1988; Shultz *et al.*, 1993; Rogers *et al.*, 1998). An unfavourable genetic correlation has also been reported between production traits, and subclinical and clinical mastitis (Mrode and Swanson, 1996; Poso and Mantysaari, 1996; Biochard and Rupp, 1997), as well as high milking speed and subclinical SCC (Seykora and McDaniel, 1986; Boettcher *et al.*, 1998). The genetic correlation between productive life, longevity, and udder conformation traits, especially with regard to clinical rather than subclinical mastitis is favourable (Shook, 1989; Rogers *et al.*, 1991; Boettcher *et al.*, 1992). Improvement in disease resistance, in addition to benefiting general animal health and welfare, has been shown to enhance the efficiency of vaccine administration (Lamont, 1989).

Selection of traits for inclusion in breeding programmes should carry sufficient genetic variability and heritability as well as provide economic benefit. The traits for inclusion should be measurable at a low or reasonable cost to maximise economic gain. Around 80% of the UK dairy farms, as well as recording BMSCC, have already opted to record individual cow SCC on a monthly basis (Mrode and Swanson, 1996). Research is being undertaken to assess the economic and welfare importance of including SCC proofs breeding programmes (McGuirk, 1998). It has been suggested that inclusion of PTA for SCC into the PIN would be of most benefit, where SCC could be directly compared and contrasted with production and other traits (Animal Data Centre).

Genetic variation associated with disease resistance in dairy cattle, with direct relevance to mastitis has been reviewed as early as 1952 (Legates and Grinnells). Direct selection of animals, which possess enhanced disease resistance with regard to mastitis, is hampered by the lack of accurate and standard recording of clinical mastitis incidence. Collection of data is both difficult and costly (Mrode *et al.*, 1998), and presently there is no routine data collection for clinical mastitis incidence within the UK. The heritability of clinical mastitis based on direct selection is estimated at approximately four percent (Mrode and Swanson, 1996).

Many of the traits used for selection of dairy cows are heritable. By definition, heritability of a trait, is the percentage of the observed variation attributed to genetics, or the proportion of the difference among individuals that should be transmitted to their progeny. The definition could also encompass the reliability associated with an individual's phenotype in predicting genetic merit for the quantitative trait in question (Hohenboken *et al.*, 1987). Type traits and production traits are classed as highly heritable, with udder traits classed as moderately heritable (Ashwell *et al.*, 1996). In comparison with type traits and production traits, health traits, such as foot angle and feet and leg scores are classed as having relatively low heritability (Starkenburg *et al.*, 1997). Lack of data on disease incidence restricts their potential for use in genetic improvement of dairy cattle (Starkenburg *et al.*, 1997).

The low heritability of clinical mastitis and the lack of reliable and available data have resulted in selection of animals with improved disease resistance based on indirect methods. Udder type traits, teat structure, SCC and SCS have been identified as traits that allow for selection of improved udder health (Seykora and McDaniel, 1986; Emanuelson *et al.*, 1988; Rogers, 1993; Philipsson *et al.*, 1995).

Somatic cell counts are considered the most suitable single trait for indirect selection of animals for mastitis resistance. A positive link between the level of SCC and clinical mastitis has resulted in this trait being classed as a genetic marker of disease resistance (Aarestrup *et al.*, 1995; Ashwell *et al.*, 1996; Dietz *et al.*, 1997a). The strong positive correlation between mastitis and SCC has been reported as 0.7 (Phillpsson *et al.*, 1995; Mrode and Swanson, 1996).

Variations in heritability estimates of SCC are reported among breeds. Heritability estimates for SCC in Holstein-Friesians approximate to 11% (Schultz *et al.*, 1990; Rogers *et al.*, 1991; Da *et al.*, 1992), greater than those reported for Ayrshires, Guernseys and Jerseys (Schults *et al.*, 1995). Variation between lactations was reported as minimal, suggesting that the occurrence of genetic and residual variation was similar across parity (Monardes and Hayes, 1985; Reents *et al.*, 1995). Other researches have reported improvements in heritability as parity increased (Coffey *et al.*, 1986) whilst others

reported reductions in heritability with increasing lactations, based on a reduction in sire variance and an increase in residual variation (Banos and Shook, 1990).

In February 1998, the Animal Data Centre published predicted transmitting ability (PTA) data for SCC. Predicted transmitting abilities reflect the effect of sire genes on the trait being measured. In terms of sire PTA for SCC they express the genetic merit of individual bulls and the predicted impact they are likely to exert on SCC of their daughters (McGuirk, 1998). Sire PTA for SCC is calculated from the mean lactational records of their daughters. Each daughter included in the evaluation requires SCC records for her first three lactations with a minimum of six test records from each lactation. An animal model, similar to that used for evaluation of production traits in the UK was used to measure the genetic potential of SCC as an indicator of resistance or susceptibility to mastitis (Animal Data Centre, 1996). The data generated from monthly milk SCC records was highly skewed, requiring a logarithmic transformation of the data prior to statistical analysis. Logarithmic transformation of the data resulted in normal distribution of the data allowing the use of statistical methodology. Various logarithmic transformations have been carried out for example transformation to $\log_e$ (Heuven *et al.*, 1988), $\log_2$ or $\log_{10}$ (Weller *et al.*, 1992; Da *et al.*, 1992). A review carried out by Mrode and Swanson (1996) reported no effect due to type of logarithmic transformation carried out on estimates of genetic parameters.

Almost all PTA for SCC fall between +/-25%, with 86% bulls and 96% cows being +/-12% (Mrode *et al.*, 1998). A negative PTA for SCC indicates that a bull should transmit a reduction in SCC to his progeny and, conversely, a positive PTA for SCC should result in a bull transmitting an increase in SCC to his progeny. Estimation of PTA for SCC based on progeny testing provides an accurate prediction of a sire's genetic merit if sufficient daughters are recorded. Using monthly SCC records to calculate this effect of sire reflects the on-farm situation, as data is based on commercial herds under typical management conditions.

Unfortunately, progeny testing schemes are both labour and cost intensive with a minimum of four years to generate reliable data. The development of new methods for evaluating a bull's potential as a sire are needed to eliminate the bulls which would

normally be removed from AI programmes after the four years of progeny testing much earlier. This would reduce costs associated with rearing as well as improving the quality of daughters produced by the bull selected for AI.

Aim of the current study

The aim of the current study is to develop an *in vitro* immunological assay to measure the proliferative response of PBM induced by *Staphylococcus aureus* in Holstein-Friesian cattle and to identify the proportion of cell types present in peripheral blood mononuclear cells (PBM) and in the proliferating cell population.

At present in the UK, progeny testing schemes are carried out to evaluate bulls' potential as sires. This method is both labour and cost intensive with a minimum of four years required to generate reliable data. The aim of the current study is to identify an *in vitro* immunological assay that may be used as a means of identifying individual bulls with strong immune responses induced by *S. aureus*, a contagious mastitis pathogen. The hypothesis to be tested is that bulls with strong *in vitro* proliferative responses induced by *S. aureus* may have daughters that have low somatic cell counts due to increased resistance to intramammary infection with *S. aureus*. The development of an immunological marker that could be measured in blood and that would identify resistance/susceptibility to mastitis pathogens would potentially reduce the time associated with progeny testing and increase the efficiency of bull selection.

In addition to testing the association between the proliferative response in bulls and their Predicted Transmitting Ability (PTA) for somatic cell count (SCC), the aim of the current study is to look for associations between the proliferative response, or PTA for SCC, and Major Histocompatibility Complex class II alleles by DNA cloning and sequencing of exon 2 and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism.

Chapter II

Materials and Methods

2.1 Animals

Over a four-year period, the proliferative response of peripheral blood mononuclear cells (PBM) to antigen was measured in 30 Holstein-Friesian cows from a commercial dairy herd.

In addition ten cows were selected as two progeny groups, five were sired by bull A and five were sired by bull B. These bulls were commercial artificial insemination (AI) dairy bulls, whose semen was purchased from Scottish Livestock Services. None of the cows were suffering from, or were recorded as having any recent cases of mastitis and all were in general good health.

The proliferative assay was also performed on bulls from four bull breeding establishments owned by Genus Ltd. These bulls were selected by age, as only those aged four years and above have PTA data for SCC, available from progeny testing. Ten bulls were chosen to represent extremes of the PTA for SCC: five bulls had the lowest PTA for SCC and five bulls had the highest PTA for SCC. A further 103 bulls were selected at random to represent the population of bulls held in bull breeding establishments owned by Genus Ltd. A random sample of 50 bulls from the total 103 bulls was re-sampled to test the repeatability of the *in vitro* proliferation assay.

2.2 Cell isolation

2.2.1 Isolation of peripheral blood mononuclear cells

Peripheral blood was collected either from the jugular or coccygeal vein of cattle into sterile ten millilitres (ml) heparinised vacutainers (Becton Dickinson UK Ltd., Cowley, Oxford). Twenty mls of blood was mixed with ten mls Hanks Balanced Salt Solution

(HBSS) without calcium chloride and magnesium sulphate (Gibco and Sigma) in a 50ml polystyrene tube (Sterilin, Bibby Sterilin Ltd, Staffordshire). This mixture was underlaid with 20ml of ficoll-isopaque, density 1.077g/l (Sigma). Samples were centrifuged for 40 minutes at 800g at 20°C with the brake off. The cells present at the interface between the ficoll-isopaque and media were aspirated using sterile plastic pastettes and washed twice in HBSS for ten minutes at 600g at 20°C. Cell pellets were re-suspended in two mls of complete BME media containing 2% autologous serum, with cell numbers counted using white cell counting fluid (WCCF) as described later.

2.2.2 Isolation of peripheral T lymphocytes using miniMACS separation

Peripheral blood mononuclear cells, isolated by density centrifugation as described later, were counted using White Cell Counting Fluid (WCCF). Thirty million cells were incubated with an anti bovine MHC class II MAb, IL-A21, (ECACC) diluted 1:4 and then mixed gently and incubated for one hour on ice. After primary incubation, the cells were washed in two ml of sterile phosphate buffered saline (PBS) for ten minutes at 600g. This centrifugation step was repeated twice more. Goat anti-mouse IgG microbeads (Miltenyi Biotech, Surrey) at a 1:4 dilution were added to the cell pellet and incubated for 30 minutes at 4°C. MiniMACS MS column (Miltenyi Biotech) with a cell capacity of 10^7, was attached to the magnetic stand. Five hundred microlitres (µl) of sterile PBS was allowed to run through the column, followed by the total volume of labelled cells. Cells were washed through with 500ul of sterile PBS and this was collected at the bottom in a separate bijou and deemed the MHC class II-negative fraction. One ml of PBS was washed though the column and collected into a separate bijou, and was classified as the wash fraction. The miniMACS column was then removed from the magnetic stand. One ml of PBS was forced through with a plunger, this procedure was repeated, to ensure removal of all bound cells from column, and was deemed the MHC class II-positive fraction. All fractions were counted using WCCF to ensure that most of the cells had been eluted by this method of positive selection. The positive fraction contained the cells used as the APC population, and the negative fraction contained the cells used as the responding lymphocyte population in the *in vitro* proliferation assay as described later.

2.2.3 Isolation of total peripheral blood leucocytes

Peripheral blood was collected either from the jugular or coccygeal veins of cattle into sterile ten mls heparinised vacutainers (Becton Dickinson UK Ltd.). Two mls of blood was mixed with 40mls of Erythrolyse (Serotec), to lyse the red blood cell population, in a 50ml polystyrene tube (Sterilin, Bibby Sterilin Ltd). Samples were mixed thoroughly, incubated at room temperature for ten minutes then centrifuged for ten minutes at 600g at 20°C. The supernatants were discarded and the cell pellets washed twice in HBSS without calcium chloride and magnesium sulphate (Gibco and Sigma) for ten minutes at 600g at 20°C. Cell pellets were re-suspended in two mls of complete BME media containing 2% autologous serum, with cell numbers counted using WCCF as described later.

2.3.4 Isolation of milk mononuclear cells

Cows' teats were washed with warm water then cleaned further with cotton wool soaked in methanol. The first few draws of foremilk were discarded, as this sample is unrepresentative of cell numbers present in the milk. Between 800ml and 1000ml of milk was collected either through hand stripping of all four quarters or by machine milking into an individual jar. One hundred mls of milk was diluted 50:50 with sterile PBS (10mM at pH 7.4, Sigma) supplemented with 15mM ethylenediaminetetraacetic acid (EDTA, Sigma) and 20% acid citrate dextrose (ACD) in polypropylene tubes (Nalgene Nunc International, Fisher Scientific Ltd, Leicestershire). Samples were centrifuged for 30 minutes at 900g at +20°C. The remaining samples were stored at +4°C as only four samples could be centrifuged at any one time. Cell pellets were transferred to sterile 50ml tubes (Sterilin) and made up to 50ml with PBS/EDTA/ACD. Samples were centrifuged for 15 minutes at 600g at +20°C, the cell pellets were re-suspended, PBS/EDTA/ACD added and centrifuged as above to remove as much milk fat and debris as possible. The cell pellets were re-suspended in 30ml PBS/EDTA/ACD and underlaid with 20ml ficoll-isopaque, density 1.077g/l (Sigma) and centrifuged for 40 minutes at 800g at +20°C with the brake off. The layer of mononuclear cells present at the interface was aspirated and washed twice in PBS/EDTA/ACD. Cell pellets were washed and re-

suspended in one or two mls of PBS/EDTA/ACD and cell viability was assessed using trypan blue as described later (Sigma).

2.3.5 Isolation of total milk leucocytes

Cells were isolated from whole milk as described for the isolation of milk mononuclear cells. The isolated cell pellet was not underlaid with ficoll-isopaque. Cell pellets were then transferred to sterile 50ml tubes (Sterilin) and made up to 50ml with PBS/EDTA/ACD. Samples were centrifuged for 15 minutes at 600g at +20°C, the cell pellets were re-suspended, PBS/EDTA/ACD added and centrifuged as above to remove as much milk fat and debris as possible. The cell pellets were washed twice in PBS/EDTA/ACD. Cell pellets were washed and re-suspended in one or two mls of PBS/EDTA/ACD and cell viability was assessed using trypan blue as described later (Sigma).

2.3 Counting and assessment of viability of cell preparations

2.3.1 Preparation of white cell counting fluid for estimation of peripheral blood mononuclear cell numbers

White cell counting fluid a 2% solution of acetic acid (BDH, Poole, Dorset) in distilled water with gentian violet for colour staining was used to perform cell counts on the PBM cell population. White cell counting fluid results in the lysis of erythrocytes permitting counting of the remaining leucocytes to be carried out accurately. Fifty µl of cell suspension was diluted in 450µl of WCCF, then mounted and counted on a Neubauer haemocytometer (Weber Scientific International Ltd., Hamilton, USA).

2.3.2 Preparation of trypan blue for estimation of viability of peripheral blood mononuclear cells

Trypan blue (Sigma, Poole Dorset) was used to assess the viability of both milk and PBM cell populations. Fifty µl of cell suspension was mixed 1:1 with trypan blue, viability and

cell numbers were assessed and counted on a Neubauer haemocytometer (Weber Scientific International Ltd.).

2.4 Identification of subclinical mastitis

2.4.1 Estimation of the infection status of milk using the California Mastitis Test

The California Mastitis Test (CMT, Genus Animal Health, Llanelli, Wales) permitted rapid semi-quantitative evaluation of cell counts in milk. The teats and udder were washed with warm water and dried with separate disposable towels. The first two or three draws of foremilk were stripped out and discarded. Approximately five to ten mls of milk, from each quarter was added to the four individual paddles of the CMT test tray. The same volume of CMT reagent was added to the milk. The test tray was swirled in a horizontal circular movement for five to ten seconds to ensure adequate mixing. A negative result indicated a low SCC with the mixture of milk and CMT reagent moving freely. A trace result indicated a slight increase in SCC, with the viscosity of the liquid altering and becoming less mobile. A positive result represented a high SCC, the viscosity of the mixture increased, characteristic of a gel rather than a liquid.

2.4.2 Somatic cell counting

Twenty mls of milk was sent to the Glasgow University Veterinary School (GUVS) haematology laboratory to determine SCC/litre of whole milk. Cells were fixed initially with Somafix® (Coulter Electronics, Luton) for 25 minutes at 56°C. Samples were allowed to cool to +20°C, then diluted 1/100 with Somaton® (Coulter) and heated for 12 minutes at +80°C. The samples were allowed to cool again to +20°C. The cells were counted on a Coulter counter, model ZF (Coulter). This acted as a gold standard for SCC, allowing an estimation of the proportion of cells isolated following the milk mononuclear cell isolation technique.

2.5 Phenotyping of peripheral blood mononuclear and milk mononuclear cells

Peripheral blood cells, PBM, milk polymorphonuclear cells and milk mononuclear cells were isolated as described previously. The cell pellet was re-suspended in four mls HBSS without calcium chloride and magnesium sulphate (Sigma) in a 50ml tube (Sterilin) and 0.4% paraformaldehyde (Sigma) was added to the cells at a ratio of 1:1 vol/vol. Samples were mixed and incubated for four minutes at +20°C. The samples were made up to 50ml with sterile PBS (Sigma) supplemented with 1% bovine serum albumin (BSA, Sigma) and 0.1% sodium azide (Sigma). Samples were centrifuged for ten minutes at 600g at +4°C. The supernatants were discarded and the cell pellets re-suspended in 50ml of PBS/BSA/sodium azide and the above centrifugation step repeated to remove any remaining debris. Cell pellets were re-suspended in three mls of PBS/BSA/sodium azide and transferred to a five mls tube (Falcon 2054, Becton Dickinson UK Ltd.). Samples were centrifuged for five minutes at 200g at +4°C. Once the supernatant was discarded, the lip of each tube was carefully blotted dry to remove any excess liquid. The cell pellets were re-suspended and the above centrifugation step repeated in fresh PBS/BSA/sodium azide. The supernatants were decanted and one ml of PBS/BSA/sodium azide added. Cell counts were carried out using trypan blue (Sigma) as described before and $0.5x10^6$ cells added to one tube for each monoclonal antibody (MAb) used. The primary MAb were added to the cell pellet at a dilution of 1/10 in PBS/BSA/sodium azide. Tubes were mixed gently and incubated for 30 minutes at +4°C. Three mls of PBS/BSA/sodium azide was added and the samples centrifuged for five minutes at 200g at +4°C, the cell pellets were re-suspended and the centrifugation repeated twice to remove any unbound primary antibody. The secondary antibody, rabbit anti-mouse fluorescein isothiocyanate (FITC, Dako A/S, Denmark) was added at 1:15 dilution in PBS/BSA/sodium azide and incubated for 40 minutes at +4°C. The cells were re-suspended in PBS/BSA/sodium azide and centrifuged twice as described above. Five hundred µl of 1% paraformaldehyde was added to each tube containing cells. Samples were stored at +4°C wrapped in tin foil and analysed by flow cytometry within two days of cell isolation.

All MAb used for flow cytometry and anti-MHC class II inhibition studies were aliquoted and stored at -20°C until required. The MAb used during the course of this study were all

purchased from the European Collection of Animal Cell Culture (ECACC, Salisbury, Wiltshire).

2.5.1 Anti-BoCD2

The cell surface protein molecule CD2 is found expressed on T lymphocytes, thymocytes, and natural killer (NK) cells and is regarded as a pan T cell marker. In addition to acting as a marker for T cells expressing a α/β T cell receptors (TCR), it is also been reported on γ/δ peripheral T cells in humans (Inghirami *et al.*, 1990), yet it has not been reported on γ/δ peripheral T cells in cattle (Clevers *et al.*, 1990). BoCD2 acts as an adherence molecule and on interaction with its ligand LFA-3 (CD58), enhances the binding of T cells to other cells. The CD2 marker has been reported previously on approximately 40-60% bovine peripheral blood T cells (Asai *et al.*, 1998). The cell line IL-A21 was used to generate the monoclonal antibody (MAb) which is directed against BoCD2 (ECACC). The antibody class of this MAb was IgG.

2.5.2 Anti-BoCD4

BoCD4 is expressed on the cell surface of T helper lymphocytes. Functionally $BoCD4^+$ cells are co-receptors for MHC class II molecules, present on the surface of antigen presenting cells (APC). The interaction between CD4, MHC class II and the TCR improves T cell binding to the APC. Cells expressing CD4 also partake in signal transduction, whereby a signal is passed from the APC via the receptor into the lymphocyte (Benjamin *et al.*, 1996). $CD4^+$ T cells account for approximately 15-35% of PBM in cattle (Bensaid and Hadam, 1991). Cell line AFRC IAH-CC30 produces the MAb CC30, which is directed against $BoCD4^+$ cells (ECACC). The antibody class of this MAb was IgG.

2.5.3 Anti-BoCD8

BoCD8 is expressed on the cell surface of T cytotoxic cells. T cytotoxic cells are responsible for elimination of cells infected with intracellular pathogens. BoCD8 acts as a co-receptor for MHC class I molecules, which as described previously, enhance binding

of APC and lymphocytes binding. $CD8^+$ T cells account for approximately 11-22% of PBM in cattle (Taylor *et al.*, 1994; Shafer-Weaver *et al.*, 1999). MAb CC58 derived from cell line AFRC IAH-CC58 was used for the detection of BoCD8 (ECACC). The antibody class of this MAb was IgG.

2.5.4 Anti-monocyte

Monocytes, present in peripheral blood, are the immature precursor of macrophages, prior to their migration into tissue. Monocytes account for approximately 20% of PBM in cattle (Shafer-Weaver *et al.*, 1999). Cell line IL-A24 was used to raise the MAb directed against bovine myeloid cells. This MAb reacts with bovine granulocytes and monocytes and some differentiated mononuclear phagocytes (ECACC). The antibody class of this MAb was IgG.

2.5.5 Anti-BoCD21

BoCD21, formerly WC3, was renamed due to similarities in size and cellular distribution as the human CD21 molecule (Naessen and Howard, 1991). It is expressed on the surface of follicular dendritic and B cells. B-lymphocytes are essentially responsible for the production of antibodies to extracellular pathogens, and possess the ability to function as APC. CD21 can act as a co-stimulatory molecule for B cell activation as well as enhancing signal transduction post antigen binding. B cells represent approximately 16-21% total lymphocyte numbers in cattle (Park *et al.*, 1992). Cell line AFRC IAH-CC21 was used to raise the antibody directed against the surface CD21 molecule (ECACC). The antibody class of this MAb was IgG.

2.5.6 Anti-BoWC1

The cell surface marker WC1 is expressed on γ/δ T cells (Mackay *et al.*, 1991). γ/δ T cells generally lack those cell surface markers associated with αβ T cells and their phenotype has been reported as $CD2^-/CD4^-/CD8^-$. This molecule remains defined as a workshop cluster, WC, as no CD homologue has yet been found. These cells represent approximately 1-3% of human and mouse peripheral T cells, however in young

ruminants, approximately 27% of T cells express a γ/δ TCR with some individual animals having greater than 50% of cells expressing γ/δ^+ TCR (Clevers *et al.*, 1990). In adult ruminants the number of γ/δ T cells declines to approximately 5-10% of PBM (Clevers *et al.*, 1990). The function of γ/δ T cells still remains unclear, although results from *in vivo* depletion studies suggest that this cell population may have a role to play in control of proliferation of other lymphocytes (Howard *et al.*, 1989). It has not been fully established if they may also have a cytolytic role in the immune system (Mackay, 1988). Cell line AFRC IAH-CC15 was used to produce the MAb CC15, which is directed against BoWC1 (ECACC). The antibody class of this MAb was IgG.

2.5.7 Anti-MHC class II

The response of T cells to antigenic stimuli has been described as MHC restricted (Zinkernagel and Doherty, 1974). This cell surface molecule is of vital importance in antigen presentation to the TCR on the corresponding lymphocyte. Only antigenic peptides expressed in association with a MHC molecule will be recognised by the TCR and its co-stimulatory molecule either CD4 or CD8. $CD4^+$ T cells recognise antigen in association with MHC class II molecules and conversely $CD8^+$ T cells recognise MHC class I molecules. MHC class II molecules are expressed on the surfaces of B cells, dendritic and thymic epithelial cells (Wiman *et al.*, 1976), however activated T cells, macrophages and endothelial cells can be induced to express these molecules on their surface (Sleeg *et al.*, 1982). Cell line IL-A21 was used to raise this monomorphic antibody directed against Bovine MHC class II molecules (ECACC). The antibody class of this MAb was IgG.

2.5.8 Control antibody

The MAb MIL-4, which detects porcine granulocytes (Haverson *et al.*, 1994), was used as a control antibody to ensure that no non-specific binding of the secondary fluorescent MAb was occurring. The antibody class of this MAb was IgG.

2.6 *In vitro* culture

2.6.1 Preparation of autologous serum for *in vitro* culture of peripheral blood mononuclear cells

Twenty mls of blood was collected either from the jugular or coccygeal vein by vacutainer (Becton Dickinson UK Ltd.), from each individual cow. The samples were allowed to clot at +20°C then incubated for 30 minutes at +37°C, followed by a further 30 minutes at +4°C, to improve clot retraction. The samples were then centrifuged for 20 minutes at 1300g, the serum collected by aspiration was heat inactivated for 30 minutes at +56°C. Sera were stored at -20°C until required.

2.6.2 Preparation of media for *in vitro* culture of peripheral blood mononuclear cells

Basal media, Eagles (BME, Gibco, Life Technologies Ltd., Paisley) with Earles salts and without L-glutamine (Gibco) was used for all antigen specific experiments. The media was supplemented with 2mM L-glutamine (Gibco), 20mM Hepes (Gibco), 50µg/50units/ml of streptomycin/penicillin (Gibco) and 2% heat inactivated autologous serum as described previously.

2.7 Preparation of antigen for *in vitro* culture

2.7.1 Isolation of *Staphylococcus aureus* from naturally occurring subclinical mastitis cases

Milk samples were taken from four adult Friesian dairy cows with subclinical mastitis, detected by the presence of high SCC. These cows were identified initially as having ICSCC (>400,000 cells/ml) through routine monthly milk sampling. Infected quarters were subsequently identified as having IQSCC (>600,000 cells/ml).

Routine bacteriological examination of milk samples from infected quarters resulted in isolation of *S. aureus*. For determination of bacterial strains present, three mls of milk was diluted in 17mls sterile distilled water (Baxters Healthcare Ltd., Baillieston).

Samples were centrifuged for 20 minutes at 4500g. Cell pellets were re-suspended in 100µl lauryl broth then 50ul was streaked onto Columbia blood and Mannitol salt agar plates (Oxoid Ltd. Ltd., Basingstoke). The agar plates were incubated overnight at +37°C. Fifteen to 20 individual colonies were tested using a coagulase slide test (Rapidec bioMerieux, France) and DNA fingerprinting. Genomic fingerprinting resulted in one strain of *S. aureus*, designated as strain A being isolated from the two cows from farm A. Colonies isolated from the cows originating from farm B, belonged to a single strain different to those from farm A, designated strain B. After identification, both strains were subcultured onto Columbia blood agar (Oxoid Ltd.) and incubated for 18 to 24 hours at +37°C. Single colonies were inoculated into ten mls of brain heart infusion (BHI) broth and incubated for 18-24 hours at +37°C, 360µl of bacterial suspension from the BHI broth was added to 60µl of glycerol in a 1.5ml micro-centrifuge tube. The glycerol stocks were mixed thoroughly then stored until further batches of bacterial antigen were required at -20°C. The bacterial suspension was then centrifuged for 15 minutes at 1000g, re-suspended, and washed four times in sterile PBS to remove any growth medium or by-products. Bacterial cell pellets were inactivated by incubation with constant stirring in 2% formalin for 48 hours at +4°C. To remove any traces of formalin, the pellets were washed four times in sterile PBS. The cell pellets were re-suspended, aliquoted and stored until required at -20°C. The fixed bacterial suspensions were streaked on to sheep blood agar (Oxoid Ltd.) for four days at +37°C, to ensure no bacterial growth occurred.

2.7.2 Preparation of *Staphylococcus aureus* from glycerol stocks

A single loop of *S. aureus* glycerol stock was streaked onto a sheep blood agar plate (Oxoid Ltd.), and incubated for 18-24 hours at +37°C. A single colony from the sheep blood agar plate was inoculated into 200ml of BHI broth and incubated for 18-24 hours at +37°C. The suspension was then treated as before.

2.7.3 Collection of milk samples from cull cows

The udders of 14 cows sent to the abattoir under the BSE cull scheme were selected at random. The age of these animals was not known although it may be assumed that they were older than 30 months and that they were mature cows. The teats were washed with an antibacterial compound (Hibiscrub, Zeneca Ltd, UK) diluted in warm water, then cleaned with methanol-soaked cotton wool. The condition of the selected udders varied considerably. Some of the udders showed obvious signs of abscessation, whilst others appeared visually normal. The first draw of milk from the teat was discarded then a ten ml milk sample was collected in a sterile universal (Sterilin) from one quarter only. These samples were collected as a possible source of additional strains of *S. aureus*, for use in the *in vitro* proliferation assay.

2.7.4 Bacteriological examination of milk samples

The milk samples obtained from the cull cows were streaked aseptically onto MacConkeys, chocolate, sheep blood and horse blood agar plates (Oxoid Ltd.). MacConkeys and sheep blood plates were incubated aerobically at +37°C, chocolate plates were incubated in the CO_2 incubator and horse blood plates were incubated anaerobically (Don Whitley, Mark 3 anaerobic workstation). After a 24-hour incubation, plates were checked for growth and those samples identified as staphylococci had single colonies removed and streaked onto fresh sheep blood agar plates. A single colony from each of the possible staphylococci was smeared onto DNAse sensitive agar (Oxoid Ltd.), allowing identification of staphylococci as a genus rather than individual strains. After 24 hours incubation, 10% hydrochloric acid was added to the DNAse plates, a transparent halo appeared around DNAse producing colonies. Non-DNAse producing bacteria were discarded at this point. Those isolates identified as DNAse producers were identified further by API staphylococcal strips (bioMerieux). Interpretation of the API staphylococcal strips creates a five-digit profile number, which can be used to determine the strain of the bacterium using the API profile index. The API profile index documents micro-organisms that have previously been identified. Once positively identified as *S. aureus*, colonies were maintained on Dorset egg slopes and prepared as described previously for use in *in vitro* culture.

2.7.5 Estimation of *Staphylococcus aureus* concentration

An aliquot of formalin fixed *S. aureus* was sent to GUVS haematology laboratory to determine bacterial cell numbers per ml of PBS. The bacterial cell preparation was allowed to settle prior to counting to eliminate the chance of floating cells being recounted. The estimation of bacterial cell numbers was carried out in a Neubauer haemocytometer (Weber Scientific International Ltd.) under X400 magnification.

2.7.6 Genomic fingerprinting of staphylococci

Sheep Blood agar plates (Oxoid Ltd.) were streaked aseptically with *S. aureus* and incubated aerobically overnight at +37°C. Ten mls of BHI broth (Oxoid Ltd.) was inoculated with a single colony from the streak plate and incubated once again overnight under the same conditions. The broth was centrifuged for ten minutes at 2000g. The supernatants were discarded and the pellets re-suspended in three mls of tris EDTA NaCl (TES) and subsequently divided into one ml aliquots. Samples were micro-centrifuged for 30 seconds at 140g and the supernatants discarded. The pellets were re-suspended in 200µl of TES supplemented with 50mM sucrose (Sigma). Six percent lysostaphin (1000units/ml, Sigma) and 31% lysozyme (40mg/ml, Sigma) were added and the samples vortex mixed, followed by incubation for 30 minutes at +37°C. Fifteen microlitres of 20% SDS was added, and the micro-centrifuge tubes inverted, gently, approximately a dozen times. Thirteen percent proteinase K (50mg/ml, Boehringer Mannheim) was added and the solution was sheared twice through a 25-gauge needle, at a constant rate. Samples were then incubated for two hours at +37°C. Twenty percent TE_{10} was added to make the aqueous layer less viscous plus 50% of 1:1 (vol:vol) phenol: chloroform (Sigma). The samples were mixed then centrifuged for ten minutes at 140g. The upper aqueous layer was aspirated and transferred to a sterile micro-centrifuge tube, which contained 500µl of isopropanol (Sigma). The samples were left for approximately one hour at +20°C, then centrifuged for ten minutes at 140g and the supernatants discarded. One hundred µl of TE_{10} was added and the samples left to re-dissolve at +20°C. The triplicate samples were pooled and approximately 10% of 7.5M ammonium acetate and 60% of 95% ethanol were added. The samples were mixed and stored overnight at -20°C.

The samples were centrifuged for ten minutes at 140g, to pellet the DNA, the supernatants were discarded and the pellets re-suspended in 300µl TE_{10} to which 6% of freshly prepared RNAse (10mg/ml, Sigma) was added. The samples were incubated for one hour at +37°C and 60% of 1:1 (vol:vol) phenol chloroform (Sigma) was added and the samples were mixed then centrifuged for ten minutes at 140g. In a fresh 1.5ml micro-centrifuge tube, 500µl isopropanol (Sigma) was added, to which the upper aqueous layer was transferred and allowed to stand for one hour at +20°C. The samples were centrifuged for ten minutes at 140g and once re-suspended in 300µl TE_{10}, 10% of 7.5M ammonium acetate (Sigma) and 60% of 95% ethanol were added. Samples were stored overnight at -20°C. The samples were centrifuged for ten minutes at 140g and the supernatants discarded. The DNA pellets were re-suspended in 60µl TE and the samples were digested further using restriction enzymes. To each reaction tube five µl of 10X buffer, two µl *Hha* I enzyme (Gibco) and 15-20µl of DNA was added. The total reaction volume was made up to 50µl with sterile water (Baxters). Three controls were set up with the following enzymes, either two µl of *Kpn* I, *Pst* I or *Hha* I (Gibco) plus two µl of bacteriophage λ DNA (Gibco), five µl of the appropriated buffer supplied with the enzyme (*Kpn* I: react 4, *Hha* 1 and *Pst* I: react 2), and the final reaction volume was made up to 50µl with sterile water (Baxters). The tubes were mixed and incubated for four hours in the +37°C water bath.

2.7.7 Gel electrophoresis of restriction digests

Agarose powder (Bioline) was mixed with 1X TBE buffer to make a 0.8% gel, ethidium bromide was not added prior to the gel setting. The gel was run overnight in 1X TBE buffer at 25mA using a DNA subcell powerpack (Biorad, Biorad Laboratories Ltd., Hemel Hempstead) to maintain a constant current and to allow clear separation of the digested fragments. Fifty µl of restriction digest was mixed with five µl of loading buffer, as described previously (Sigma). Approximately 40µl of both sample and control mixtures were loaded into individual wells.

2.7.8 Ethidium bromide staining of genomic gel

The gel was removed from the gel tank, transferred to a plastic tupperware dish and immersed in one litre of 1X TBE containing 3% ethidium bromide solution (stock solution 10mg/ml). The Tupperware was placed on a plate rocker (Luckham 4RT rocking table, Denley Instruments, UK) for one hour on its lowest setting. The gel was destained using normal tap water for 20 minutes on the plate rocker. The restriction digest products were visualised under UV light and Polaroid pictures were taken with the exposure time increased to two minutes on the Polaroid T224 land camera (Sigma). It was important to include the wells of the gel in any photographs that were taken to allow the results to be digitised at a later date.

2.7.9 Digitisation of Restriction Enzyme Fragmentation Pattern Results

Interpretation of electrophoretic gels was a skill-intensive task. Digitisation of gel photographs was an objective yet flexible technique that allowed the accurate assessment of electrophoretic data. Data was stored in numerical form (molecular weight of restriction fragments). The graphical output was used to present the data in a familiar format, which allowed visual comparisons with the original gel photograph. Digitisation of REFP isolates allowed comparisons of restriction fragments generated by the different strains of *S. aureus*. Strains were analysed using a commercially available programme (Platt and Sullivan, 1992) with restriction digested λ DNA using enzymes *Kpn* I and *Pst* I as calibration controls.

2.7.10 Detection of Staphylococcal Enterotoxins (SE) using Reverse Passive Latex Agglutination (RPLA)

A single colony of *S. aureus* was added to ten mls of tryptone soya broth (Oxoid Ltd.) and incubated for 18-24 hours at +37°C in an orbital shaker. The samples were centrifuged for 20 minutes at 900g at +4°C. The supernatant was retained for the detection of SE. The diluents, anti-SE antibody, which consists of polystyrene particles coated in purified antiserum of the various SE, and latex solutions were supplied as part of the toxin detection kit (TD900, Oxoid Ltd.). The used of a latex control ruled out non-specific

agglutination between sample and the anti-SE antibody. Each sample to be tested was added to five rows and eight wells on a microtitre V well plate. Twenty-five µl of diluent was added to each well, 25µl of sample supernatant was added to the first well of each row. Once mixed 25µl was taken from well one and serial dilutions carried out until well seven, well eight contained only diluent as a negative control. To every well of rows one to five, 25µl of anti-SEA or SEB or SEC or SED or latex control was added respectively. Samples were mixed thoroughly prior to an adhesive clear cover being fixed to the top of each plate to eliminate evaporation. The plates were left on a sheet of black card for 18-24 hours at +20°C, on a vibration free surface.

As defined in the working instructions (Oxoid Ltd.), if SE was present in the sample the latex particles formed a lattice structure, and once settled on the bottom of the V well plate they formed a diffuse layer. If SE was absent there was no cross linkage and therefore a tight pinhead formed at the bottom of the plate. Results were scored visually from negative to three plus depending on the level of agglutination.

The RPLA method was also used for the detection of Toxic Shock Syndrome Toxin-1 (TSST-1), using a TSST-1 detection kit (TD940, Oxoid Ltd.). One difference in the methodology being that the first two wells of each row had sample supernatant added and the serial dilutions subsequently started from well two leaving well one at the original concentration. Incubation times, conditions and interpretation of results were, otherwise, as described for SET-RPLA.

2.8 *In vitro* proliferation assays induced by antigen and enterotoxin

2.8.1 *In vitro* proliferation assays of peripheral blood mononuclear cells induced by *Staphylococcus aureus*

Peripheral blood mononuclear cells were isolated from heparinised blood, as described previously. Proliferative responses to particulate and soluble antigens were assayed in two ml volumes in 24 well tissue culture grade flat bottomed plates (Nunclon™, Nunc). Each test well contained one ml of PBM at a final concentration of $2x10^6$/ml and one ml

of antigen at an optimal dilution, in complete media containing 2% autologous serum. Control wells contained the same concentration of PBM, and in replace of antigen, one ml of complete media containing 2% autologous serum was added. Culture plates were incubated at +37°C with 5% CO_2 and 100% humidity for up to 11 days. At specific time points during culture, wells were sampled and cells pulsed with ^{3}H-thymidine as described below.

Each well of the *in vitro* cell culture was mixed thoroughly by pipetting prior to 100µl being transferred to a fresh 96 well plate, in triplicate (Corning Costar Corporation, Cambridge). One microcurie of methyl-tritiated (^{3}H) thymidine (5mCi, Amersham International plc, Buckinghamshire) in 20µl of complete media was added to each well. The plates were incubated for six hours at +37°C with 5% CO_2 and 100% humidity. Samples were harvested onto GFC bonded unifilter plates (Canberra Packard, Berkshire) using a filtermat 196™ (Canberra Packard) and dried for at least one hour at +65°C. Once dried, the filtermats were sealed on the under side and ten µl of Microscint O™ (Canberra Packard) was added to each well of the filtermat. A clear seal was adhered to the top of the filter plate to prevent spillage of the radioactive scintillation fluid. The ^{3}H-thymidine incorporated into the newly synthesised DNA of the proliferating cells was measured by liquid scintillation counting using a Topcount Microplate Scintillation and Luminescence Counter™ (Canberra Packard). The data produced was recorded as counts per minute (cpm), and means of the triplicate wells were calculated. Stimulation indices (SI) were calculated by dividing the cpm of the wells with antigen by the cpm of the wells in the absence of antigen.

2.8.2 *In vitro* proliferation assays of peripheral blood mononuclear cells induced by Staphylococcal Enterotoxin B

The *in vitro* culture of PBM and Staphylococcal Enterotoxin B (SEB, Sigma) was designed to study the difference in the kinetics of the proliferative response when stimulated by antigen or staphylococcal enterotoxin. Staphylococcal Enterotoxin B from *S. aureus* was diluted in sterile water (Baxters) to a stock concentration of 1ug/ml.

Staphylococcal Enterotoxin A content was estimated to be $\leq$ 0.25%, as per data sheet (Sigma). The stock solution of SEB was aliquoted and stored at -20°C until required.

Peripheral blood mononuclear cells isolated by density centrifugation were diluted in complete media with 2% autologous serum as described previously, at a final concentration of $2x10^6$ cells/ml media. Staphylococcal Enterotoxin B (Sigma) was added at dilutions ranging from 1:100 to 1:1,000,000. Cultures were incubated under the same conditions and pulsed with ^{3}H-thymidine as described previously.

2.8.3 *In vitro* proliferation assays of peripheral blood mononuclear cells induced by recall antigen

After the peak day of proliferation, day 10 or 11, the cells were removed from the 24 well plate and transferred to a 50ml centrifuge tube (Sterilin). The empty wells were washed with complete media heated to +37°C, to ensure no cells remained in the wells. The cells were then centrifuged twice in warm complete media for ten minutes at 600g to remove any antigen and debris. The cell pellets were re-suspended in a small volume of complete media and cell numbers and viability were assessed using Trypan Blue (Sigma). The cells were re-cultured in 24 well plates in two ml volumes at varying cell concentrations. Irradiated PBM were added as a source of fresh antigen presenting cells, and antigen concentrations remained as in the primary culture. The ratio of PBM: APC: antigen was $1x10^6$:$0.5x10^6$:$1x10^4$. Again the culture was incubated at +37°C with 5% CO_2 and 100% humidity. After 24, 48 and 72 hours the culture was pulsed with ^{3}H-thymidine as described previously.

2.8.3.1 Preparation of antigen presenting cells for *in vitro* proliferation assays of peripheral blood mononuclear cells to recall antigen

Additional APC were required, as the number of cells expressing the monocyte marker fall dramatically post *in vitro* primary stimulation. Irradiation of the APC removes the ability of the cells to proliferate but not the ability to present antigen to the responding lymphocyte population. Peripheral blood collected in EDTA-coated vacutainers (Becton Dickenson UK Ltd.) was irradiated with 5000rads in the central position of the Co^{60}

source (Glass *et al.*, 1991). Once irradiated, blood was diluted in HBSS without calcium carbonate and magnesium sulphate (Sigma) and underlaid using ficoll-isopaque 1.077g/l (Sigma) as described previously. The cells aspirated from the interface were washed and used as antigen presenting cells (APC) at ratios of 1:2 and 1:8 respectively to the responding cell population.

2.8.4 *In vitro* proliferation assays of peripheral blood mononuclear cells induced by antigenic stimuli in the presence of anti-MHC class II monoclonal antibody

To determine if the proliferative ability of PBM of cows induced by *S. aureus* strain A was dependent on MHC class II for processing and presentation of antigen, an anti-MHC class II MAb, IL-A21, was added to PBM and *S. aureus* strain A during the culture *in vitro*. Peripheral blood mononuclear cells were isolated from heparinised blood, as described previously. The proliferative responses of PBM induced by *S. aureus* strain A in the presence and absence of anti-MHC class II MAb (IL-A21) at dilutions of 1:5 and 1:10 was recorded. The test wells were set up as described previously for *in vitro* proliferation of PBM induced by *S. aureus* strain A. Culture plates were incubated at +37°C with 5% CO_2 and 100% humidity for up to 11 days. At specific time points during culture, wells were sampled and cells pulsed with ^{3}H-thymidine as described previously.

2.9 DNA isolation

2.9.1 DNA extraction from peripheral blood cells

Ten mls of blood was taken from either the jugular or coccygeal veins into an EDTA-coated vacutainer (Becton Dickinson UK Ltd.). The whole blood was transferred to a 50ml centrifuge tube (Sterilin) containing 40ml of nucleic lysis mix. After a gentle mix, the tubes were submerged in ice for ten minutes to improve cell lysis. Samples were centrifuged for ten minutes at 1900g at +20°C. The supernatants were discarded and the tube rims blotted with absorbent tissue to remove excess liquid. Cell pellets were re-suspended in three mls of nucleic lysis mix, 6% sodium-lauryl-sulphate (SDS, Sigma) and 1.6% proteinase K (Boehringer Mannheim). The samples were then incubated overnight at +37°C or for two hours at +55°C. To aid precipitation of the DNA, one ml of 6M NaCl

was added and each sample shaken vigorously. Samples were further centrifuged for ten minutes at 1900g at +20°C. The supernatants were transferred to sterile 15ml centrifuge tubes (Sterilin), to which three ml of 1:1 (vol:vol) phenol chloroform (Sigma) was added. The tubes were inverted to ensure proper mixing and then centrifuged as before. Tubes were removed carefully from the centrifuge so as not to disturb the interface. The upper aqueous layers were transferred to 30ml sterile universals and care was taken to prevent aspiration of the bottom fraction, which contained unwanted protein. One hundred percent ethanol was added to each universal at approximately three times the volume of the top fraction, the contents were mixed and the precipitated DNA was spooled out using a heat sealed glass pipette. The spooled out DNA was re-suspended in 300µl of sterile water (Baxters) in screw cap tubes (Starstedt Ltd., Leicester). If the DNA did not precipitate out immediately, one tenth of the original volume of the upper aqueous layer of sodium acetate (Sigma) was added and incubated for one hour at –20°C. Finally the DNA was allowed to redissolve for approximately one hour at +20°C and stored at –20°C until required.

2.9.2 Estimation of DNA concentration by spectrophotometry

One µl of stock DNA was added to 99µl of sterile water (Baxters). One hundred µl of sterile water (Baxters) was used as a blank for the spectrophotometer. Optical density of the samples was determined at wavelengths of 260nm, 280nm and 320nm. Concentrations of DNA per sample was determined using the following equation.

$$\text{DNA concentration } (\mu g/\mu l) = \frac{\text{nucleic acid reading x dilution}}{\text{working DNA concentration}}$$

The working concentration of DNA for future use was 0.5µg/µl, stock samples were diluted with sterile water if necessary. Ten mls of the 0.5µg/µl DNA stock was transferred to 0.5ml micro-centrifuge tubes to prevent repeated freeze-thawing of stock samples.

2.10 Cloning and sequencing of MHC class II DQB region

2.10.1 Polymerase Chain Reaction amplification of MHC class II DQB alleles

To avoid contamination with extragenous template, the work area was decontaminated by depurination. Surfaces and pipettes were wiped sequentially with 1N HCl, 1N NaOH, and finally, Tris buffer as a neutralising agent. The surfaces and pipettes were rinsed with distilled water and wiped down with 100% ethanol. A master mix, enough for all samples, was made up to avoid contamination of stock PCR solutions. For each sample five µl of PCR enzyme buffer, one µl dNTP, nine µl of 0.4µM LA40, 5' (TCCCCCGCAGAGGA TTTCGT) and 12µl of 0.4µM LA41, 3' (ATAGAATTCACCT(A/T)GCC GCTGCCAGGT), primers published previously for MHC class II in cattle (Sigurdardottir *et al.*, 1992) were used. The master mix was vortexed and irradiated by ultra violet light (UV) for five minutes, prior to addition of DNA and DNA polymerase (Dynazyme, Flowgen, Novara Group Ltd., Leicestershire) to each sample to eliminate possible contaminants. One µg of genomic DNA plus 0.25µl of DNA polymerase was added to the above master mix, and the total reaction volume made up to 50µl with sterile water (Baxters). A negative control containing no DNA was set up as part of a standard protocol. Two drops of mineral oil (Sigma) were added to each micro-centrifuge tube prior to loading onto a 480 thermal cycler (Perkin Elmer, Birchwood Science Park North, Cheshire) to prevent evaporation of the sample. PCR amplification involved an initial hot start for five minutes at +95°C straight from ice, followed by 30 cycles for one minute at +94°C, for one minute at +65°C, for one minute at +72°C and a final extension for 30 minutes at +72°C.

Gel electrophoresis of the PCR products was carried out as follows. Agarose powder (molecular grade, Bioline, The Edge Business Centre, London) was mixed with 1X tris boric acid EDTA (TBE) buffer to make a 1.5% gel. The gels were run in submarine agarose gel units (Hoefer Scientific Instruments, San Francisco) with 1X TBE as the running buffer at 100 volts. Prior to the gel setting, five mls of ethidium bromide (10mg/ml, Sigma) was added to the dissolved agarose for visualisation of product bands under UV light. Ten µl of PCR product was taken from below the oil layer and mixed

with five µl of loading buffer (Sigma), which consisted of 40% sucrose and 1% bromophenol blue. Twelve µl of this mixture was loaded into each well and eight µl of a 100 base pair (bp) marker (Gibco) was loaded to allow estimation of size of the PCR product. The desired product size was approximately 300bp (Sigurdardottir *et al.*, 1992; Nasir *et al.*, 1997). Once the bromophenol blue had run approximately half the length of the gel, it was removed and analysed under UV light on a transilluminator T2201 (Sigma), connected to a Polaroid MP4 land camera to allow photographs to be taken and results retained in picture and negative form.

2.10.2 Molecular cloning of MHC class II DQB alleles

The 300bp product generated by PCR amplification was used in conjunction with Novagen PT7 Blue Perfectly Blunt™ cloning kit (Novagen, Inc., 601 Science Drive, Madison). The PCR product was removed from beneath the mineral oil layer and transferred to a 500µl micro-centrifuge tube. The PCR product was mixed 1:1 (vol:vol) with chloroform then centrifuged for two minutes at 140g in a micro-centrifuge tube 5402 micro-centrifuge (Micro-centrifuge tube, M^{C}Quilken and Co., Polmadie Ave, Glasgow). The top aqueous layer was removed and run through Qiaquick PCR purification kit (Qiagen, Boundary Court, Crawley) to inactivate residual enzyme, dNTP, and any double stranded DNA that may be present within the PCR product that would prevent successful cloning. Twenty-two nanograms of amplified product were mixed with five µl of an end conversion mix (Novagen). The total volume was made up to ten µl with nuclease-free water provided as part of the kit, forming the end conversion reaction. Samples were incubated for 40 minutes at +22°C, as these were amplified with polymerases that lacked 3'→5' activity. To localise the kinases at the bottom of the tube, the samples were centrifuged briefly for 30 seconds at 140g in a micro-centrifuge. The samples were subsequently heated for ten minutes to +75°C to terminate the reaction and stop the kinases reducing the vector backgrounds. To prevent inactivation of the ligase enzyme, samples were cooled on ice for two minutes, followed by centrifugation to collect any condensation that had formed on the tube walls. The ligation reaction involved combining the blunt phosphorylated insert to the blunt dephosphorylated pT7 Blue vector. One µl of T4 DNA ligase (4U) and one µl of pT7 Blue vector (50ng/ul) were added to the

ten µl of the end conversion reaction. Samples were mixed gently with a pipette tip and incubated overnight at +22°C. One µl of the ligation reaction mixture was used in the transformation reaction to create recombinants. Twenty µl of Novablue *E. coli* competent cells were removed from the –70°C freezer and transferred directly onto ice. The cells had been previously aliquoted into pre-chilled micro-centrifuge tubes to eliminate a reduction in transformation efficiencies due to repeated freeze thawing. One µl of the ligation reaction was added to the cells. Samples were stirred gently and incubated for a further 30 minutes on ice. The samples were heated for exactly 40 seconds to +42°C, followed by a further incubation for two minutes on ice. Eighty µl of SOC media (Novagen), which had been allowed to warm to +20°C, was added and the total volume transferred to a 20ml universal (Sterilin). Samples were transferred to an orbital shaker to improve the cloning efficiency, for one hour at +37°C with the lids slackened slightly to allow aeration. A further 170µl of SOC was added to the cells. Two LB broth agar plates which had been previously seeded with, 50µg/ml carbenicillin (Sigma), 15µg/ml tetracycline (Sigma), 70µg/ml Xgal (5-Bromo-4-chloro-3-indolyl beta-D-galactopyranoside, Gibco) and 80µM IPTG (Isopropyl beta-D-thiogalactopyranoside, Gibco), were spread with either 80µl or 50µl of the transformation mixture. The different volumes of the transformation mixture were spread to ensure single colonies would be obtainable in the case of blanket coverage of the plate or under growth of the spread cells. Spread plates were incubated inverted overnight at +37°C, under aerobic conditions. The presence of Xgal and IPTG allowed for selection of recombinants with a white phenotype and non-recombinants that possessed a blue phenotype. Enhancement of the blue phenotype can be achieved by incubating the streak plates for 30 minutes at +4°C (Novagen). Approximately ten single white colonies, of medium size, were picked off the agar plates under aseptic conditions and inoculated individually into three mls of LB broth in a 50ml centrifuge tube. The LB broth contained 100mg/ml carbenicillin and 15mg/ml tetracycline, which were added post-autoclaving. The lids of the tubes were slackened and placed into the orbital shaker overnight at +37°C. The samples were checked the following morning visually for signs of growth indicated by cloudiness. Any colonies, which had not grown, were returned to the orbital shaker until later on the same day. Twenty µl of the cell culture was transferred to a 0.5ml micro-centrifuge tube for PCR to check for the presence of an insert. Three hundred and sixty µl of cell culture was

mixed with 60µl of glycerol to provide a glycerol stock should samples be required for further sequence analysis. To check for the presence of the insert, 20µl of the sample was heated for five minutes at +95°C, followed by centrifugation for two minutes at 140g. Ten µl of the supernatant was mixed with 10mM dNTP mix, 5pmol/µl T7, 5pmol/µl U19, 1X buffer (10X), 30µl nuclease free water and 0.3µl of DNA polymerase (Dynazyme). The mastermix was irradiated prior to DNA and DNA polymerase (Dynazyme) being added. Finally two drops of mineral oil (Sigma) was added to the top of the tube. Samples were amplified for 35 cycles for one minute at +94°C, for two minutes at +72°C and a final extension for five minutes at +72°C. Samples were run out on a 1.5% agarose gel as described previously. Once the presence of the 450bp insert was confirmed samples were subjected to further purification using Wizard ™ Minipreps DNA purification kit (Promega Ltd., Chilworth Research Centre, Southampton) as described below, to remove any residual dNTP, enzyme, and primers.

2.10.3 Purification of clones for commercial sequencing

Purification involved using the Wizard™ Minipreps DNA purification kit (Promega). Broth samples were centrifuged for ten minutes at 1200g and the supernatants discarded. Two hundred µl of cell re-suspension solution was added to the cell pellets and transferred to a 1.5ml micro-centrifuge tube. Two hundred µl of cell lysis solution was added and the micro-centrifuge tube continually inverted until the solution cleared. To this, 200µl of neutralising solution was added, and the micro-centrifuge tube was again inverted. The sample was centrifuged for five minutes at 140g and the supernatant was transferred to a fresh two ml micro-centrifuge tube.

Plasmid purification of the clones was carried out as follows. One ml of the Wizard minipreps DNA purification resin was added to the supernatant sample and mixed by inverting the micro-centrifuge tube. The plunger from a three ml syringe was removed prior to the attachment of the miniprep column, to ensure the membrane was not damaged. The resin / DNA mix was pipetted into the syringe, the plunger replaced and the solution pushed through slowly. The syringe was detached from the column prior to removal of the plunger. Once the plunger was removed the column was reattached and

two mls of column wash was added and pushed gently through. The membrane within the column was centrifuged for two minutes at 140g to dry the resin retained. For elution of the DNA from the membrane, 50µl of sterile water (Baxters), that had been preheated to +55°C, was added direct to the column and allowed to stand for two minutes followed by centrifugation for a further 20 seconds. The plasmid DNA collected was stored at -20°C. These samples were then sent to Cambridge Bioscience (Cambridge Bioscience Ltd., Cambridge) for commercial sequencing.

2.11 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

2.11.1 Polymerase Chain Reaction amplification of MHC class II DQB exon 2 alleles for analysis by Restriction Fragment Length Polymorphism.

Polymerase Chain Reaction amplification of BoLA MHC class II DQB exon 2 was carried out using DNA isolated from peripheral blood by standard methods as described previously. To avoid contamination of stock PCR solutions, a master mix, enough for all samples was made up. This master mix contained per sample five µl of 10X PCR buffer, 1.5µl 50mM Mg Cl, one µl 10mM dNTP, one µl 25pmol/µl DQBEX2fwd, 5'(GGGCC[TA]GTGTTA [CT] TTCAC [CT] AA)3', and one µl 25pmol/µl DQBEX2rev, 5'(TTGT[GT] TCTGCACACC [CG]TGTCC)3', primers published previously for MHC class II in cattle (Russell, 2000) were used. To avoid contamination with extraneous template the work area was decontaminated by depurination as described previously. The master mix was vortexed and irradiated by UV light for five minutes, prior to addition of DNA and DNA polymerase (Dynazyme) to each sample to eliminate possible contaminants. One µg of genomic DNA plus 0.5µl of DNA polymerase was added to the above master mix, and the total reaction volume made up to 50µl with sterile water (Baxters). A negative control containing no DNA was set up as part of a standard protocol. Polymerase Chain Reaction amplification involved an initial hot start for three minutes at +94°C straight from ice, followed by 30 cycles for one minute at +94°C, for one minute at +57°C, for three minutes at +72°C and a final extension for five minutes at +72°C.

Gel electrophoresis of the PCR products was carried out as follows. Briefly, ten µl of the 50µl PCR reaction was mixed with five µl of loading buffer (Sigma) and run on a 1.5% agarose gel. The expected size of the PCR product generated was 214bp in length. The product was analysed under UV light on a transilluminator T2201 (Sigma), to ensure the DQB fragment of the correct size had been amplified.

2.11.2 Polymerase Chain Reaction amplification of MHC class II DRB alleles for analysis by Restriction Fragment Length Polymorphism.

The same basic principle for generation of a PCR product was used to amplify the MHC class II second exon in DRB3. Unlike DQB, PCR amplification of DRB3 exon 2 involved a hemi-nested PCR, whereby the product of a first stage amplification is used to generate the final product using one nested on first stage products. In the first stage, one µl of 5pmol/µl HLO30, 5'(ATCCTCTCTCTGCAGCACATTTCC)3' and one µl of 5pmol/µl HLO31, 5'(TTTAAATTCGCGCTCACCACGCCGCT)3' (van Eijk *et al.*, 1992), were added with the DNA and other PCR reagents. PCR amplification involved an initial hot start for four minutes at +94°C straight from ice, followed by 15 cycles for one minute at +94°C, for two minutes at +60°C, for one minute at +72°C and a final extension for five minutes at +72°C. The first stage PCR product was not run out on an agarose gel, as a 15 cycle amplification would not generate enough product to visualise by ethidium bromide staining.

For the second stage amplification, two µl of the first stage product was added to one µl of 25pmol/µl HLO30, 5'(ATCCTCTCTCTGCAGCACATTTCC)3', and one µl of 25pmol/µl HLO32, 5'(TCGCCGCTGCACAGTGAAACTCTC) 3' (van Eijk *et al.*, 1992), and made up to 50µl with the other PCR reagents. PCR amplification involved 30 cycles at +94°C for one minute, +65°C for 30 seconds and a final extension at +72°C for five minutes. The PCR product generated was anticipated to be 284bp in length. Gel electrophoresis was carried out as described previously.

2.11.3 Restriction Fragment Length Polymorphism enzymatic digestion of MHC class II DQB and DRB3 exon 2 Polymerase Chain Reaction products.

The products of the DQB and DRB3 PCR amplification were divided into three 15µl aliquots and transferred into 0.5ml micro-centrifuge tubes. For the DQB samples, the three enzymes used were *Rsa* I, *Hae* III and *Brs* I. To ten µl of PCR product, 0.5µl of one of the enzymes was added to 4.5µl 1X enzyme buffer. The samples containing *Rsa* I and *Hae* III were incubated for eight to ten hours at +37°C. The samples containing *Brs* I were incubated for eight to ten hours at +65°C. The samples were then transferred directly onto ice prior to analysis on a polyacrylamide gel.

For the DRB3 samples, the three enzymes used were *Rsa* I, *Hae* III and *Bst* YI. To ten µl of PCR product, 0.5µl of one of the enzymes was added to 4.5µl 1X enzyme buffer. The samples containing *Rsa* I and *Hae* III were incubated for eight to ten hours at +37°C. The samples containing *Bst* YI were incubated for eight to ten hours at +50°C. The samples were then transferred directly onto ice prior to analysis on a polyacrylamide gel.

2.11.4 Visualisation of Restriction Fragment Length Polymorphism by gel electrophoresis

Fifteen mls of 40% acrylamide (Easigel, Scotlab, Coatbridge) was mixed with 100µl of n, n, n', n'-tetramethylethylelediamine (TEMED), ten mls of 10X TBE, 100µl of 25% ammonium persulphate and made up to 100ml with distilled water, to give a final concentration of 6% polyacrylamide. The solution was mixed thoroughly and poured slowly between the two glass plates to ensure no air bubbles formed. Once set, the wells were flushed out with the buffer used to run the gel prior to samples being loaded. The enzymatic digestion products were mixed with bromophenol blue (Sigma) and run along with a pBR322/*Hae* III (Sigma) and 20/100bp ladder size standards mixed, to allow easier determination of fragment sizes. The gel was run in 1X TBE buffer at 400volts/60mA until the bromophenol blue was almost at the bottom.

Once the run was complete, the gels were stained in 100µg/ml ethidium bromide for 15-30 minutes. The gels were then rinsed in tap water to remove excess ethidium bromide, before visualisation of the digestion patterns under UV light. Digital images of the gels were recorded and printed using a Bio-Rad Gel Doc 1000 system running multi-analyst software.

2.11.5 Analysis of Restriction Fragment Length Polymorphism generated by Polymerase Chain Reaction

The RFLP patterns generated were compared with previously published patterns, which were already attributed to sequenced alleles by prediction of restriction sites (van Eijk *et al.*, 1992; Gelhaus *et al.*, 1995, appendix D).

2.12 Statistical analysis

2.12.1 Correlation

The correlation coefficient was calculated using a general linear mixed model (SAS, Cary, North Carolina) where the model used was:

Stimulation Index (SI) mean = mean (SI1 + SI2 + SI3 + SI4)
LogSI mean = $\log_{10}$ (SI mean + 1)

2.12.2 Repeatability

Repeatability used the statistical model:

$$Y = \mu + \alpha_k + e_{km}$$

Where μ is the common mean, α_k is the effect of the individual and e_{km} is the environmental deviation of the Mth measurement.

Repeatability was calculated as:

$$R = \frac{\hat{\sigma}_w^2}{\sigma_w^2 + \hat{\sigma}_e^2}$$

Where R is the permanent environmental effect plus the genetic effects; w is the variation between individuals and e is the variation within individuals

2.12.3 T test

A two-tailed unpaired T test was used to compare the means of two independent groups of observations and was calculated as:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

Where n is the number of observations, x is the sample mean and s is the estimated standard deviation.

A two tailed paired T test was used to compare the means of two related groups of observations and was calculated as:

$$\frac{\bar{d}}{SE(\bar{d})} = \frac{\bar{d}}{Sd\sqrt{n}}$$

Where n is the number of pairs in the sample, d is the mean of the difference in the sample, $SE(d)$ is the estimate of the standard error of the differences and Sd is the estimate of the standard deviation of the differences

The difference in the proportion of cell subpopulations between bulls and cows was tested using Hotelling's T square test (Minitab, Release 13 for Windows).

2.12.4 Hardy-Weinberg equation

The probability of the MHC DRB3.2*16 RFLP type being identified in all five bulls chosen as having the strongest proliferative response induced by *S. aureus* strain A was calculated assuming a gene frequency of 0.1 (Kelm *et al.*, 1997; Sharif *et al.*, 1998b) and the Hardy-Weinberg principle that an allele p will be carried by p^2 homozygotes and 2pq heterozygotes. Allele frequencies of p and q can be calculated as follows:

$p^2 + 2pq + q^2 = 1$, where

$0.01 + 0.18 + 0.81 = 1$

Chapter III

Results

3.1 Restriction Enzyme Fragmentation Pattern Analysis

Staphylococcus aureus strains A, C and F, used to induce the proliferative response of PBM isolated from cows and bulls, were characterised by REFP analysis. Two further isolates, strains D and E, which were not used to induce the *in vitro* proliferative response were also characterised by REFP analysis to demonstrate the variation in strains of *S. aureus* present in the dairy cow's environment. The *S. aureus* strains A, C, D and E were all isolated from the mammary gland. The *S. aureus* strains C and D were isolated from the mammary gland of the same cow but from different quarters. The *S. aureus* strain F was isolated from the vagina of the same cow as strains C and D.

The band patterns generated following enzymatic digestion highlighted genomic differences between the strains. Differences in patterns were seen between strains isolated from the vagina (strain F) and the mammary gland, but also among isolates from different mammary glands (strain A, C, D, and E), (Figure: 1).

Digestion of the isolates generated a variety of fragment sizes. For *S. aureus* strain A, isolated from the mammary gland, sizes ranged from 8.36kilobases (kb) to 4.38kb (Figure 1: lane 1). *Staphylococcus aureus* strain C, isolated from the mammary gland, sizes ranged from 8.36kb to 4.47kb (Figure 1: lane 2). *Staphylococcus aureus* strain D, isolated from the mammary gland, sizes ranged from 8.63kb to 4.47kb (Figure 1: lane 3). *Staphylococcus aureus* strain E, isolated from the mammary gland, sizes ranged from 8.63kb to 4.38kb (Figure 1: lane 4).

Digestion of strain F, isolated from the vagina of same cow as mammary isolates C and D, generated fragment sizes that ranged from 26.37 to 4.36kb (Figure 1:lane 4).

Estimation of the band sizes was performed using enzymatic standards, *Kpn* I, *Pst* I and *Hha* I. These were run simultaneously with the *S. aureus* isolates. Lambda DNA incubated with *Kpn* I generated fragments of 30.04kb and 18.21kb (Figure 1: lane 9).

Lambda DNA incubated with *Pst* I generated fragments of 14.13kb, 11.95kb, 4.61kb, 4.42kb, 4.27kb and 3.68kb (Figure 1: lane 10).

Analysis of the fragmentation patterns showed individual bands for *S. aureus* strain A, isolated from the mammary gland, of sizes; 8.63kb, 7.68kb, 6.13kb, 5.79kb, 5.5kb, 5.03kb, 4.9kb, 4.73kb, 4.69kb, 4.58kb, 4.49kb, 4.43kb and 4.38kb (Figure 2: lane 1). Estimation of the band sizes generated by the REFP showed that *S. aureus* strains C and D, isolated from the mammary gland of the same cow but different quarters were identical with sizes of; 8.63kb, 7.68kb, 6.62kb, 5.92kb, 5.82kb, 5.49kb, 4.9kb, 4.83kb, 4.72kb, 4.59kb, 4.49kb and 4.47kb (Figure 2: lanes 2 and 3 respectively), for *S. aureus* strain E, isolated from the mammary gland; 8.36kb, 7.68kb, 6.13kb, 6.19kb, 5.45kb, 4.93kb, 4.67kb, 4.57kb, 4.38kb (Figure 2: lane 4). Estimation of the band sizes generated by REFP showed that *S. aureus* strain F although isolated from the vagina of the same cow as strain C and D was unique with sizes of; 26.37kb, 12.47kb, 8.75kb, 7.64kb, 6.55kb, 5.53kb, 5.3kb, 4.96kb, 4.82kb, 4.65kb, 4.55kb and 4.36kb (Figure 2: lane 5).

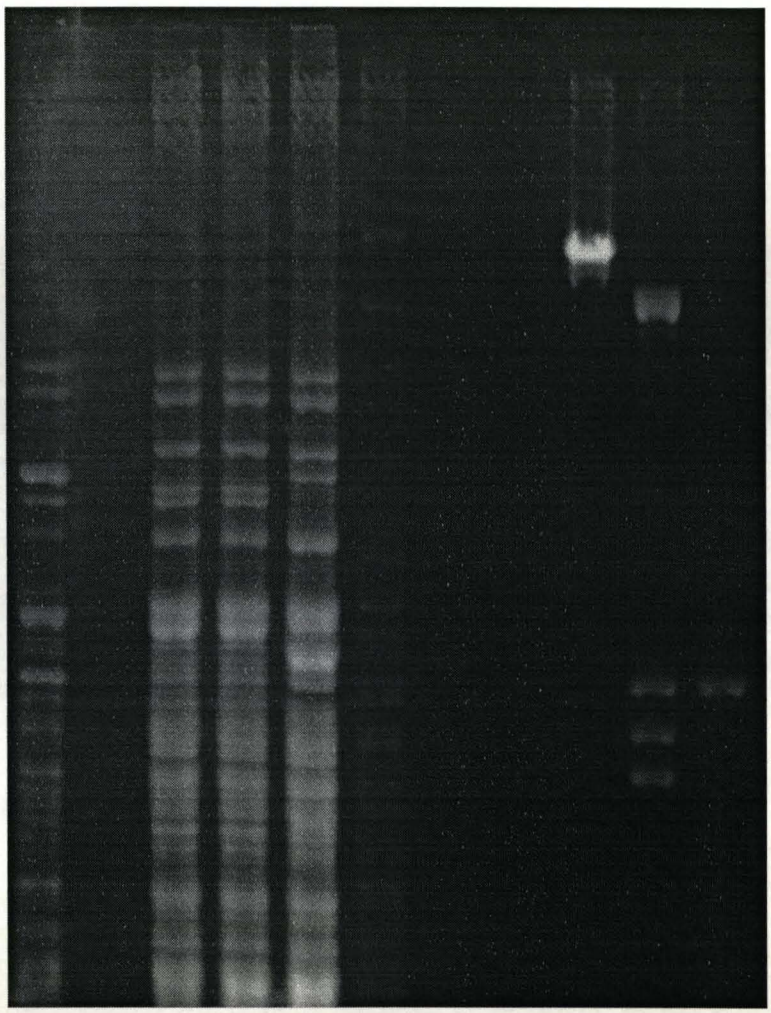
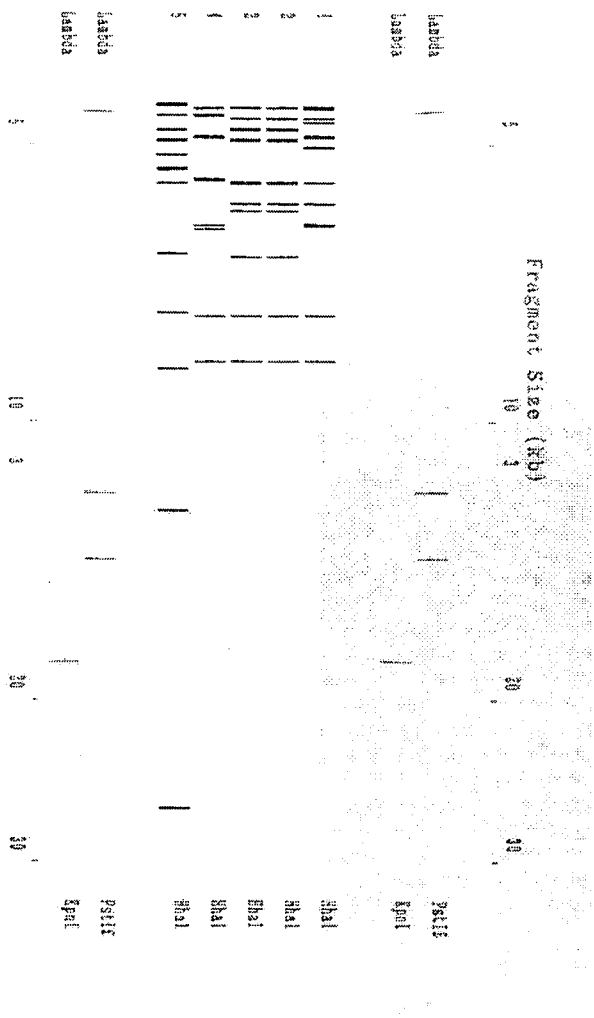


Figure 1: Results of restriction enzymatic digestion of various strains of *S. aureus* with *Hha* I. Lane 1: *S. aureus* strain A isolated from the mammary gland, lane 2: *S. aureus* strain C isolated from the mammary gland, lane 3: *S. aureus* strain D isolated from the mammary gland, lane 4: *S. aureus* strain E isolated from the vagina and lane 5 shows *S. aureus* strain F isolated from the mammary gland. Lambda DNA controls were digested with *Kpn* I and *Pst* I lanes 9 and 10 respectively.



Lanes: 5 4 3 2 1

Figure 2: Graphical output of digitised image of figure 1, results of restriction enzymatic digestion of various strains of *S. aureus* with *Hha* I. Lane 1: *S. aureus* strain A isolated from the mammary gland, lane 2: *S. aureus* strain C isolated from the mammary gland, lane 3: *S. aureus* strain D isolated from the mammary gland, lane 4: *S. aureus* strain E isolated from the mammary gland, and lane 5: *S. aureus* strain F isolated from the vagina. Lambda DNA controls were digested with *Kpn* I and *Pst* I and the fragment sizes used on the left and right hand side of the digitised image.

3.2 Detection of Staphylococcal Enterotoxins (SE) using Reverse Passive Latex Agglutination (RPLA)

Investigation of enterotoxin production from *S. aureus* strains A, C, D and F, used to induce the proliferative response of PBM *in vitro,* was undertaken. The results would allow comparison of strain phenotype and genotype, as pre-determined by REFP analysis, and provide additional information on the strains of *S. aureus* used to induce the proliferative response of PBM *in vitro.*

Four isolates confirmed as *S. aureus*, three of which were isolated from the mammary gland (strains A, C, and D) and one of which was isolated from the vagina (strain F), by use of selective and differential media, coagulase testing and serotyping were tested for enterotoxin and TSST1 production using RPLA toxin detection kits (Oxoid; Matsunaga *et al.*, 1993).

Results showed variation in the types of enterotoxin produced by the four different strains of *S. aureus*. Enterotoxin production varied among mammary isolates and between the mammary and vaginal isolates. Strain A, isolated from the mammary gland, produced SED only (Table: 1), this differed from the other two mammary isolates, strains C and D, which produced SEC and TSST1 (Table: 1). The results from strain C and D supports those reported in the REFP genomic analysis, which showed that these two strains had identical restriction enzyme digestion patterns. Strain F, isolated from the vagina, although originating from the same cow as strain C and D, produced two different enterotoxins, SEA and SEB (Table: 1).

Staphylococcal enterotoxin	**SEA**	**SEB**	**SEC**	**SED**	**TSST1**
S. aureus strain					
strain A mammary	-	-	-	+	-
strain C mammary	-	-	+	-	+
strain D mammary	-	-	+	-	+
strain F vaginal	+	+	-	-	-

Table 3: Results of RPLA toxin detection of three mammary gland strains and one vaginal strain of *S. aureus*. *Staphylococcus aureus* strain A was isolated from the mammary gland of a cow from a different farm from strains C, D and F. *Staphylococcus aureus* strain C and D were isolated from the same mammary gland but different quarters of the same cow. *Staphylococcus aureus* strain F was isolated from the vagina of the same cow as strains C and D.

3.3 *In vitro* proliferation of peripheral blood mononuclear cells

Modification of the antigen presentation assay described by Fitzpatrick (1992) during the current study attempted to isolate a pure population of peripheral T cells using miniMACS. Cell yield for this technique was in the range of 85-95% of the total number of cells loaded onto the column. Incubation of an anti-CD2 MAb with PBM prior to loading onto the column resulted in positive selection where the labelled T cell population was retained in the column until removed from the magnetic stand when the positive fraction was eluted. Cell viability of the positive fraction was poor at 50-60%. When T cells isolated by positive selection, at various concentrations were cultured *in vitro* with *S. aureus* strain A and fresh irradiated APC at varying ratios to the T cell population, they failed to proliferate. A possible explanation for the lack of proliferative response induced by *S. aureus* strain A was that positive selection of T cell may have damaged the isolated cells, altering their functional capabilities. Peripheral blood mononuclear cells were subsequently labelled with an anti-MHC class II MAb, which retained the APC population within the column and allowed the negative fraction to pass through. When T cells isolated by negative selection were culture *in vitro* with fresh irradiated APC and induced by *S. aureus* strain A, again, they failed to proliferate.

As a result of the failure to induce T cells isolated by positive and negative miniMACS selection to proliferate *in vitro*, development of the current study led to total blood mononuclear cells being isolated by centrifugation with media of a specific density. The isolated PBM population were then use as the responding cell population in the current *in vitro* proliferation assay induced by *S. aureus*.

Peripheral blood mononuclear cells were isolated from six cows, by density centrifugation using ficoll-isopaque (density 1.077g/l). To determine the optimal concentrations of formalin-killed whole *S. aureus* strain A and PBM that would induce maximal proliferative responses, a range of *S. aureus* strain A and PBM concentrations were employed. *Staphylococcus aureus* strain A at a stock concentration of $1.4x10^8$ bacteria/ml complete media was diluted 1:100-1:800, and concentrations of PBM ranged from $1.0\text{-}4.0x10^6$ PBM/ml complete media. These initial experiments resulted in a final dilution of *S. aureus* strain A as 1:300 ($0.5x10^6$ bacteria/ml complete media) and a final

concentration of $2.0x10^6$ PBM/ml complete media being chosen (Table: 4). The proliferative response was measured over an eleven-day period of culture *in vitro*.

PBM concentration	**$1.0x10^6$/ml**	**$2.0x10^6$/ml**	**$4.0x10^6$/ml**
***S. aureus* dilution**			
1:100	**0**	**0+/-2**	**0+/-1**
1:200	**8+/-3**	**14+/-20**	**12+/-10**
1:300	**30+/-11**	**70+/-25**	**45+/-15**
1:400	**25+/-6**	**55+/-10**	**50+/-8**
1:500	**18+/-4**	**40+/-9**	**30+/-7**
1:600	**5+/-2**	**29+/-13**	**10+/-3**
1:700	**3+/-2**	**20+/-14**	**15+/-5**
1:800	**9+/-3**	**20+/-7**	**10+/-4**

Table 4: The mean proliferative response (SI+/-SD) of PBM isolated from Holstein-Friesian cows (n=6) at concentrations ranging from 1.0-$4.0x10^6$PBM/ml, induced by *Staphylococcus aureus* strain A at dilutions ranging from 1:100-1:800 from a stock concentration of $1.4x10^8$bacteria/ml.

Incubation of peripheral blood mononuclear cells at a concentration of $2.0x10^6$ PBM/ml in the presence of *S. aureus* strain A, at a dilution of 1:100 ($1.4x10^6$ bacteria/ml), failed to induce a proliferative response throughout the *in vitro* culture period. *Staphylococcus aureus* strain A at dilutions of 1:300 ($0.5x10^6$ bacteria/ml), 1:500 ($0.3x10^6$ bacteria/ml), and 1:800 ($0.18x10^6$ bacteria/ml), induced a proliferative response with the day of peak proliferation measured on day 7 of culture (Figure: 3). Results presented in the current study have been expressed as SI and counts per minute to illustrate the extent of the proliferative response induced by the *S. aureus* strain A.

Peripheral blood mononuclear cells, in the absence of *S. aureus* strain A, had a very low background proliferation. Results showed that the mean proliferative response (^{3}H-thymidine incorporation +/-SD) of PBM cultured in the absence of *S. aureus* strain A was; 407+/-207 cpm on day 5, 336+/-94 cpm on day 7, 436+/-143 cpm on day 9, and 363+/-102 cpm on day 11 (n=4). A proliferative response above the background levels was apparent

in the presence of *S. aureus* strain A from day five of culture. The proliferative response continued to increase at day seven, peaked at day nine, and had begun to diminish by day eleven. The mean proliferative response (^{3}H-thymidine incorporation +/-SD) of PBM induced by *S. aureus* strain A was; 10,452+/-11,515 cpm on day 5, 13,606+/-17,677 cpm on day 7, 23,278+/-17,598 cpm on day 9, and 10,100+/-6,266 cpm on day 11 (Figure: 4).

Variation in the magnitude of the proliferative response of PBM at a concentration of $2.0x10^6$ PBM/ml, induced by *S. aureus* strain A at a concentration of $0.5x10^6$bacteria/ml, was apparent among individual animals (n=21), (Figure: 5). As can be noted from figure 5, there was little response detected between days two and four of *in vitro* culture. Variation in the response induced by the *S. aureus* strain A was seen among animals with some cows showing an increase in proliferation to a peak on day 9, with the day of peak proliferative response occurring later in some cows. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain A was; 0 on day 2; 9+/-4 on day 3; 1+/-1 on day 4; 35+/-30 on day 5; 12+/-9 on day 6; 45+/-37 on day 7; 34+/-26 on day 8; 61+/-53 on day 9; 36+/-44 on day 10 and 22+/-20 on day 11 (Figure: 6). The SI on day 9, the day of peak proliferation, ranged from 0 to 164.

To investigate if different strains of *S. aureus* resulted in different kinetics associated with a proliferative response, two strains of *S. aureus*, strain A and strain B, characterised by genomic fingerprinting, were isolated and used to induce proliferation of PBM *in vitro*. The *S. aureus* strain A was isolated from a case of subclinical mastitis on one farm, and the *S. aureus* strain B was isolated from a case of subclinical mastitis on another farm. Variation in the proliferative response of PBM to *S. aureus* strain A and strain B at concentrations ranging from 1.4-0.14 $x10^6$ bacteria/ml was measured in the same cows and a representative result for one cow shown in Figure 7. Results showed that the proliferative response induced by *S. aureus* strain A was stronger than that induced by *S. aureus* strain B at concentrations of $0.5x10^6$ bacteria/ml, $0.35x10^6$ bacteria/ml, $0.14x10^6$ bacteria/ml (n=4). However, as shown previously with *S. aureus* strain A, *S. aureus* strain B at a concentration of $1.4x10^6$bacteria/ml, failed to induce a proliferative response throughout the culture period. Results showed the mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain A at the previously chosen optimal dilution of $0.5x10^6$ bacteria/ml was; 49+/-43 on day 5, 94+/-59 on day 7, 75+/-20 on day 9, and 45+/-

29 on day 11. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain B was; 1+/-2 on day 5, 1+/-1 on day 7, 1+/-1 on day 9, and 2+/-1 on day 11. The day of peak proliferation was day 7 of culture for *S. aureus* strain A. The difference in proliferative response in cows (n=4) between strain A and strain B was highly significant ($p<0.01$).

The within assay repeatability of the proliferative response induced by *S. aureus* strain A, measured as SI, was shown to be 99.0% on day 5, 87.0% on day 7, 99.0% on day 9, 99.8% on day 11 and 92.0% (n=4), (Figure: 8). When statistical analysis was carried out on log-transformed SI the within assay repeatability was shown to be 99.8% on day 5; 98.3% on day 7; 96.1% on day 9; 98.6% on day 11 and 98.4%, with $p=0.01$ for all days tested.

Considerable variation in the proliferative response of PBM of cows, kept in the same environmental conditions, to *S. aureus* strain A, suggested that the difference in ability to induce a proliferative response may be under genetic control. To investigate the hypothesis, ten cows from a commercial dairy herd were chosen. The cows were sired by one of two commercial Holstein-Friesian bulls; five were sired by bull A and five were sired by bull B. The mean proliferative response (SI+/-SD) induced by *S. aureus* strain A of the progeny sired by bull A was, 13+/-14 on day 7; 17+/-27 on day 9; and 11+/-16 on day 11. The mean proliferative response (SI+/-SD) induced by *S. aureus* strain A of the progeny sired by bull B was, 41+/-45 on day 7; 77+/-69 on day 9; and 20+/-19 on day 11. Results showed that on day 9 the mean proliferative response of the progeny of sire B was significantly higher than the mean proliferative response of the progeny of sire A ($p=0.03$), (Figure: 9). On day 7 and day 11 of culture, the proliferative response of progeny of sire B was again greater than that of the progeny of sire A, but did not reach statistical significance (day 7: $p=0.08$, and day 11: $p=0.19$), (Figure: 10). The between assay repeatability of the proliferative response induced by *S. aureus* strain A in these ten cows, measured as SI, was shown to be 79% for all days tested.

The variation in the proliferative response of PBM in cows induced by the two strains of *S. aureus*, strain A and strain B, resulted in investigation of the proliferative response induced by further strains of *S. aureus* isolated from the mammary gland and from

different areas of the cows' anatomy. *Staphylococcus aureus* strain C, was isolated from the mammary gland, whilst *S. aureus* strain F, was isolated from the vagina of the same cow. The isolate from the mammary gland, strain C, allowed a direct comparison of the ability of three different *S. aureus* strains, A, B and C, all originating from the mammary gland of different cows to induce a proliferative response. The vaginal isolate allowed comparison of the ability of two different strains of *S. aureus* isolated from different mucosal sites in a single animal, strain C and strain F. The concentration of each strain of *S. aureus* used to induce a proliferative response was $0.5x10^6$ bacteria/ml.

Results showed the proliferative response of PBM (n=8) induced by *S. aureus* at a concentration of $0.5x10^6$ bacteria/ml, was again greatest for *S. aureus* strain F, the vaginal strain (Figure: 11). The mean proliferative response (SI+/-SD) of *S. aureus* strain A, at a concentration of $0.5x10^6$ bacteria/ml, isolated from the mammary gland, was; 2+/-1 on day 2; 29+/-40 on day 4; 45+/-74 on day 6; 40+/-54 on day 8; and 27+/-32 on day 10. The mean proliferative response (SI+/-SD) of *S. aureus* strain C, at a concentration of $0.5x10^6$ bacteria/ml, isolated from the mammary gland, was; 2+/-1 on day 2; 5+/-2 on day 4; 1+/-1 on day 6; 0 on day 8; and 0+/-1 on day 10. The mean proliferative response (SI+/-SD) of *S. aureus* strain D, at a concentration of $0.5x10^6$ bacteria/ml, isolated from the vagina, was; 1+/-1 on day 2; 38+/-50 on day 4; 86+/-137 on day 6; 34+/-43 on day 8; and 12+/-11 on day 10. The day of peak proliferation was day six rather than day four.

The response of PBM induced by different strains of *S. aureus* highlighted the variation in the ability of different strains isolated from a common site, the mammary gland, strain A, strain B and strain C, to induce a proliferative response. Similarly, *S. aureus* strains isolated from the same animal, but from different mucosal sites, strain F and strain C, also showed different abilities to induce proliferation of PBM.

Whole bacterial formalin-fixed *S. aureus* was purchased from Sigma (*Staphylococcus aureus* Wood 46 strain), a non-protein A producing strain. The proliferative response of PBM induced by *S. aureus* Wood 46 strain was measured in cows (n=4). Dilutions of the *S. aureus* Wood 46 strain ranged from 1:100-1:1,000,000. Variation in proliferative response of PBM induced by *S. aureus* Wood 46 strain was noted at the various dilutions (Figure: 12). Peak proliferation of PBM induced by *S. aureus* Wood 46 strain was at

dilutions of 1:1,000 or 1:10,000. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* Wood 46 strain (1:1,000) was; 18+/-22 on day 3; 61+/-91 on day 5; 58+/-65 on day 8 and 30+/-36 on day 10. The mean proliferative response (SI+/-SD) induced by *S. aureus* Wood 46 strain (1:10,000) was; 14+/-19 on day 3; 48+/-78 on day 5; 47+/-34 on day 8 and 39+/-38 on day 10. Variation among animals in the proliferative response induced by *S. aureus* Wood 46 strain was noted, as found previously with *S. aureus* strain A, C and D, with some animals generating a higher proliferative response than others (Figure: 13). It can be seen (Figure: 13) that cow 1 had a higher proliferative response induced by the *S. aureus* Wood 46 Strain At both dilutions of 1:1,000 and 1:10,000 compared to cows 2, 3 and 4. The day of peak proliferation for cow 1 was day five compared to day eight for cows 2, 3 and 4.

Generally, as was noted with the other strains of *S. aureus* tested, those animals with a strong proliferative response induced by one dilution of *S. aureus* also showed a strong proliferative response at the other dilutions tested.

The proliferative response of PBM to staphylococcal enterotoxin B was measured in cows (n=3). Enterotoxin B is one of a number of exoproteins secreted by strains of *S. aureus* that has been shown to induce a mitogenic polyclonal T cell response. To demonstrate that the kinetics of the proliferative response of PBM, induced by *S. aureus* in the current study were induced by an antigenic stimulus rather than a mitogenic stimulus, PBM, at the predetermined optimal concentration, were incubated with SEB (Sigma) at dilutions ranging from 1:100-1:100,000. The mean proliferative response (SI+/-SD) induced by SEB at a dilution of 1:100 was; 41+/-14 on day 2; 63+/-43 on day 4; 2+/-4 on day 6; and 0 on day 8. The mean proliferative response (SI+/-SD) induced by SEB at a dilution of 1:1000 was; 43+/-10 on day 2; 54+/-34 on day 4; 10+/-8 on day 6; and 1+/-2 on day 8. The mean proliferative response (SI+/-SD) induced by SEB at a dilution of 1:10,000 was; 40+/-8 on day 2; 49+/-32 on day 4; 9+/-5 on day 6; and 1+/-2 on day 8. The mean proliferative response (SI+/-SD) induced SEB at a dilution of 1:100,000 was; 32+/-13 on day 2; 46+/-25 on day 4; 8+/-4 on day 6; and 1+/-2 on day 8. Results showed that a strong proliferative response induced by all dilutions of SEB was detectable on day 2 of culture. The day of peak proliferation for all dilutions of SEB was day 4, by day 6 the proliferative response

induced by the SEB had begun to decline and by day 8 the response was negligible (data not shown), (Figure: 14).

As was reported when studying the different strains of *S. aureus*, variation in proliferative response of PBM induced by SEB was observed among cows (Figure: 15). The magnitude of the proliferative response induced by SEB on day 4 was greater in cow 3 compared to cows 1 and 2, which had quite similar levels of response. The results show clear differences in the kinetics of the proliferative response of PBM of cows induced by antigen and SEB when *S. aureus* strain A was added to the culture at a concentration of $0.5x10^6$ bacteria/ml, peak proliferation occurred at day 7 or day 9. In contrast, when SEB was added to the culture peak proliferation occurred much earlier on day 4.

In vivo, cell proliferation, induced by mammary gland pathogens would probably occur in cells found within the local mammary gland environment, including the interstitial tissue, and possibly in cells in the alveoli or gland cistern of the udder. To investigate the possible effect of milk on the proliferative response of PBM induced by SEB, an experiment was designed where proliferation induced by SEB was compared in the presence and absence of complete media containing 7% and 14% normal filtered milk. Peripheral blood mononuclear cells and SEB were also cultured in the presence of the same dilutions of filtered milk from a mastitic cow, which had been identified as being infected with *S. aureus* by routine bacteriology. Staphylococcal Enterotoxin B was used at a dilution of 1:10,000. Results showed that the day of peak proliferation in the presence of SEB was on day three, which confirmed the difference in kinetics of proliferative response, induced by SEB and *S. aureus* as shown previously. The mean proliferative response (SI+/-SD) of PBM induced by SEB was; 2+/-2 on day 1; 69+/-47 on day 2; 162+/-156 on day 3; 107 +/-95 on day 4; and 18+/-19 on day 5. The mean proliferative response (SI+/-SD) induced by SEB in 14% normal milk was; 0 on day 1; 8+/-3 on day 2; 28+/-18 on day 3; 28+/-30 on day 4; and 10+/-6 on day 5. The mean proliferative response (SI+/-SD) of PBM induced by SEB in 7% normal milk was; 1+/-1 on day 1; 19+/-1 on day 2; 33+/-5 on day 3; 108+/-161 on day 4; and 38+/-56 on day 5 (n=3). Using a paired T test, the presence of 14% normal milk in the culture system significantly reduced the proliferative response of PBM of cows induced by SEB compared to the proliferative response induced by SEB in the absence of milk (p=0.025).

Again using a paired T test, a similar trend could also be seen in the proliferative response induced by SEB in the presence of 7% normal milk, however this was not statistically significant (p=0.28), (Figure: 16). Incubation of PBM in the presence of milk infected with *S. aureus*, as determined by routine bacteriology, induced no proliferative response over background when added to the *in vitro* culture at concentrations of 7% and 14%.

Presentation of antigen to $CD4^+$ cells is dependent on expression of MHC class II on antigen presenting cells. To determine if the proliferative ability of PBM of cows induced by *S. aureus* strain A was dependent on MHC class II, an anti-MHC class II MAb, IL-A21, was added to PBM and *S. aureus* strain A during culture *in vitro*. Dilutions of the anti-MHC class II used were 1:5 and 1:10. The mean proliferative response (SI+/-SD) of PBM of cows induced by *S. aureus* strain A was; 4+/-1 on day 2; 66+/-61 on day 4; 225+/-111 on day 6; 81+/-31 on day 8; and 75+/-27 on day 10. The mean proliferative response (SI+/-SD) of PBM of cows induced by *S. aureus* strain A in the presence of anti-MHC class II (1:5) was; 1+/-1 on day 2; 29+/-16 on day 4; 138+/-91 on day 6; 81+/-61 on day 8; and 19+/-18 on day 10. The mean proliferative response (SI+/-SD) of PBM of cows induced by *S. aureus* strain A in the presence of anti-MHC class II (1:10) was; 3+/-1 on day 2; 45+/-28 on day 4; 182+/-90 on day 6; 105+/-43 on day 8 and 31+/-9 on day 10 (Figure: 17). Results showed that the proliferative response of PBM induced by *S. aureus* strain A was significantly reduced in the presence of anti-MHC class II at a dilution of 1:5 (p=0.05) on day 6 of culture (n=5). A similar trend in reduction of proliferative response of PBM was also seen in the presence of anti-MHC class II at a dilution of 1:10 on day 6 of culture, however this was not statistically significant (p=0.06).

During the course of the study, additional stocks of *S. aureus* strain A were required for use in the proliferative assay. Variation in the ability of two newly prepared batches of *S. aureus* strain A and the original batch to generate a proliferative response of PBM of cows (n=8) was noted. The comparison was based on the *S. aureus* strain A at the original concentration of $0.5x10^6$ bacteria/ml that was chosen from the primary results. The mean proliferative response (SI+/-SD) of PBM induced by the second batch of *S. aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml was; 1+/-1 on day 2; 4+/-3 on day 4; 3+/-5 on day 6; 22+/-15 on day 8 and 14+/-12 on day 10. The mean proliferative response (SI+/-SD) of PBM induced by the third batch of *S. aureus* strain A at a

concentration of $0.5x10^6$ bacteria/ml was 1+/-2 on day 2; 4+/-4 on day 4; 9+/-11 on day 6; 11+/-19 on day 8 and 6+/-1 on day 10. These were compared with the mean proliferative response (SI+/-SD) of PBM induced by the original strain A at a concentration of $0.5x10^6$ bacteria/ml (n=21), (Figure: 18). The day of peak proliferation for the newly prepared batches of *S. aureus* strain A at a concentration of $0.5x10^6$bacteria/ml was slightly later, measured on day 10 of culture, whereas the day of peak proliferation of the original batch was day 8 of culture.

The initial concentration of bacteria present in the newly prepared batches of *S. aureus* strain A was greater than that of the original batch. Titration experiments were carried out to determine the optimum concentration of the new batches of *S. aureus* strain A. Concentrations ranged from 0.35-$5x10^8$ bacteria/ml. Results of the titration experiments showed at a concentration of $5x10^8$ bacteria/ml, *S. aureus* strain A failed to induce a proliferative response in PBM of cows. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain A, at a concentration of $2.5x10^8$ bacteria/ml was; 40+/-42 on day 2; 59+/-157 on day 4; 9+/-15 on day 6; 8+/-14 on day 8 and 3+/-3 on day 10. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain A, at a concentration of $1.4x10^8$ bacteria/ml was; 48+/-42 on day 2; 219+/-240 on day 4; 35+/-28 on day 6; 20+/-22 on day 8 and 8+/-8 on day 10. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain A, at a concentration of $0.7x10^8$ bacteria/ml was; 32+/-29 on day 2; 158+/-194 on day 4; 71+/-66 on day 6; 44+/-49 on day 8 and 21+/-32 on day 10. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain A, at a concentration of $0.35x10^8$ bacteria/ml was; 17+/-13 on day 2; 87+/-35 on day 4; 113+/-76 on day 6; 87+/-98 on day 8 and 29+/-24 on day 10 (n=9), (Figure: 19). These results showed that the optimal dilution of *S. aureus* strain A altered from $0.5x10^6$ bacteria/ml to either $1.4x10^8$ bacteria/ml or $0.7x10^8$ bacteria/ml, and that the day of peak proliferation had changed compared to the original batch. The alteration was from peak proliferation occurring on day eight or nine to the day of peak proliferation to day four at a concentration of $1.4x10^8$ bacteria/ml (Figure: 20), and either day four or day six at a concentration of $0.7x10^8$ bacteria/ml (Figure: 21). As the concentration of *S. aureus* strain A reduced, the day of peak proliferation occurred later. Variation in the proliferative response of PBM induced by *S. aureus* strain A among cows at the new concentrations studied was noted. In general, cows with a strong proliferative response of

PBM induced by *S. aureus* strain A had similar responses at the other concentrations of *S. aureus* strain A tested. This can be seen in figures 20, 21 and 22, at concentrations of $1.4x10^8$ bacteria/ml, $0.7x10^8$ bacteria/ml and $0.35x10^8$ bacteria/ml in cows 2, 3 and 4, which responded better than the other cows to the *S. aureus* strain A.

The variation in proliferative response induced by the original batch of *S. aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml in cows led to investigation of the effect of sire on the proliferative response induced by *S. aureus* strain A in two progeny groups. The significant difference between the progeny groups sired by two different bulls led to investigation of the proliferative response of PBM of Holstein-Friesian dairy bulls induced by *S. aureus* strain A. Initially, ten bulls were selected from the list of UK artificial insemination bulls owned by Genus Ltd. Plc. These bulls were chosen based upon available information for PTA for SCC, and they represented the extremes within the population: five bulls were chosen as having the lowest PTA for SCC, and five were chosen as having the highest PTA for SCC. The PTA for SCC, recorded in 1997, for the ten extreme Holstein-Friesian bulls ranged from –12.9 (a mean reduction of 12.9% in SCC in the daughters of that sire, compared to the mean of the population), to +19.6 (a mean increase of 19.6% in SCC in the daughters of that sire, compared to the mean of the population). The PTA for SCC data is not static and as more daughters and more lactation records are generated these are incorporated into the data set from which PTA for SCC is calculated. As more data is included the reliability of the PTA for SCC value increases, adding power to the predicted increase or decrease in progeny SCC. The PTA for SCC in 1998 for the same ten Holstein-Friesian bulls ranged from –23 to +35, and in 1999 the PTA for SCC ranged from –2 to +23. Two of the bulls did not have PTA for SCC available in November 1999 (Figure: 23). The reliabilities for the 1997 values for PTA for SCC ranged from 36% to 94%, for the 1998 values, from 49% to 98%, and for the 1999, from 49% to 98%. In addition to the PTA for SCC data on the ten extreme bulls in 1997, PTA for the production traits of milk, fat and protein were available (Table: 5). On comparison of the bulls with low PTA for SCC with the group of bulls with high PTA for SCC it was noted that PTA for milk ($p=0.7$), fat ($p=0.96$) and protein ($p=0.81$) was not affected by the PTA for SCC. This indicates that selection of sires with a low PTA for SCC should not result in reduced milk yield, fat or protein content in their progeny.

		PREDICTED TRANSMITTING ABILITIES 1997			
BULL	**SI**	**SCC**	**MILK (kg)**	**FAT (kg)**	**PROTEIN (kg)**
A	37	-12.9	504	21.5	N/A
B	736	-12.2	544	15.5	19.2
C	516	-8.1	666	26.3	21.9
D	12	-7.8	242	2.3	11.2
E	75	-5.8	520	12.4	14.8
F	38	10.6	653	14.3	16.1
G	5	12.0	645	18.7	15.6
H	16	12.9	474	14.3	17.8
I	8	13.6	678	26.6	19.7
J	11	19.6	273	5.7	11.2

Table 5: Predicted Transmitting Abilities of SCC, milk, fat and protein in 1997 and the proliferative response (SI) induced by *S. aureus* strain A for the five bulls recorded as having the lowest PTA for SCC (coloured blue) and for the five bulls recorded as having the highest PTA for SCC (coloured red)

The proliferative response (SI) of PBM isolated from the initial ten bulls induced by *S. aureus* strain A ranks bull A as having the lowest PTA for SCC in 1997, and bull J as having the highest PTA for SCC in 1997 (Figure: 24). The range of SI for those bulls selected as having the lowest PTA for SCC in 1997 was; 12 to 726 on day 9, and the range of SI for those bulls selected as having the highest PTA for SCC in 1997 was; 5 to 38 on day 9. There was a strong negative correlation (r= -0.7) between the log SI and PTA for SCC in 1997. Results showed that the proliferative response of PBM of bulls induced by *S. aureus* strain A was statistically significant between the two groups of bulls (p=0.05), with the regression of response on SCC proof also being significant (p=0.05). The results however were not dichotomous but bimodal and overlapping. Bull D, recorded as having a low PTA for SCC, showed a weak proliferative response induced by *S. aureus* strain A, and bull F, recorded as having a high PTA for SCC, showed a strong proliferative response induced by *S. aureus* strain A (Figure: 24). The within assay repeatability based on log SI was shown to be 97% for these ten bulls (p=0.03).

The strong negative correlation between SI and PTA for SCC in 1997 in the extreme ten bulls resulted in the study being extended. One hundred and three Holstein-Friesian dairy bulls were sampled and the proliferative response of the PBM induced by *S. aureus* strain A at a concentration of $0.5x10^6$bacteria/ml was measured. These bulls were sufficiently old to have generated sufficient daughters to undergo progeny testing. A total of 59 proofs for PTA for SCC were available in 1998 when the 103 bulls were first sampled, the mean +/-SD for PTA for SCC at that time was 4.8+/-6.0. The range of PTA for SCC was from –7.5 to 22.6. The following year in 1999, more proofs for PTA for SCC became available, a total of 98, and the mean +/-SD altered to 4.9+/-8.4. The range of PTA for SCC was from –12.0 to 27.0. The alteration in proofs for PTA for SCC is due to the bulls maturing, increasing in the number of daughter lactation records on which the PTA for SCC is based and improving the reliability. On statistical analysis of the different years for PTA for SCC there was shown to be a good correlation between the data sets (r=0.75), this is not surprising as the data sets are not independent. Analysis of the 1998 PTA for SCC in relation to proliferative response of the 103 bulls induced by *S. aureus* strain A (mean log SI) resulted in a correlation (r=) of –0.07. Analysis of the 1999 PTA for SCC in relation to immunological response of the 103 bulls induced by *S. aureus* strain A (mean log SI) resulted in a correlation (r=) of –0.01. Both of these results show that the significant negative correlation found in the ten extreme bulls (r=-0.7) was not present in the randomly selected sample of 103 bulls.

Variation in the proliferative response of PBM of Holstein-Friesian bulls induced by *S. aureus* strain A was noted among animals with SI ranging from 131 to 0 on day nine of culture (Figure: 25). The most extreme SI value was 131, followed by the next highest SI value of 49. Thirty-four of those bulls sampled failed to generate a proliferative response induced by *S. aureus* strain A. From the sample population of 103 Holstein-Friesian bulls, 53 were resampled to test between assay repeatability. Additionally nine bulls were sampled for a third time and six bulls were sampled for a fourth time. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain A for the bulls tested once was 9+/-16; the SI ranged from 0 to 31. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain A for the bulls tested twice was 7+/-12; the SI ranged from 0 to 48. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus*

strain A for the bulls tested three times was 8+/-12; the SI ranged from 0 to 33. The mean proliferative response (SI+/-SD) of PBM induced by *S. aureus* strain A for the bulls tested four times was 6+/-5; the SI ranged from 0 to 14. Variation in the proliferative response of PBM induced by *S. aureus* strain A was noted among those bulls, which were sampled on more than one occasion. The proliferative response (SI) of PBM induced by *S. aureus* strain A of the bulls sampled on four occasions range was; bull one: 5 to 45; bull two: 4 to 14; bull three: 0 to 5; bull four: 5 to 15; bull five: 2 to 32 and for bull six: 2 to 30 (Figure: 26).

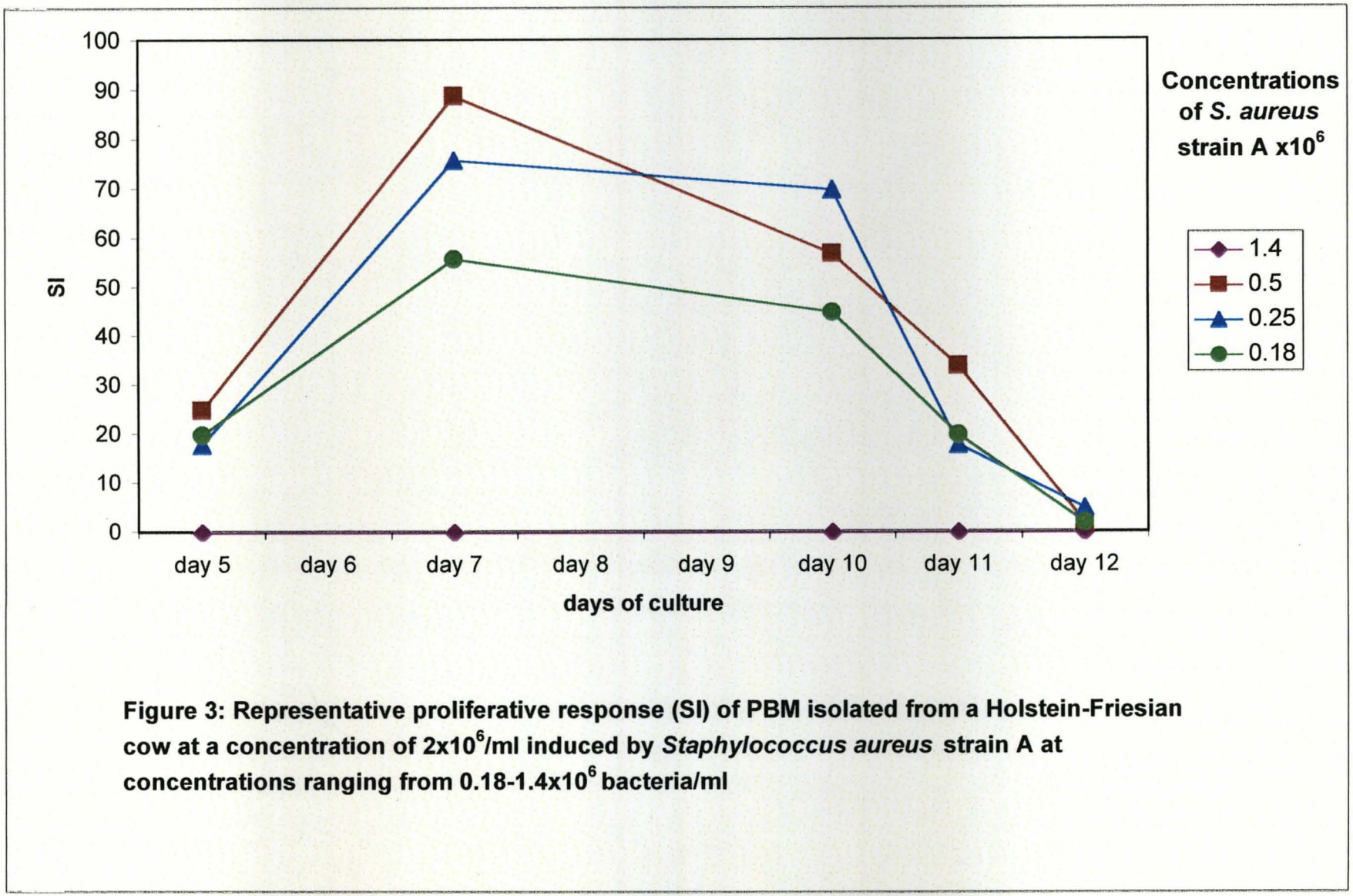


Figure 3: Representative proliferative response (SI) of PBM isolated from a Holstein-Friesian cow at a concentration of $2x10^6$/ml induced by *Staphylococcus aureus* strain A at concentrations ranging from $0.18\text{-}1.4x10^6$ bacteria/ml

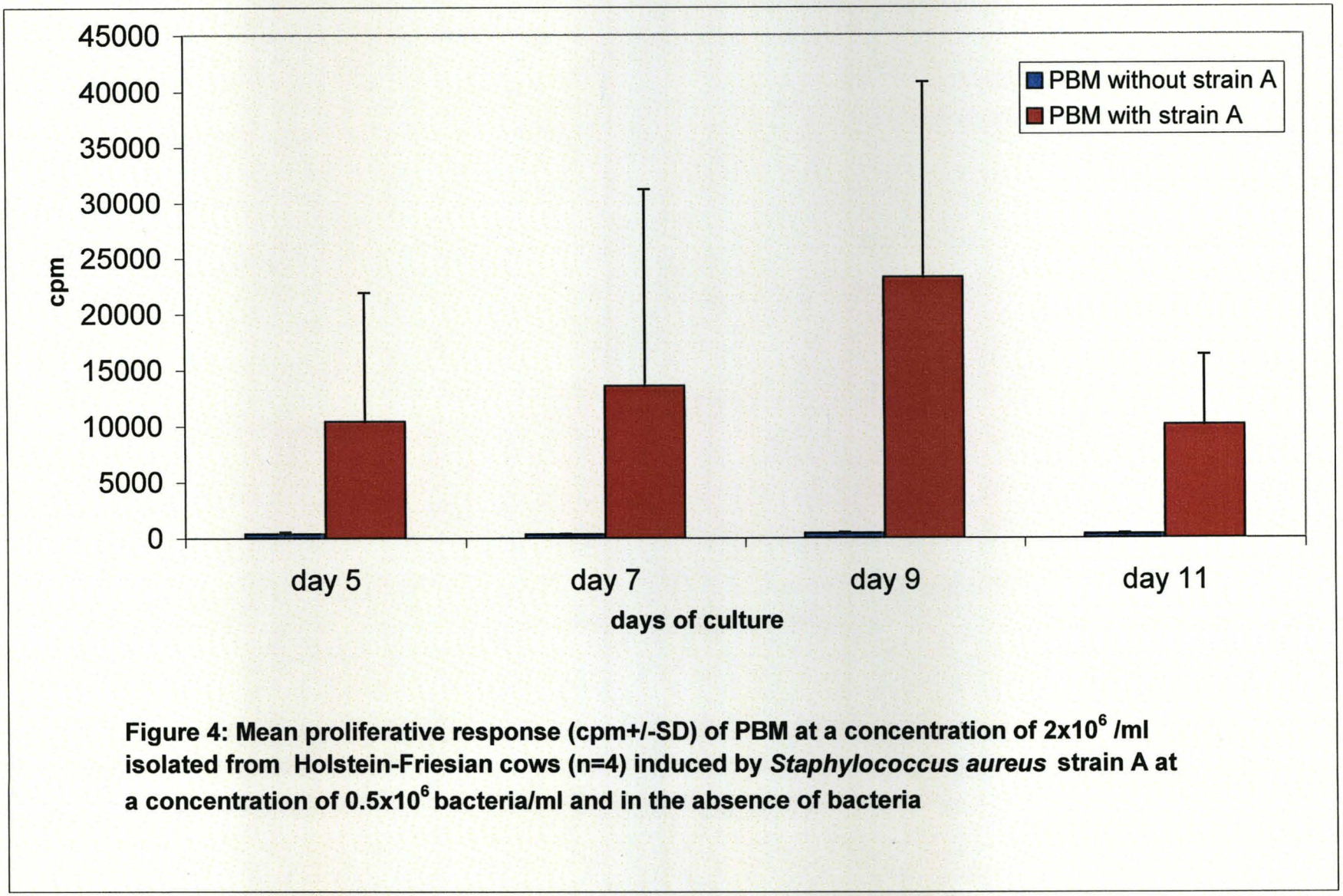


Figure 4: Mean proliferative response (cpm+/-SD) of PBM at a concentration of $2x10^6$ /ml isolated from Holstein-Friesian cows (n=4) induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml and in the absence of bacteria

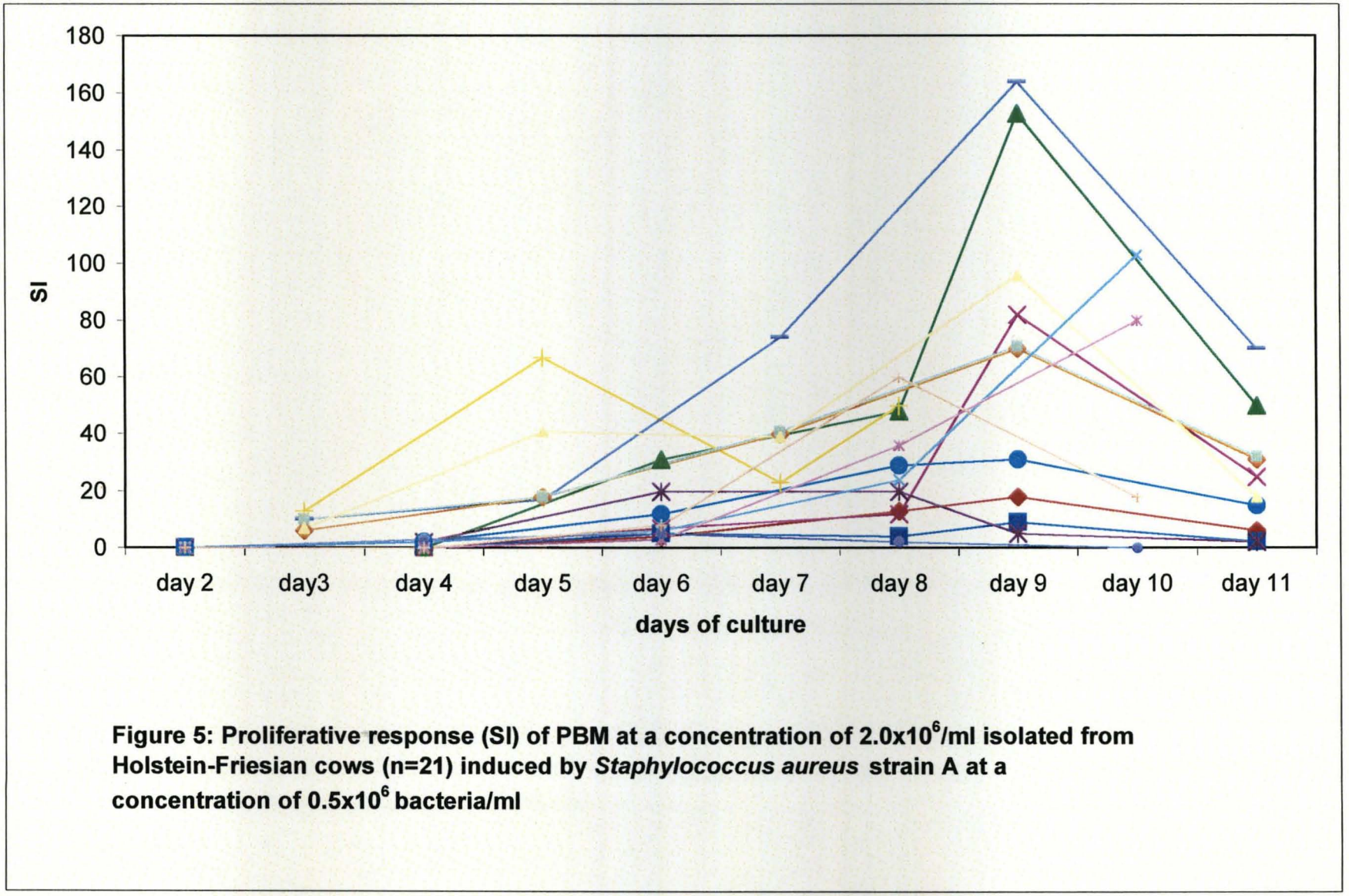


Figure 5: Proliferative response (SI) of PBM at a concentration of $2.0x10^6$/ml isolated from Holstein-Friesian cows (n=21) induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml

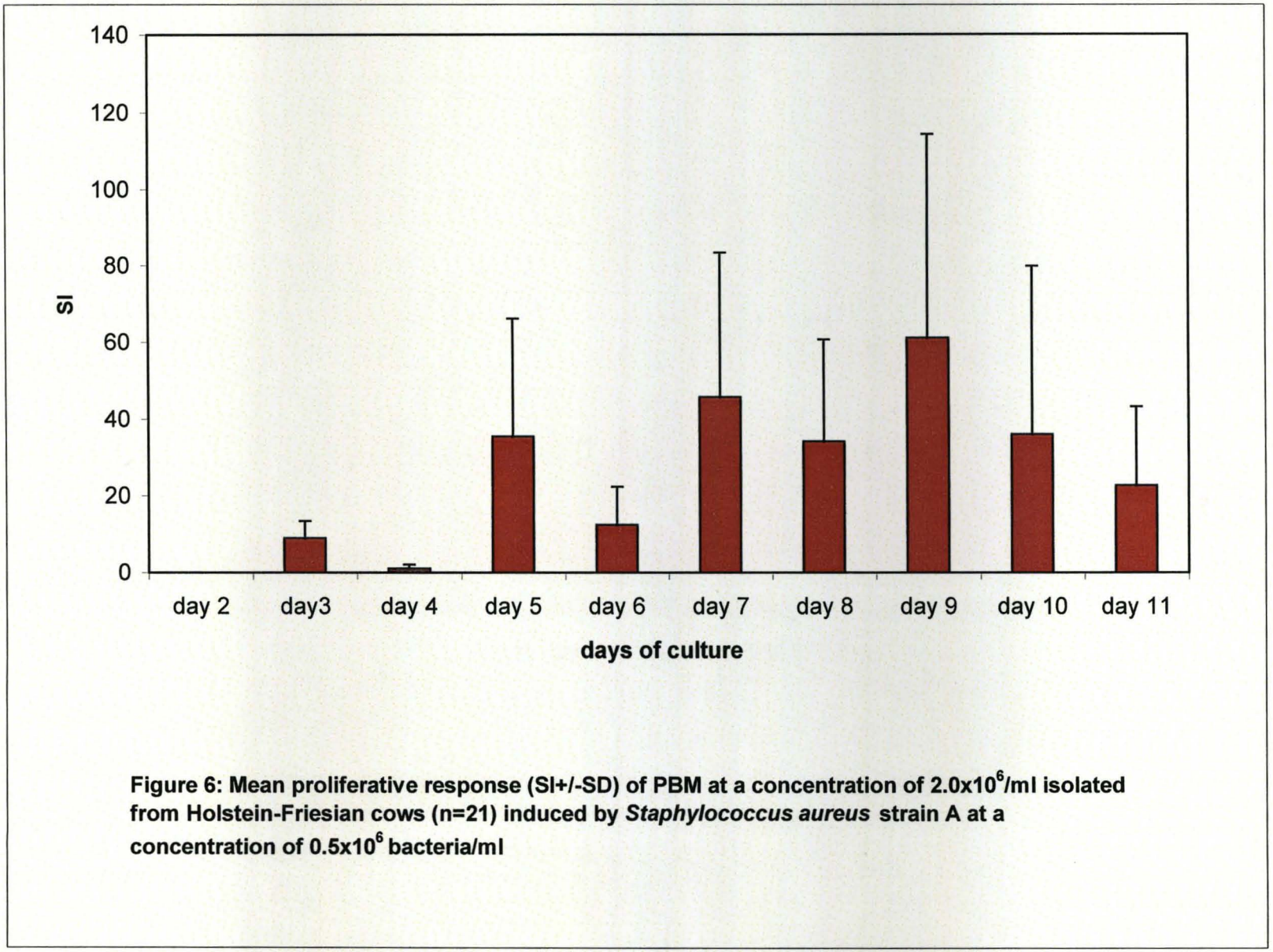


Figure 6: Mean proliferative response (SI+/-SD) of PBM at a concentration of $2.0x10^6$/ml isolated from Holstein-Friesian cows (n=21) induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml

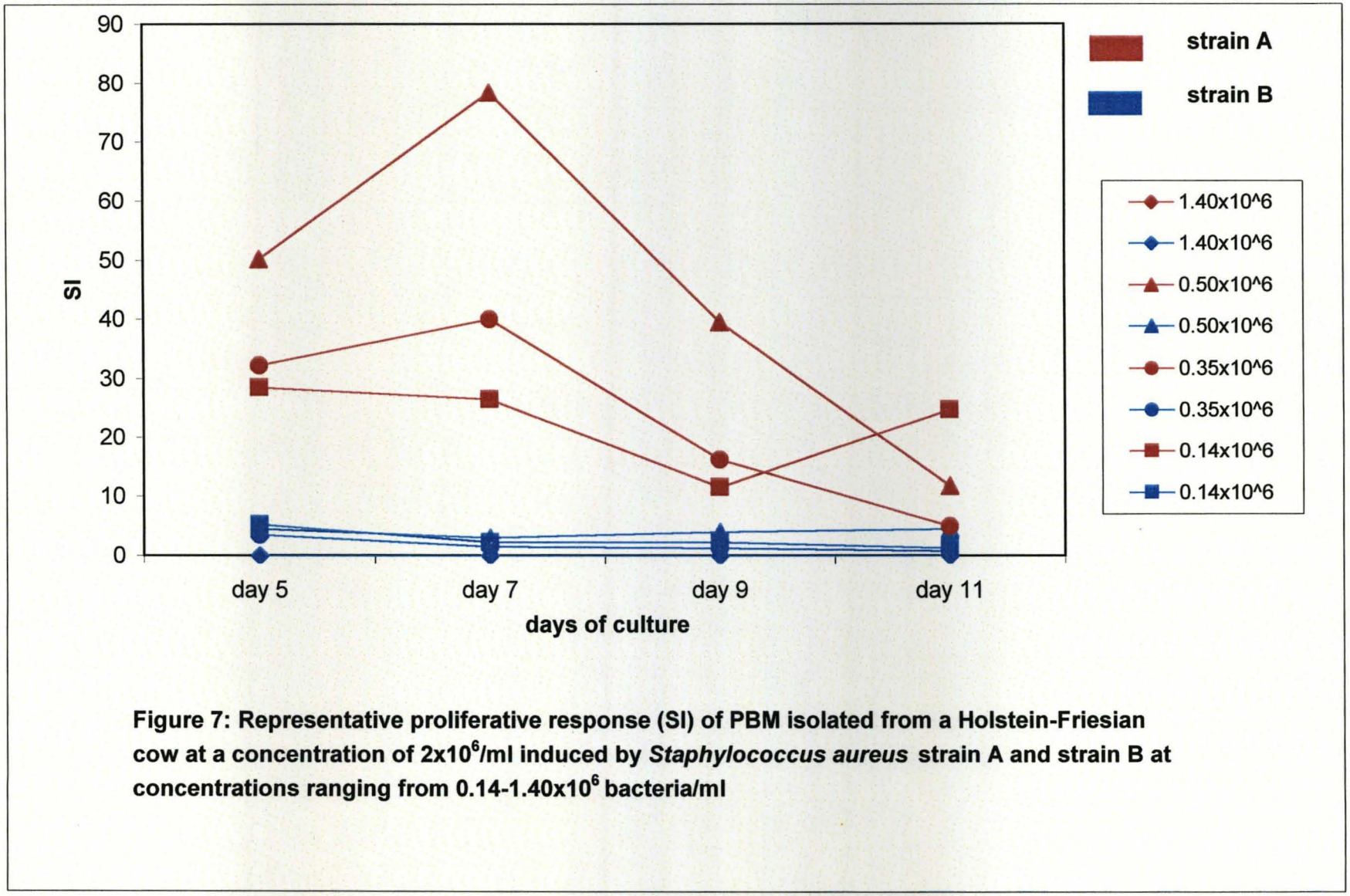


Figure 7: Representative proliferative response (SI) of PBM isolated from a Holstein-Friesian cow at a concentration of $2x10^6$/ml induced by *Staphylococcus aureus* strain A and strain B at concentrations ranging from 0.14-1.40x10^6 bacteria/ml

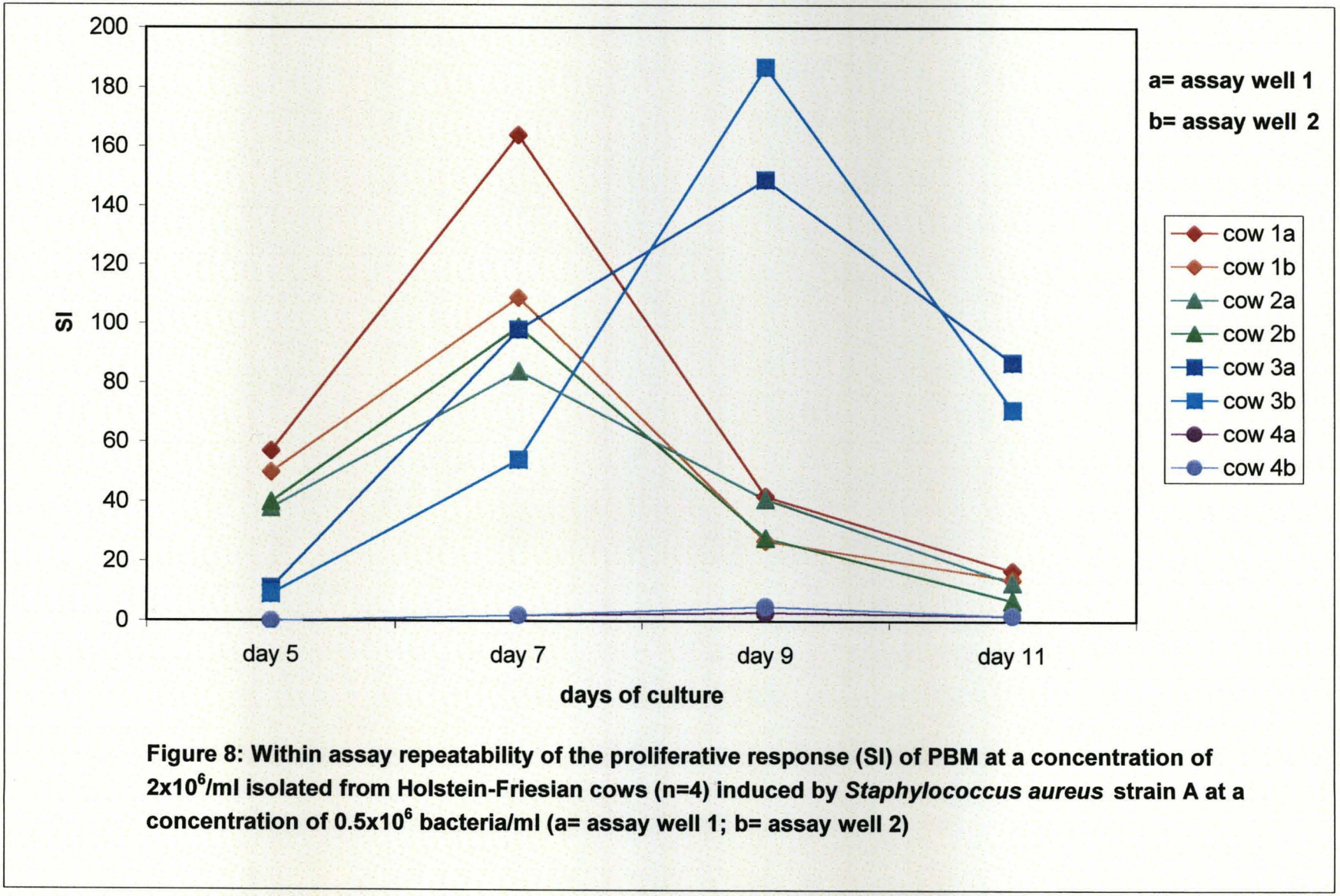


Figure 8: Within assay repeatability of the proliferative response (SI) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=4) induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml (a= assay well 1; b= assay well 2)

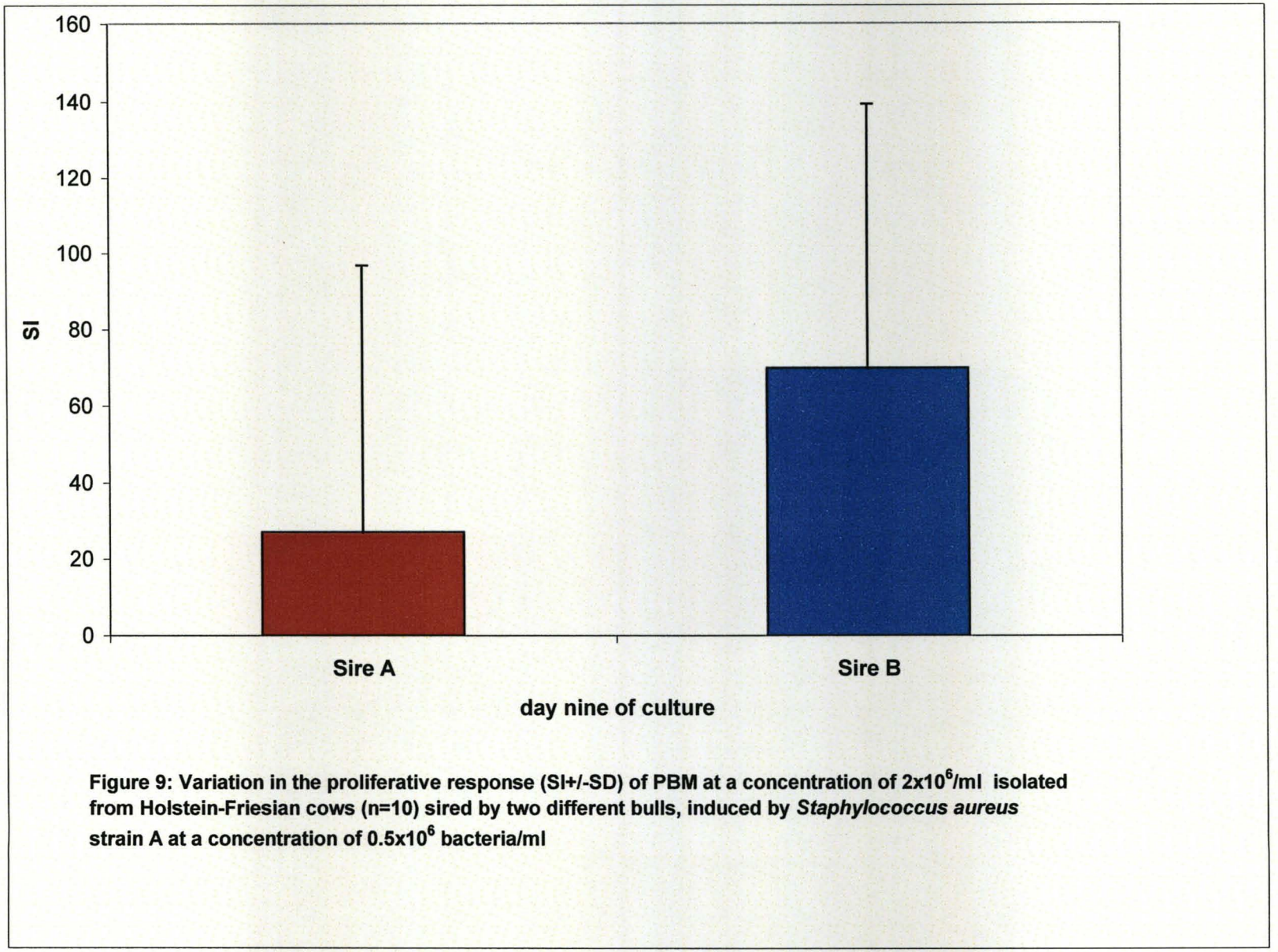


Figure 9: Variation in the proliferative response (SI+/-SD) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=10) sired by two different bulls, induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml

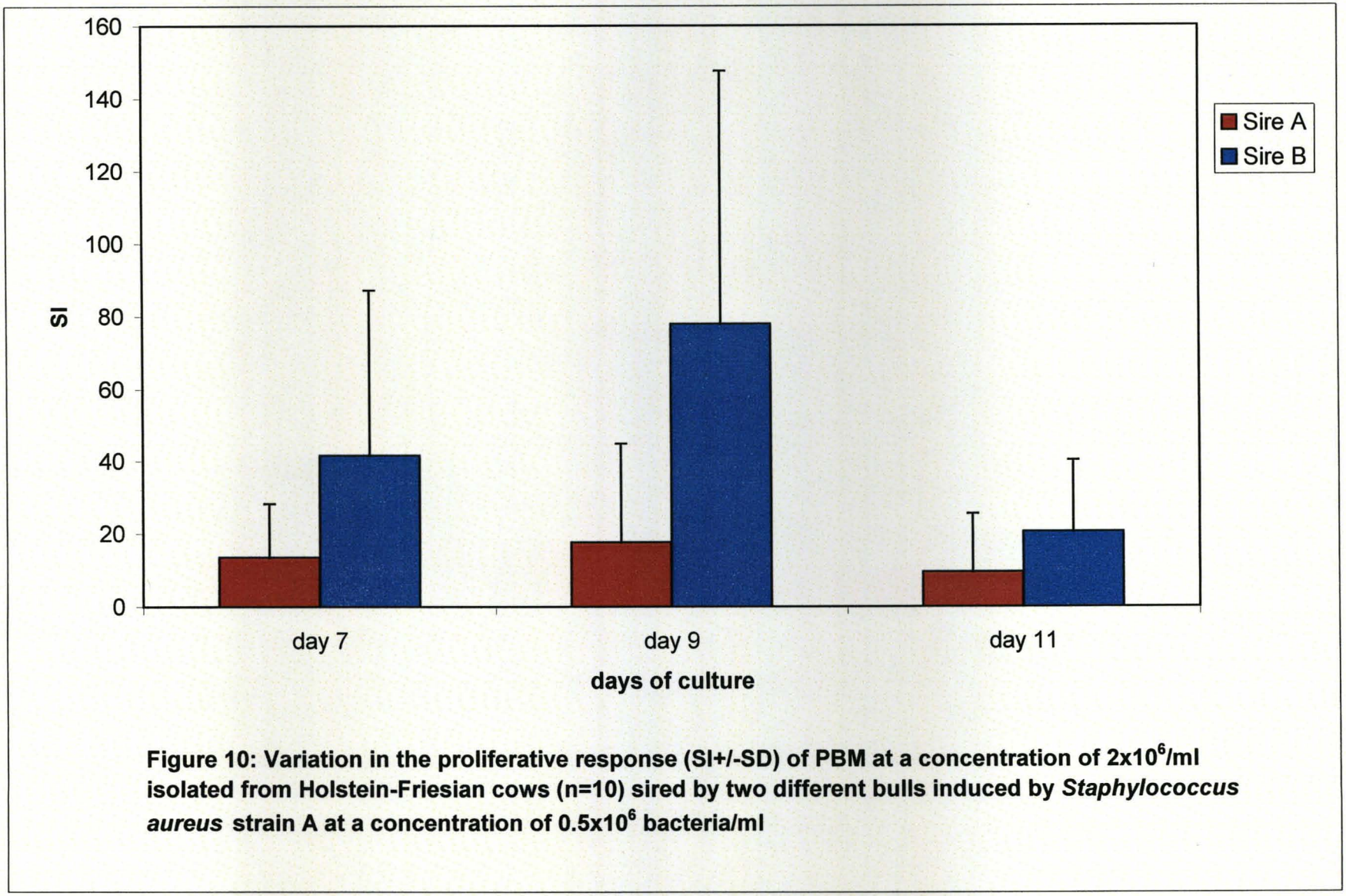


Figure 10: Variation in the proliferative response (SI+/-SD) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=10) sired by two different bulls induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml

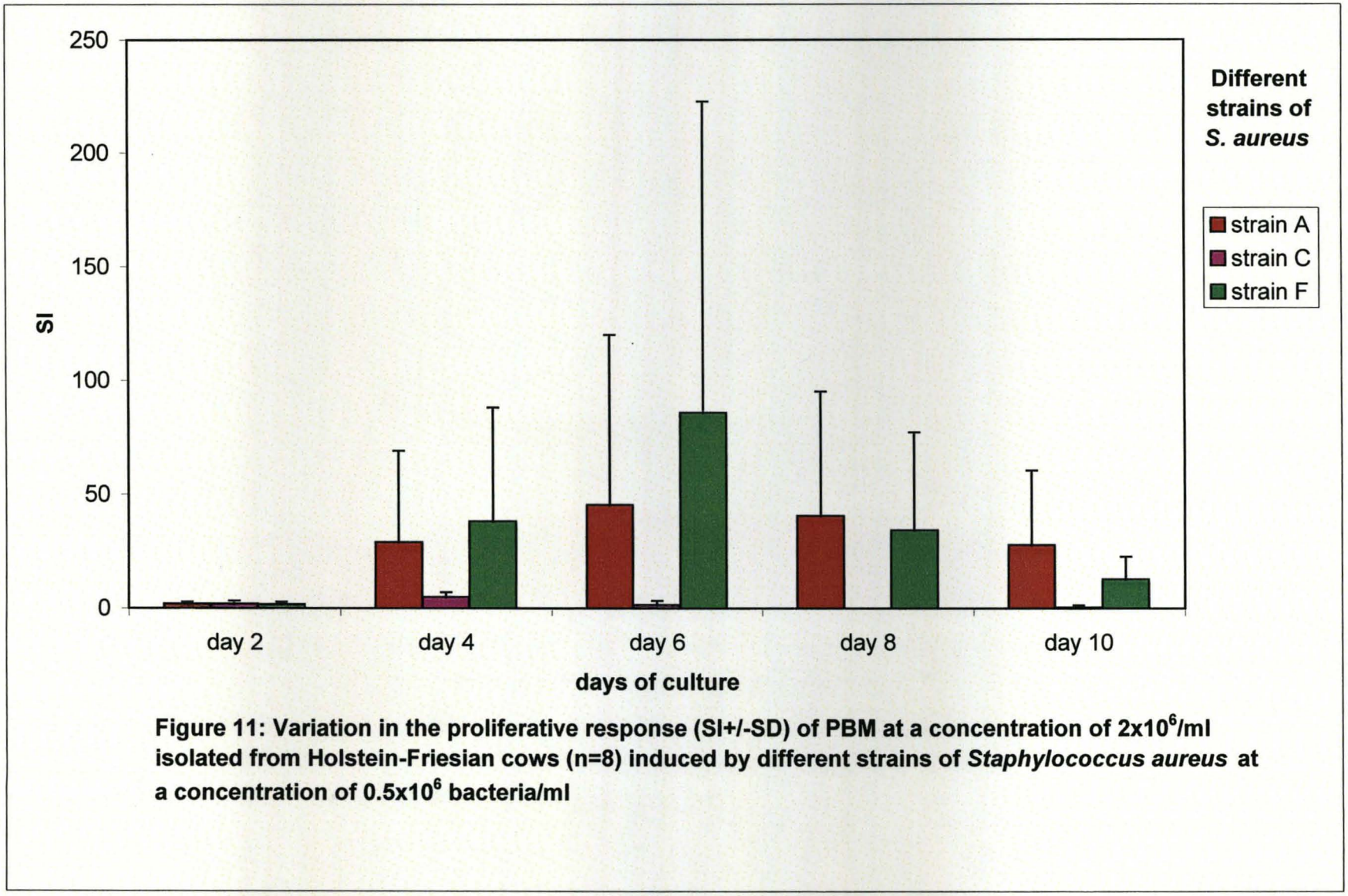


Figure 11: Variation in the proliferative response (SI+/-SD) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=8) induced by different strains of *Staphylococcus aureus* at a concentration of $0.5x10^6$ bacteria/ml

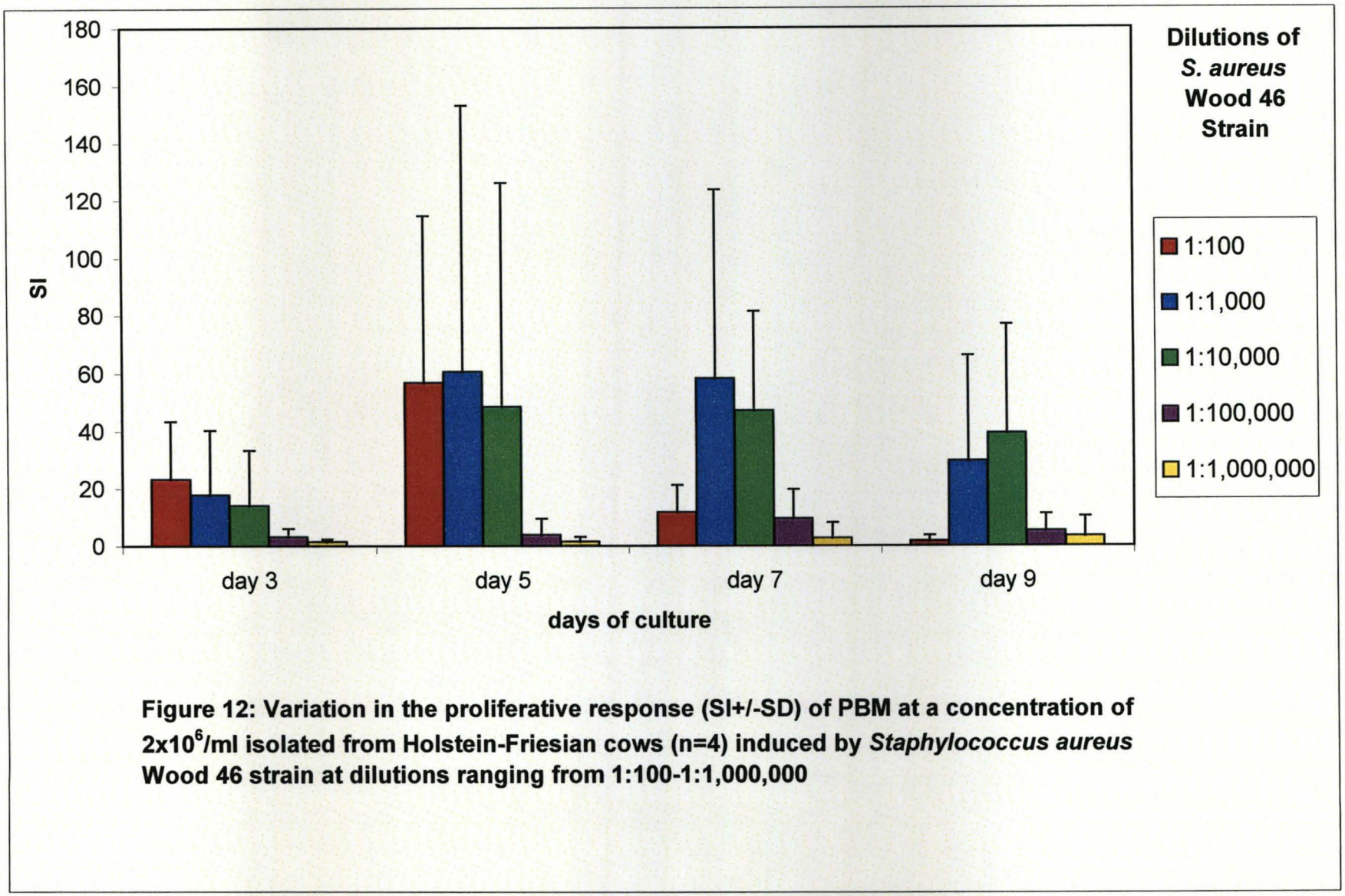


Figure 12: Variation in the proliferative response (SI+/-SD) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=4) induced by *Staphylococcus aureus* Wood 46 strain at dilutions ranging from 1:100-1:1,000,000

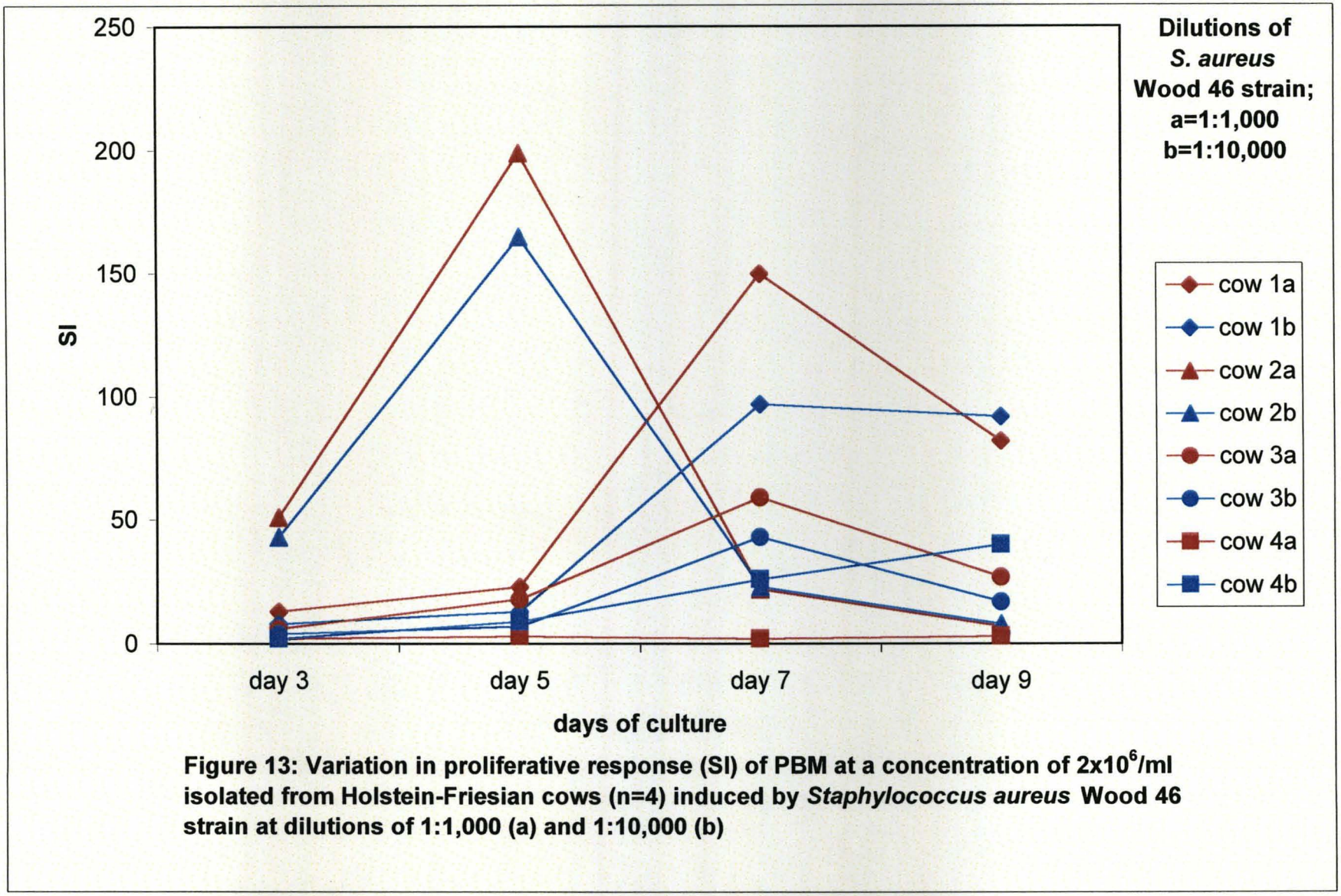


Figure 13: Variation in proliferative response (SI) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=4) induced by *Staphylococcus aureus* Wood 46 strain at dilutions of 1:1,000 (a) and 1:10,000 (b)

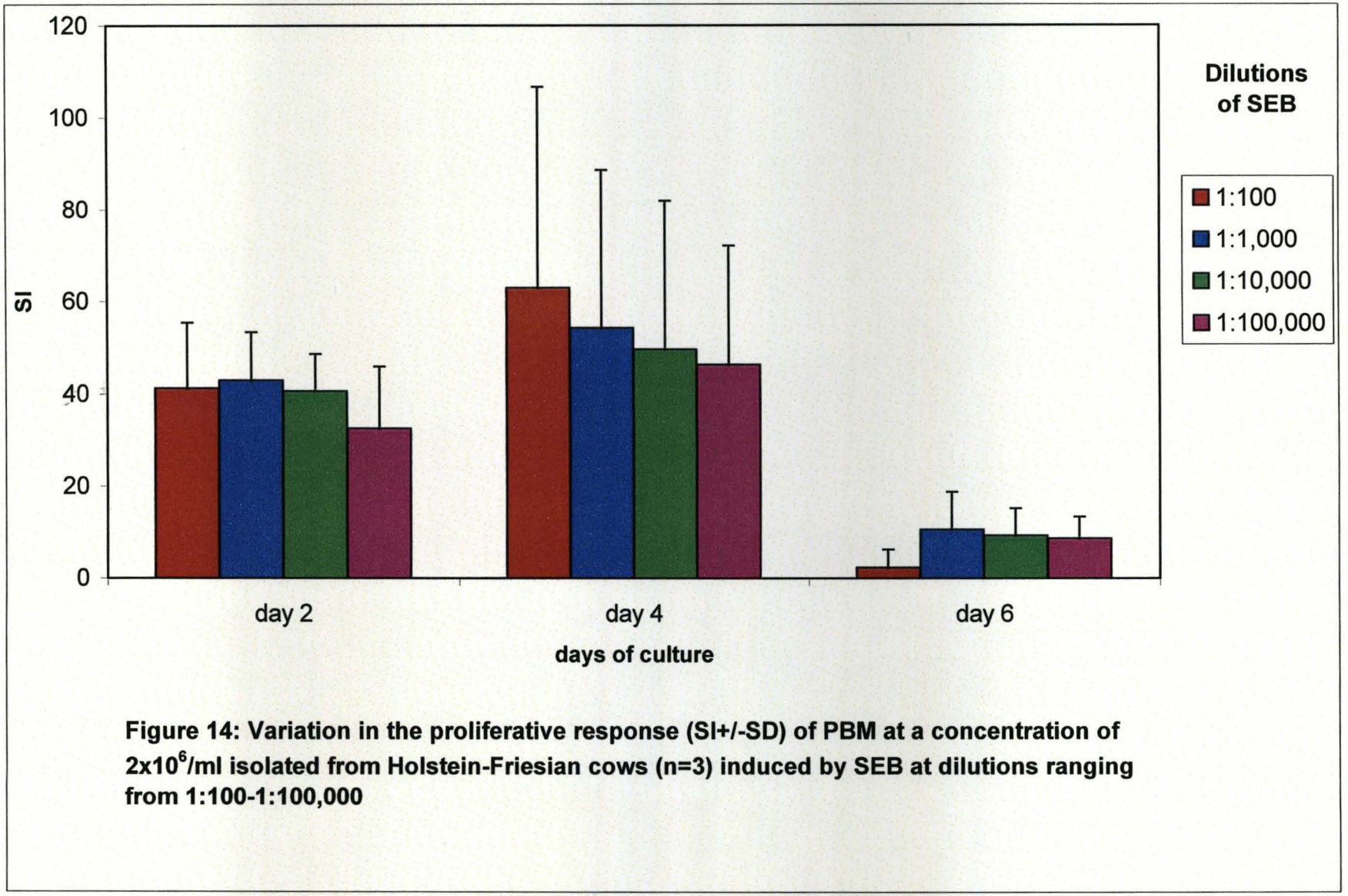


Figure 14: Variation in the proliferative response (SI+/-SD) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=3) induced by SEB at dilutions ranging from 1:100-1:100,000

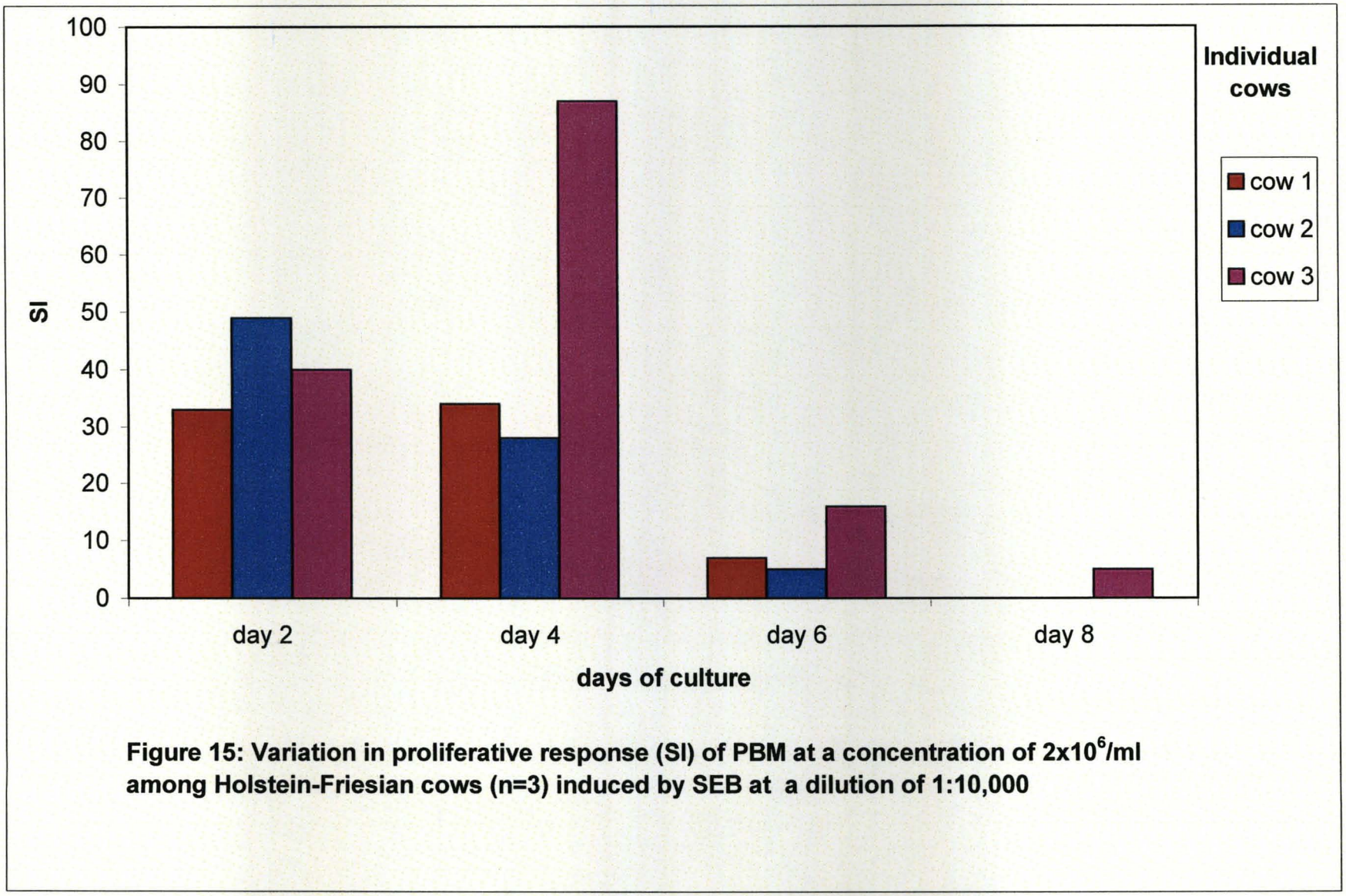


Figure 15: Variation in proliferative response (SI) of PBM at a concentration of $2x10^6$/ml among Holstein-Friesian cows (n=3) induced by SEB at a dilution of 1:10,000

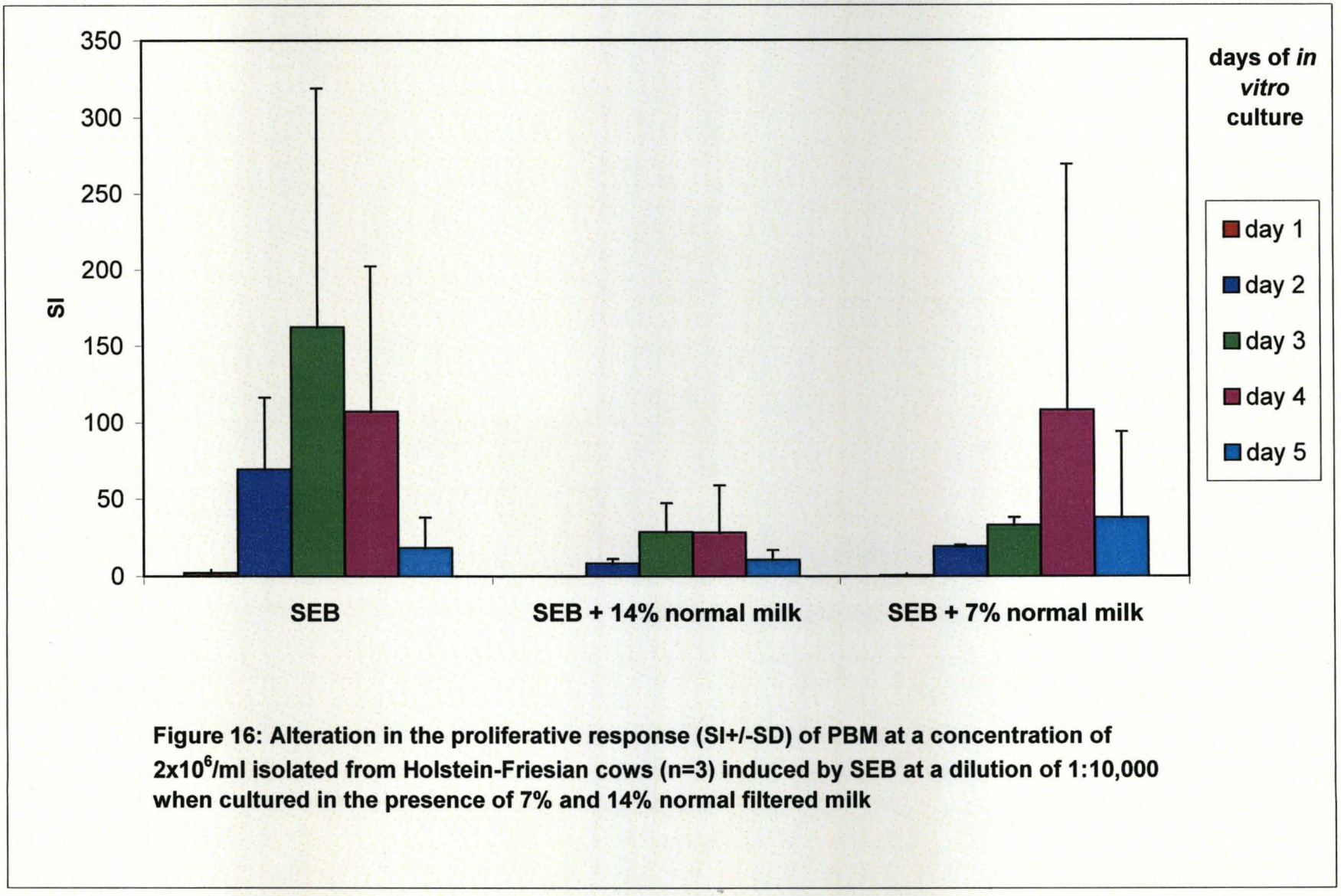


Figure 16: Alteration in the proliferative response (SI+/-SD) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=3) induced by SEB at a dilution of 1:10,000 when cultured in the presence of 7% and 14% normal filtered milk

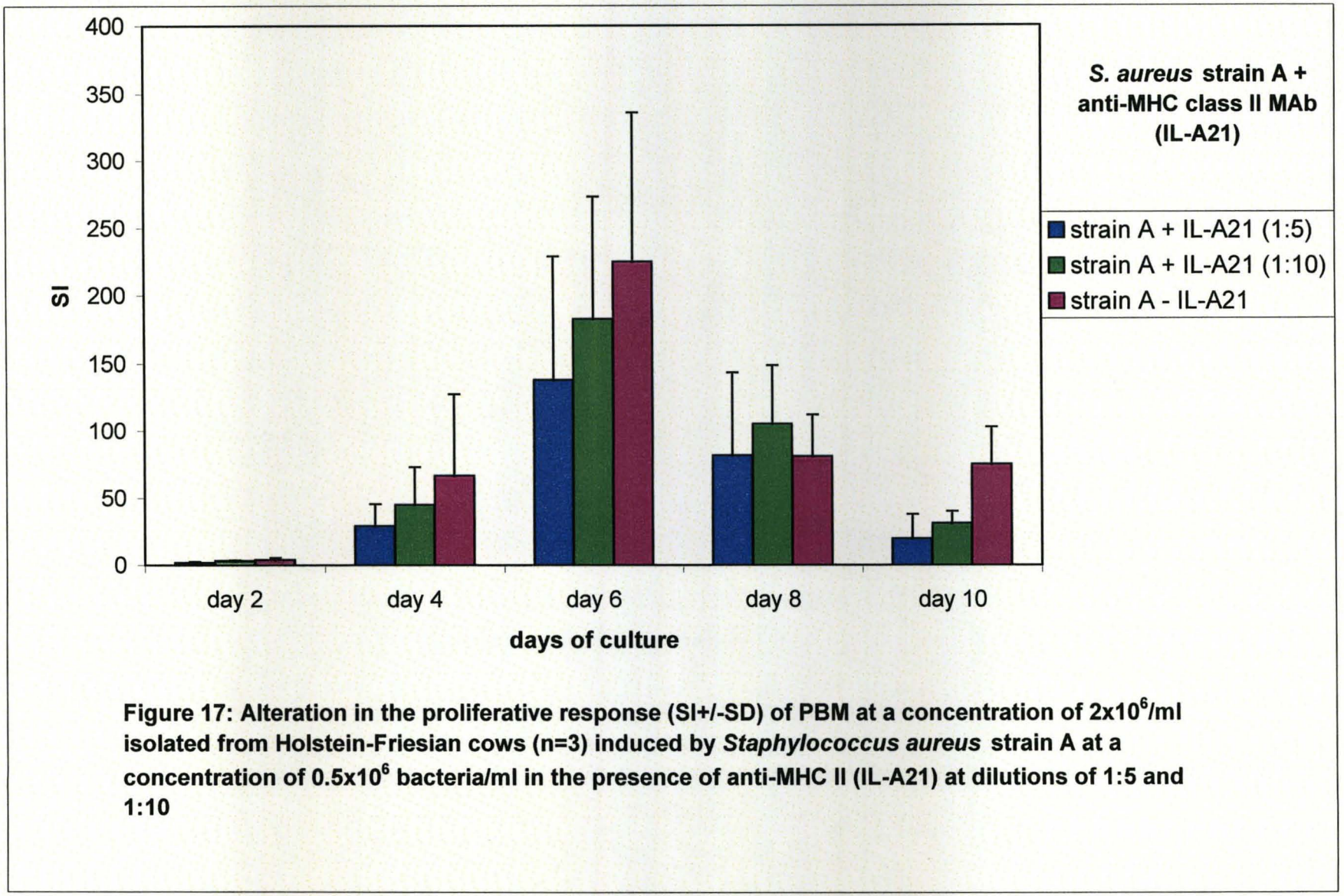


Figure 17: Alteration in the proliferative response (SI+/-SD) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=3) induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml in the presence of anti-MHC II (IL-A21) at dilutions of 1:5 and 1:10

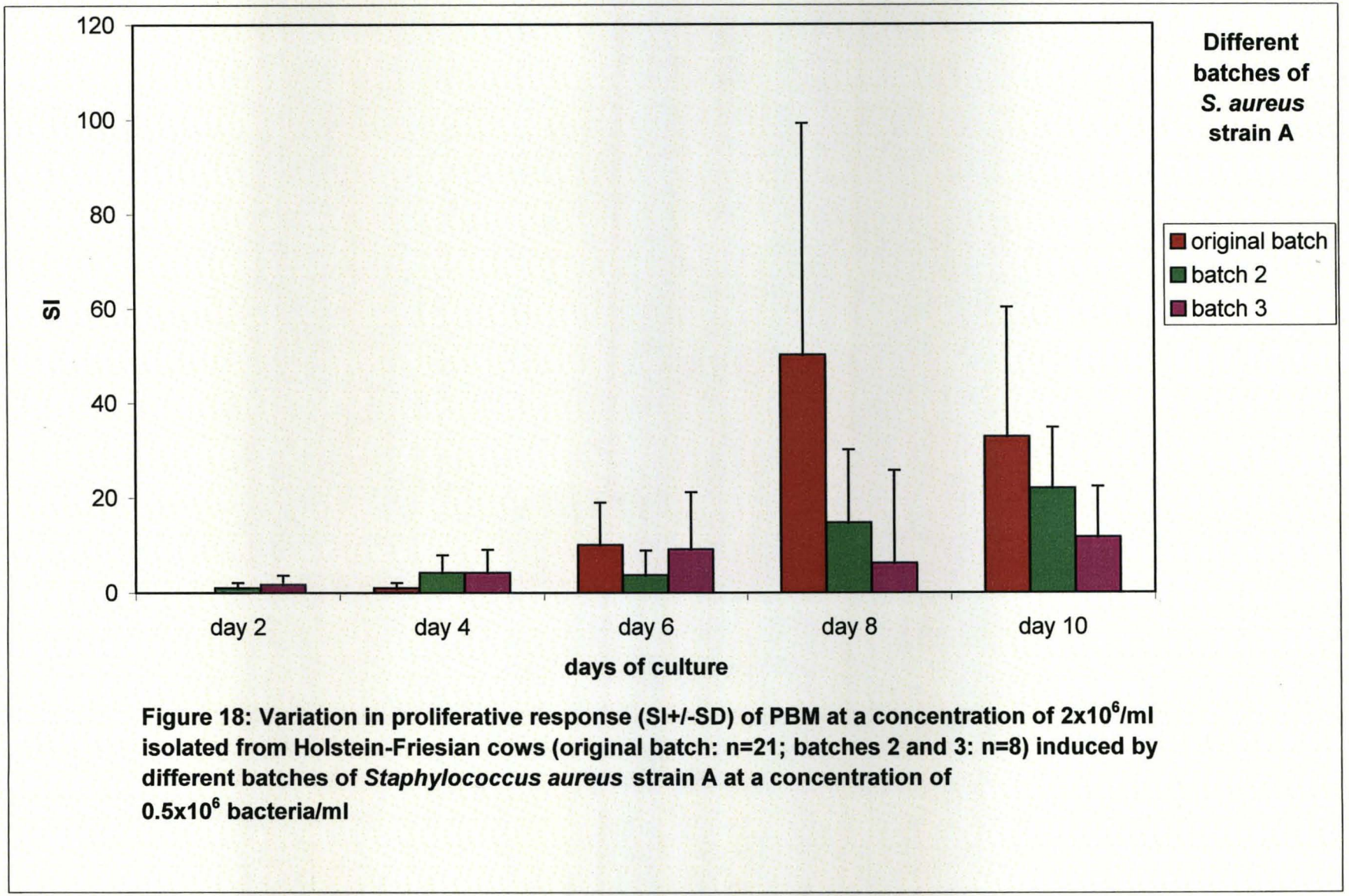


Figure 18: Variation in proliferative response (SI+/-SD) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (original batch: n=21; batches 2 and 3: n=8) induced by different batches of *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml

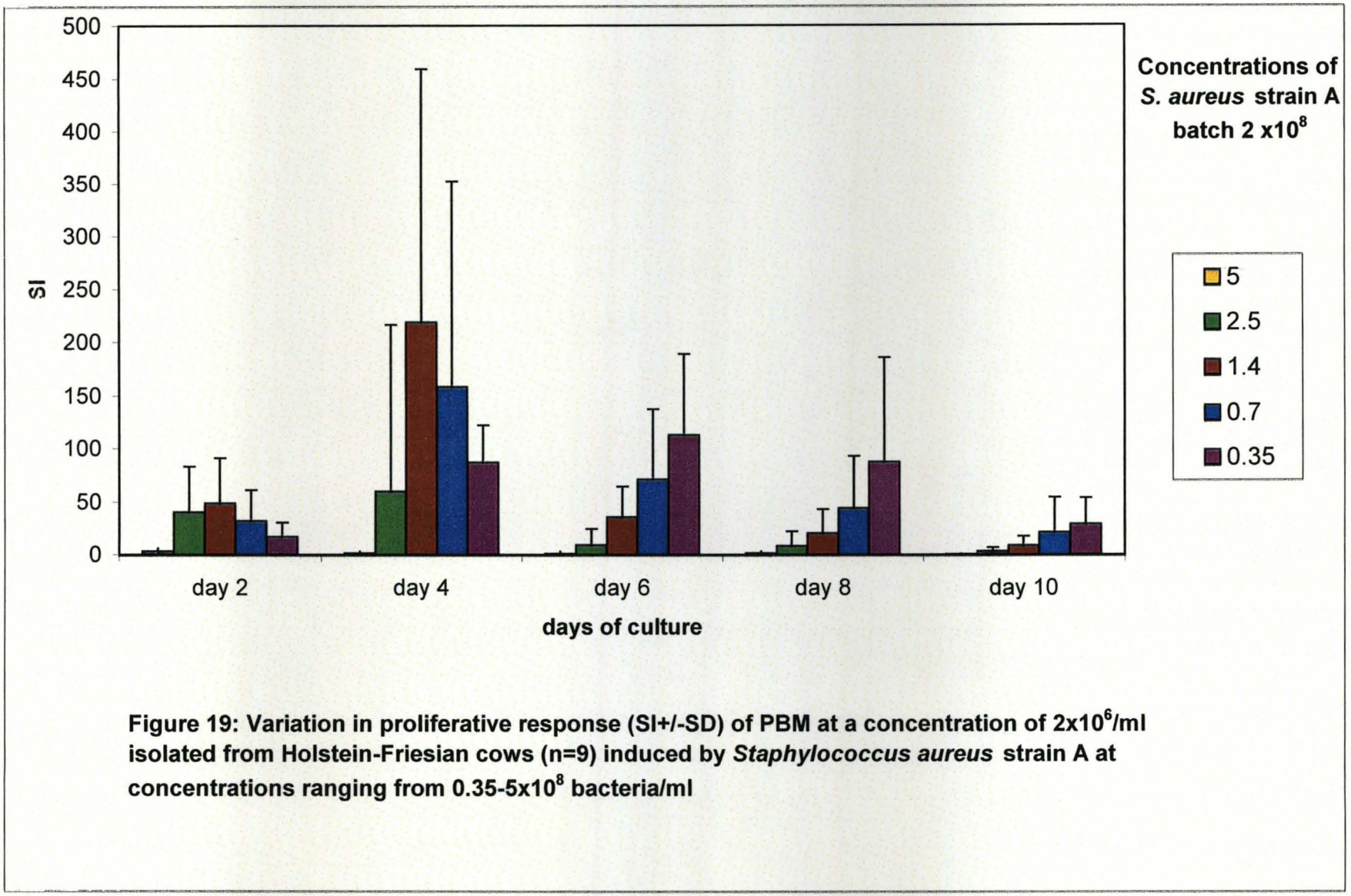


Figure 19: Variation in proliferative response (SI+/-SD) of PBM at a concentration of $2x10^6$/ml isolated from Holstein-Friesian cows (n=9) induced by *Staphylococcus aureus* strain A at concentrations ranging from 0.35-$5x10^8$ bacteria/ml

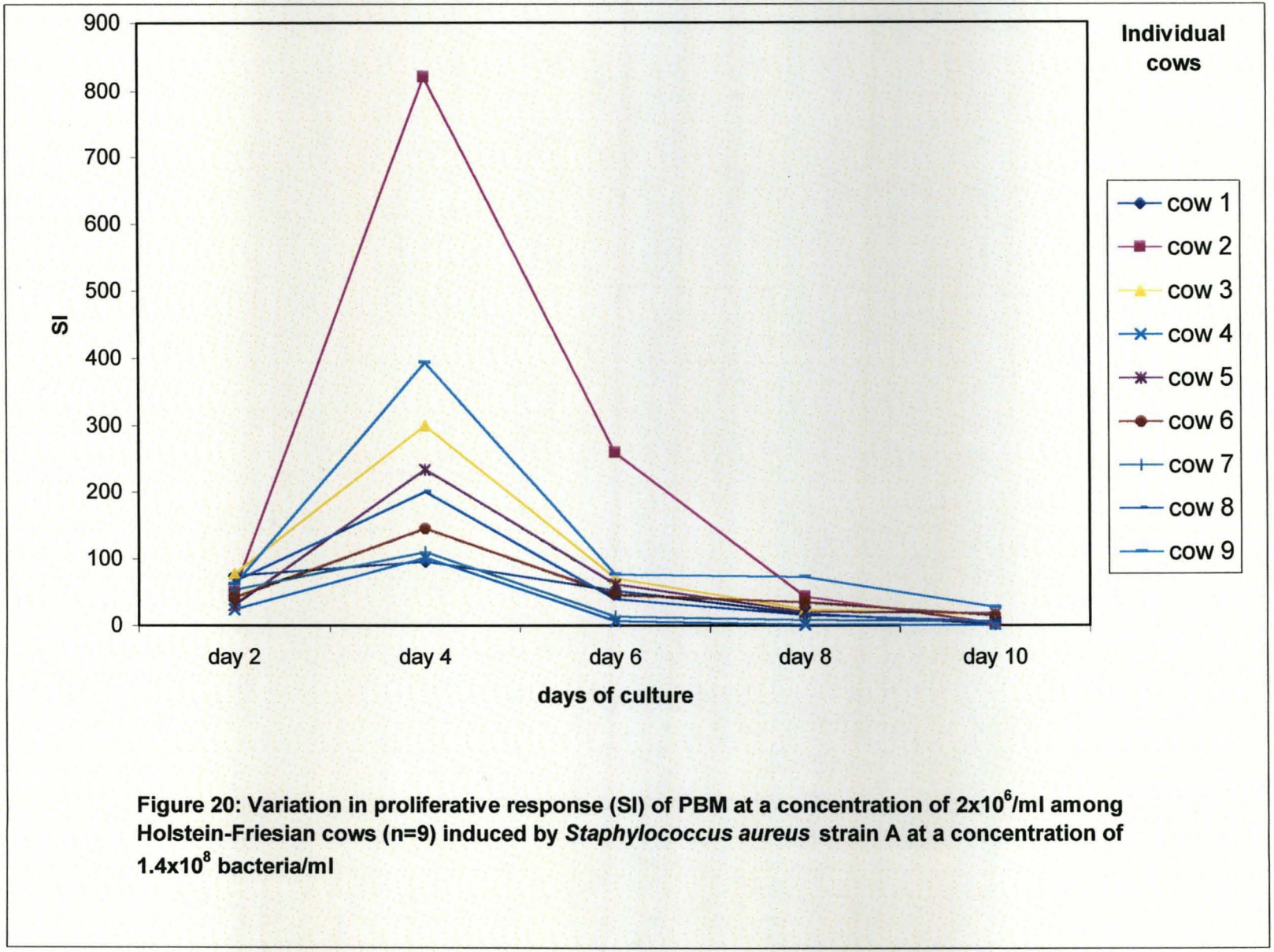


Figure 20: Variation in proliferative response (SI) of PBM at a concentration of $2x10^6$/ml among Holstein-Friesian cows (n=9) induced by *Staphylococcus aureus* strain A at a concentration of $1.4x10^8$ bacteria/ml

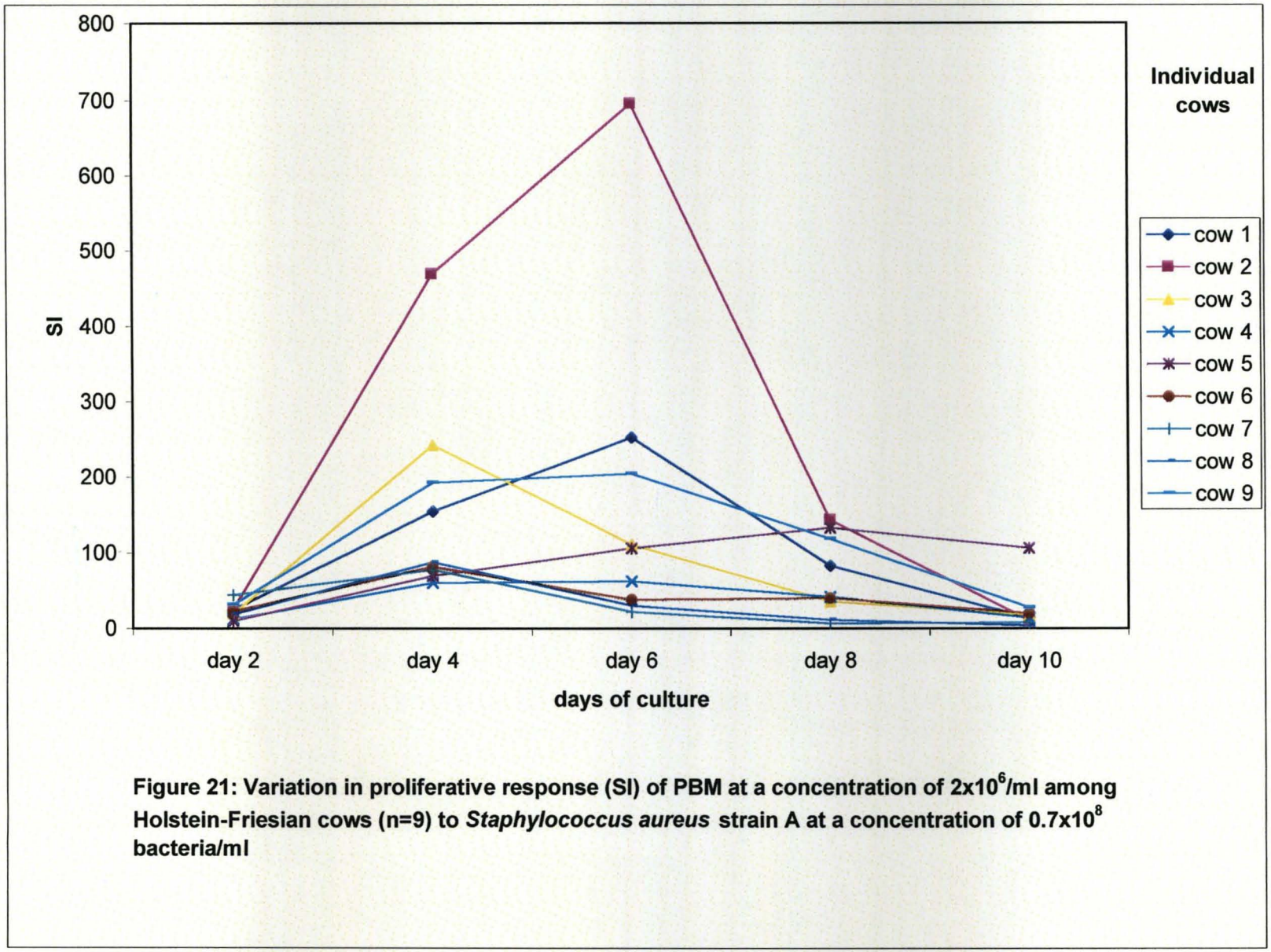


Figure 21: Variation in proliferative response (SI) of PBM at a concentration of $2x10^6$/ml among Holstein-Friesian cows (n=9) to *Staphylococcus aureus* strain A at a concentration of $0.7x10^8$ bacteria/ml

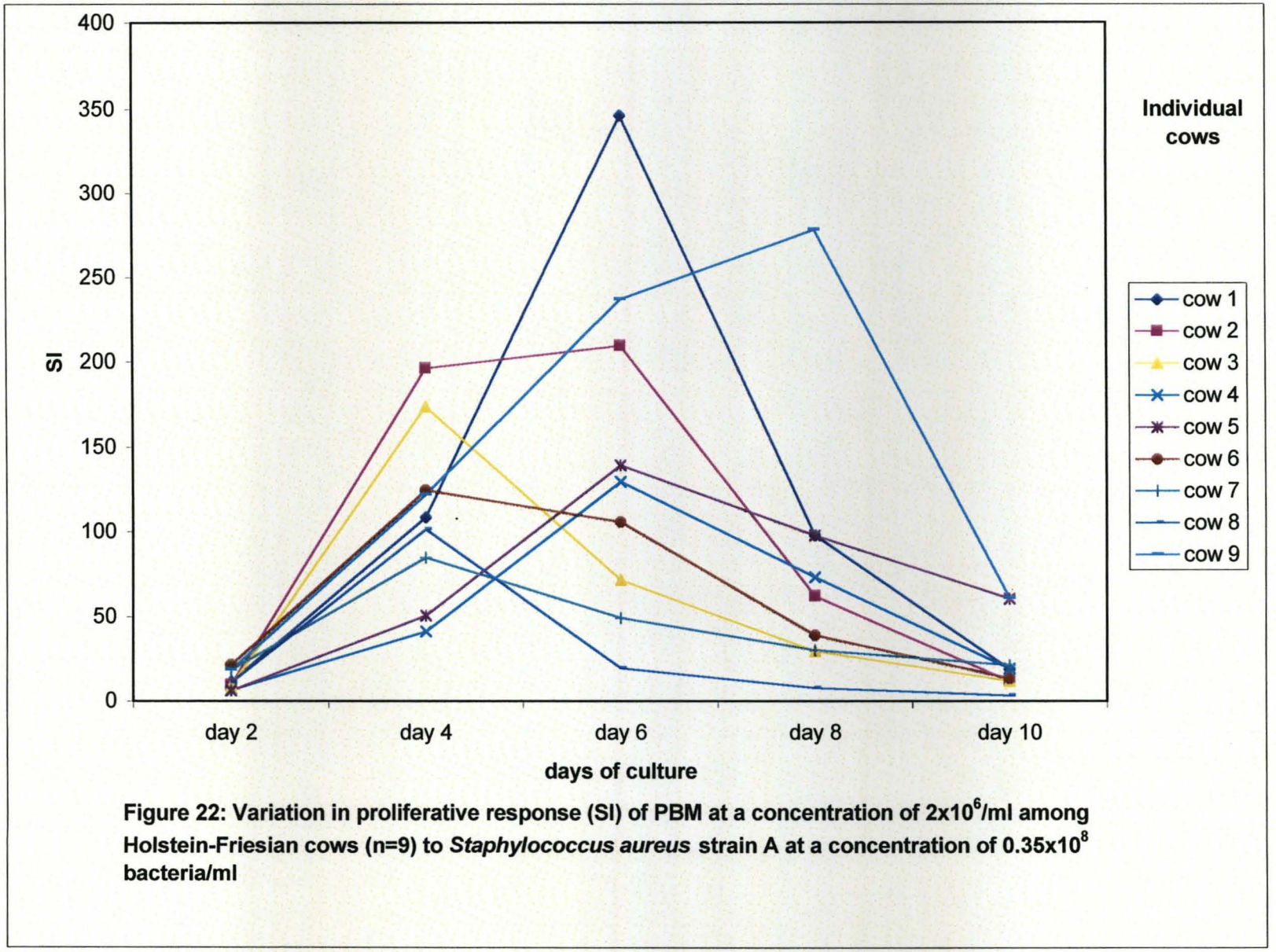


Figure 22: Variation in proliferative response (SI) of PBM at a concentration of $2x10^6$/ml among Holstein-Friesian cows (n=9) to *Staphylococcus aureus* strain A at a concentration of $0.35x10^8$ bacteria/ml

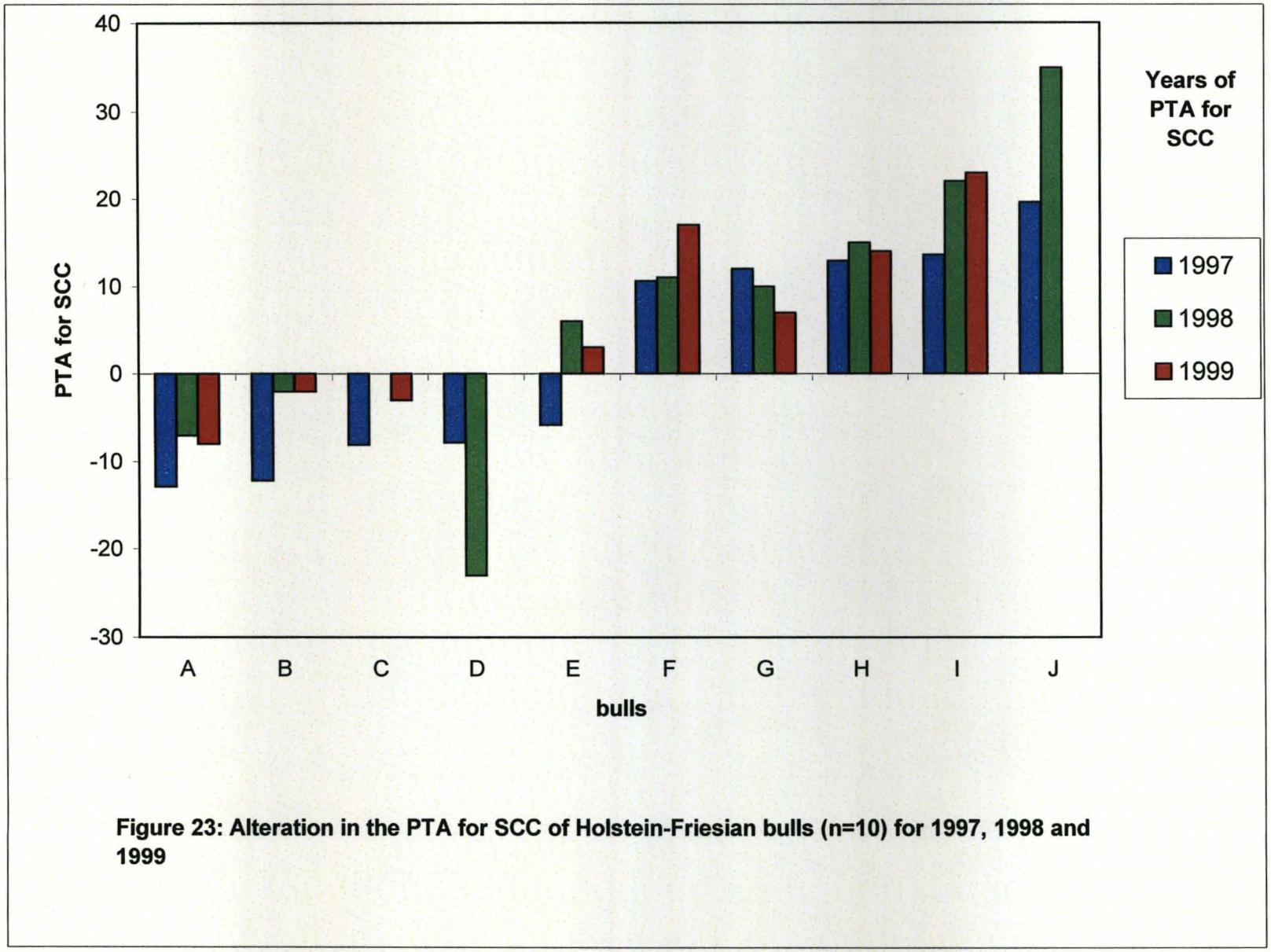


Figure 23: Alteration in the PTA for SCC of Holstein-Friesian bulls (n=10) for 1997, 1998 and 1999

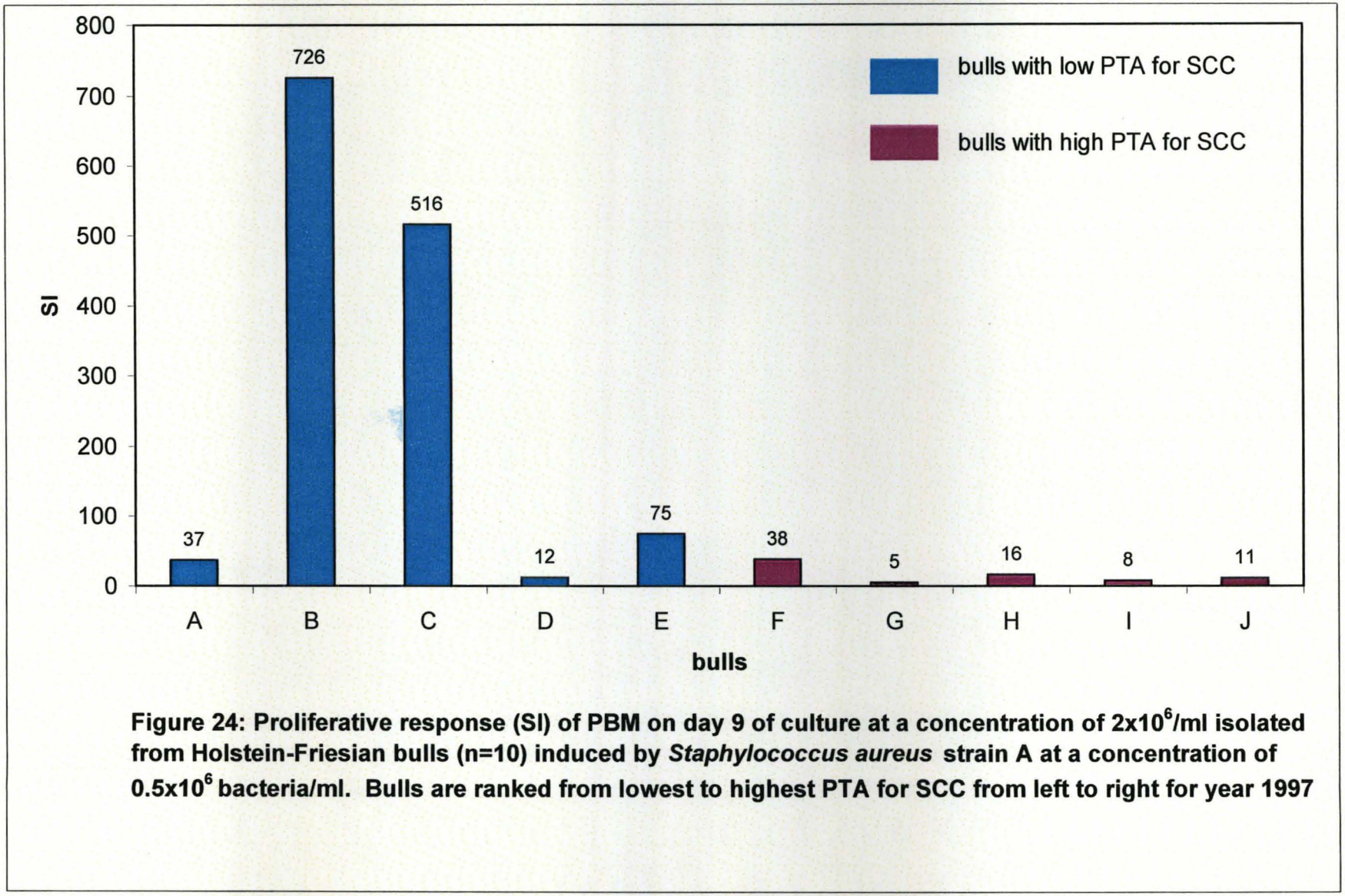


Figure 24: Proliferative response (SI) of PBM on day 9 of culture at a concentration of $2x10^6$/ml isolated from Holstein-Friesian bulls (n=10) induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml. Bulls are ranked from lowest to highest PTA for SCC from left to right for year 1997

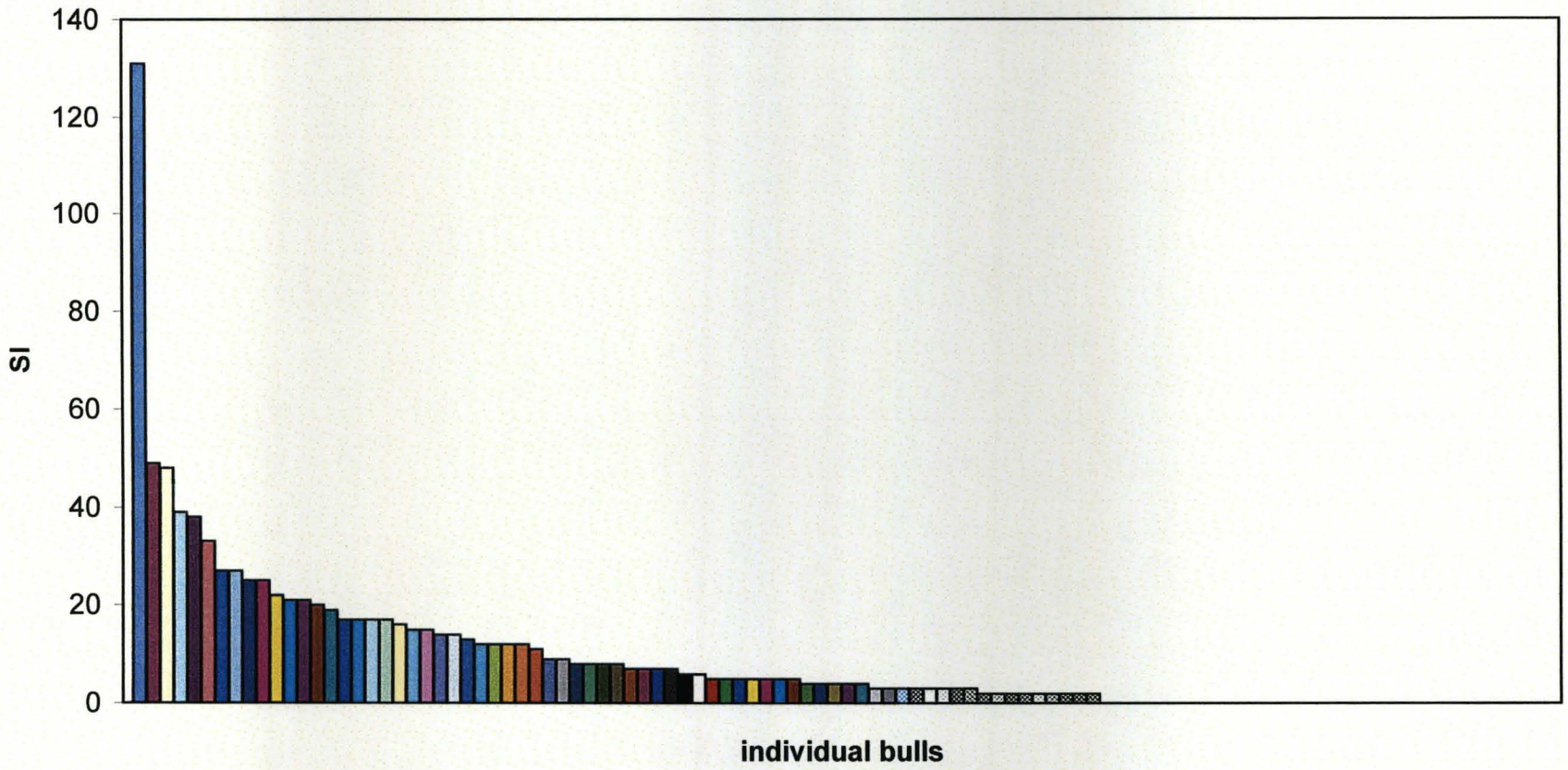


Figure 25: Variation in the proliferative response (SI) of PBM on day 9 of *in vitro* culture at a concentration of $2x10^6$/ml isolated from Holstein-Friesian bulls (n=103) induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml

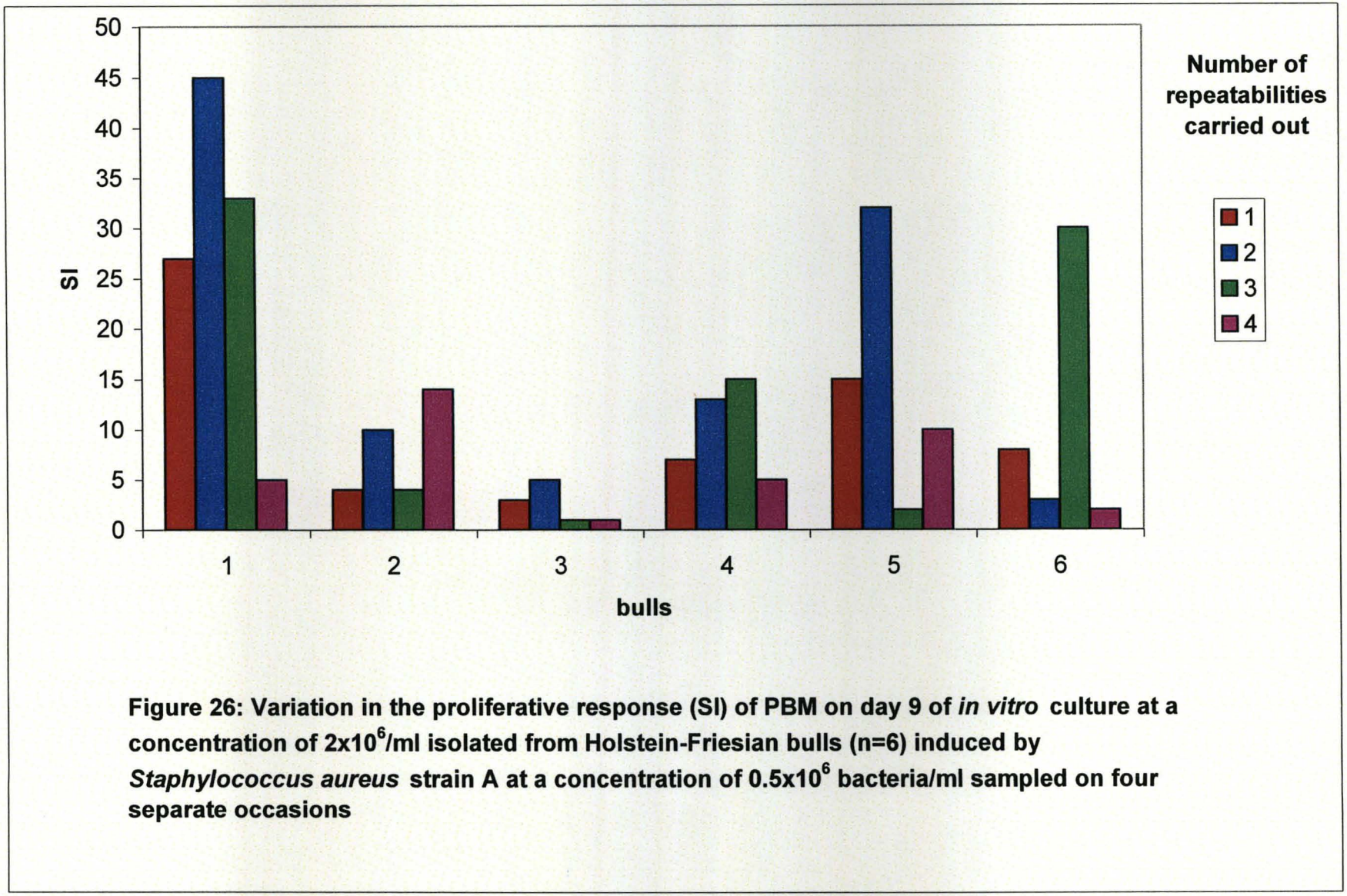


Figure 26: Variation in the proliferative response (SI) of PBM on day 9 of *in vitro* culture at a concentration of $2x10^6$/ml isolated from Holstein-Friesian bulls (n=6) induced by *Staphylococcus aureus* strain A at a concentration of $0.5x10^6$ bacteria/ml sampled on four separate occasions

3.4 Phenotyping of peripheral blood mononuclear cells and total blood leucocytes

The phenotyping studies on PBM isolated from cows showed that the mean percentage (+/-SD) of cells expressing CD4 was 26+/-8%; expressing CD8 was 16+/-10%; expressing γδ was 7+/-4%; expressing the B cell marker was 17+/8%; expressing the monocyte marker was 38+/-17% (n=10), and expressing MHC class II was 34+/-9% (n=8), (Figure 27).

Considerable variation in the percentage of different cell phenotypes was observed among cows. The percentages of stained cells ranged from 16 to 44% for $CD4^+$ cells; 7 to 41% for $CD8^+$ cells; 1 to 17% for $\gamma\delta^+$ cells; 9 to 39% for cells expressing the B cell marker; 12 to 61% for cells expressing the monocyte marker; and 23 to 49% for MHC class II^+ cells (Figure 28).

The phenotyping studies on the PBM isolated from bulls showed that the mean percentage +/-SD of cells expressing CD4 was 17+/-3%, expressing CD8 was 7+/-3%, expressing γδ was 1+/-0.5%, expressing the B cell marker was 10+/-3%, expressing the monocyte marker was 0.5+/-0.5%, and expressing MHC class II was 32+/-12% (n=10), (Figure: 29).

Analysis of these results using Hotelling's T square test showed a statistically significant difference in the proportions of cell subpopulations between cows and bulls ($p<0.05$).

As was noted in cows, considerable variation in the percentage of different cell phenotypes was observed among bulls. The percentage of stained cells isolated from the bulls ranged from; 11 to 23% for $CD4^+$ cells; 2 to 15% for $CD8^+$ cells; 1 to 2% for $\gamma\delta^+$ cells; 6 to 18% for cells expressing the B cell marker; 0.6 to 1% for cells expressing the monocyte marker; and 16 to 55% for MHC class II^+ cells (Figure: 30).

Density centrifugation allows the separation of mononuclear cells from whole blood by retention of cells of a specific density at the interface between the density centrifugation media and the diluted blood sample. Media of various densities are available, and the one most commonly used for isolation of PBM in cattle is ficoll-isopaque, with a density of 1.077g/l. (Shafer-Weaver, 1997; McCole *et al.*, 1998; Asai *et al.*, 1998). To ensure that certain cell subtypes in bulls were not being lost due to the density of the centrifugation media chosen, ficoll-isopaque with a greater density of 1.083g/l, was used to isolate the cells from whole blood collected from bulls. The mean cell yield (+/-SD) with ficoll-isopaque of density 1.077g/l was $8+/-2x10^5$ cells/ml whole blood, and with ficoll-isopaque of density 1.083g/l was $13+/-4x10^5$ cells/ml whole blood (n=5). Using a paired T test the difference in cell yield between the two media was shown to be significantly different (p=0.01) indicating that some cell loss was occurring when ficoll-isopaque of the lower density was used.

The phenotype of cells isolated from the two density media was then compared. The phenotyping studies on the PBM isolated from bulls with ficoll-isopaque of density 1.077g/l showed that the mean percentage +/-SD of cells expressing CD4 was 19+/-6%, expressing CD8 was 6+/-2%, expressing γδ was 1+/-0.4%, expressing the B cell marker was 10+/-3%, expressing the monocyte marker was 0.6+/-0.6%, and expressing MHC class II was 37+/-11% (n=5). The phenotyping studies on the PBM isolated from bulls with ficoll-isopaque of density 1.083g/l showed that the mean percentage +/-SD of cells expressing CD4 was 6+/-3%, expressing CD8 was 2+/-1%, expressing γδ was 0.9+/-0.7%, expressing the B cell marker was 3+/-2%, expressing the monocyte marker was 0.4+/-0.3%, and expressing MHC class II was 13+/-8% (n=5), (Figure: 31). Results indicated that the percentage of stained cells with all monoclonal antibodies employed were lower from the samples separated with ficoll-isopaque of density 1.083g/l, compared to those samples separated with ficoll-isopaque of density 1.077g/l. Using a paired T test a statistically significant reduction in the proportion of PBM expressing CD4 (p=0.01), CD8 (p=0.03), the γδ marker (p=0.02), expressing the B cell marker (p=0.006), and MHC class II (p=0.004) was reported. There was shown to be no statistically significant difference in the expression of the monocyte marker (p=0.47) between PBM isolated by ficoll-isopaque of density 1.077g/l and 1.083g/l.

In further experiments with ficoll-isopaque of density 1.077g/l, PBM were aspirated from the interface of the diluted blood sample. The red blood cell pellet at the bottom of the centrifuge tube was treated with a red cell lysis buffer and the cells remaining were then phenotyped. This approach was to ensure that certain cell subtypes were not being lost due to the media of lower density, which may have resulted in cells being deposited into the pellet below. Results showed very few stained cells were present within the cell pellet, with only 5% of $CD4^+$cells, 1% of $CD8^+$cells, 1% of $\gamma\delta^+$ cells, 2% of cells expressing the B cell marker, 0.8% of cells expressing the monocyte marker, and 7% of MHC class II^+ cells being detected (n=2).

Results of the phenotyping studies carried out in bulls on the cell pellet, after centrifugation with a ficoll-isopaque of density 1.077g/l, showed that the percentage of stained cells was approximately 18% of the total cells. Seventy two percent of the cell population that comprised the cell pellet was unlabelled. Isolation of PBM from bulls by centrifugation with ficoll-isopaque of density 1.083g/l resulted in approximately 80-90% of the cells from the cell pellet being unlabelled. One explanation may be that these two unlabelled cell populations are the same cells. Those present within the cell pellet may have been deposited through the less dense ficoll-isopaque (1.077g/l), while these cells may be retained at the interface of the higher density ficoll-isopaque (1.083g/l).

The methodology of density centrifugation to isolate mononuclear cells from whole blood may result in a proportion of cell lysis, consequently some differential cell loss may occur. To test this, PBM isolated by density centrifugation and PBM isolated from whole blood treated with Erythrolyse (Serotec), a red cell lysis buffer, from cows were compared (n=2). The whole blood treated with the lysis buffer produced a cell pellet that still contained neutrophils, whereas the cell pellet produced by density centrifugation contained only mononuclear cells. Results indicated that the percentage of cells expressing CD4, CD8, cell expressing the B cell marker, cells expressing the monocyte marker, and MHC class II were lower in whole blood compared with cells isolated by centrifugation with media of density 1.077g/l. In samples where cells were isolated by density centrifugation the percentage of cells expressing CD4 was 31%; CD8 was 10%; the B cell marker was 11%; the monocyte marker was 24%; and MHC class II was 36%.

In samples where cells were isolated by use of the red blood cell lysis buffer the mean percentage of cells expressing CD4 was 21%; CD8 was 5%; the B cell marker was 3%; the monocyte marker was 3%; and MHC class II was 26%. The proportion of cells expressing γδ was similar in both samples, the proportion of cells expressing γδ isolated by centrifugation with a density media (1.077g/l) was 2% compared to 3% of the cells isolated by red blood cell lysis (Figure: 32). In normal blood, lymphocytes account for approximately 70% of cells and neutrophils for approximately the remaining 30% (Sordillo *et al.*, 1997). The difference in percentage of stained cells between cells isolated by density centrifugation and those isolated by red blood cell lysis buffer is expected due to the neutrophil population altering the proportion of mononuclear cells identified.

During the current study PBM isolated from cows and induced by *S. aureus* strain A *in vitro* were removed on day nine of culture, the day of peak proliferation, to identify those cells that were proliferating in response to *S. aureus* strain A. The phenotyping studies on the responding PBM isolated from cows on day nine of the *in vitro* proliferation assay showed that 53+/-8% of cells expressed CD4, 21+/-5% of cells expressed CD8, 13+/-4% of cells expressed the γδ cell marker, 11+/-4% of cells expressed the B cell marker, and that 1+/-1% of cells expressed the monocyte marker (Figure: 33).

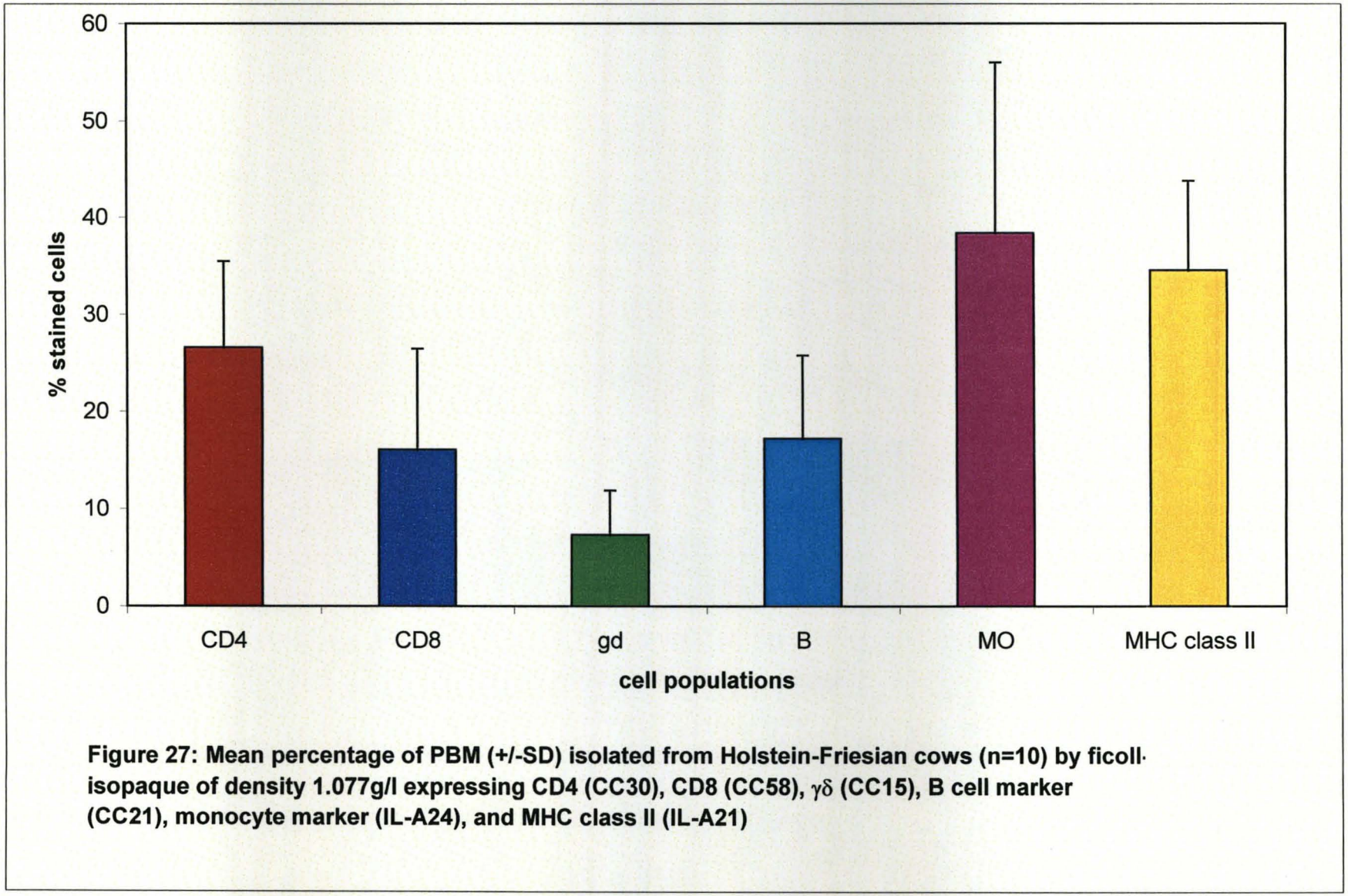


Figure 27: Mean percentage of PBM (+/-SD) isolated from Holstein-Friesian cows (n=10) by ficoll· isopaque of density 1.077g/l expressing CD4 (CC30), CD8 (CC58), γδ (CC15), B cell marker (CC21), monocyte marker (IL-A24), and MHC class II (IL-A21)

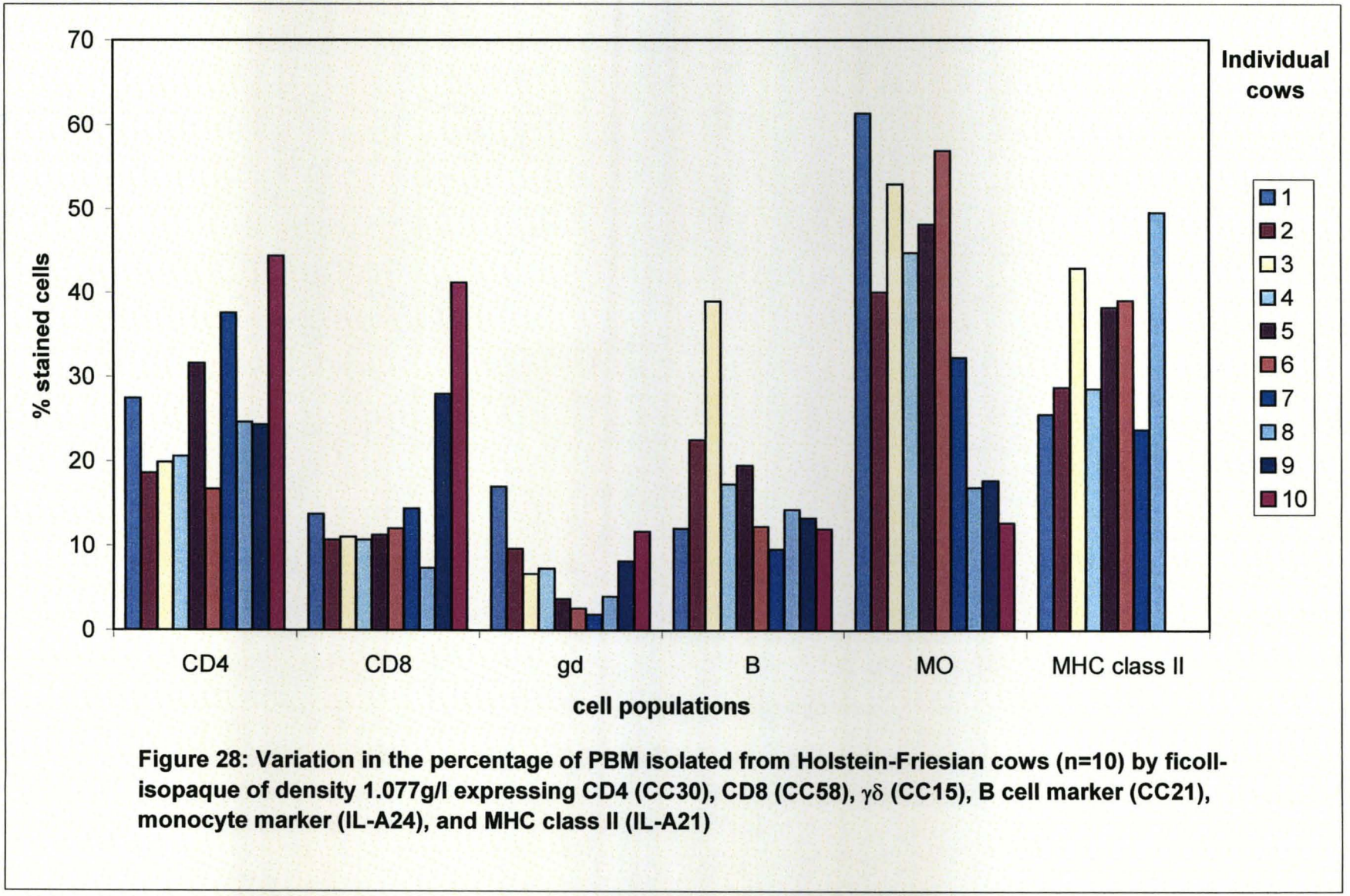


Figure 28: Variation in the percentage of PBM isolated from Holstein-Friesian cows (n=10) by ficoll-isopaque of density 1.077g/l expressing CD4 (CC30), CD8 (CC58), γδ (CC15), B cell marker (CC21), monocyte marker (IL-A24), and MHC class II (IL-A21)

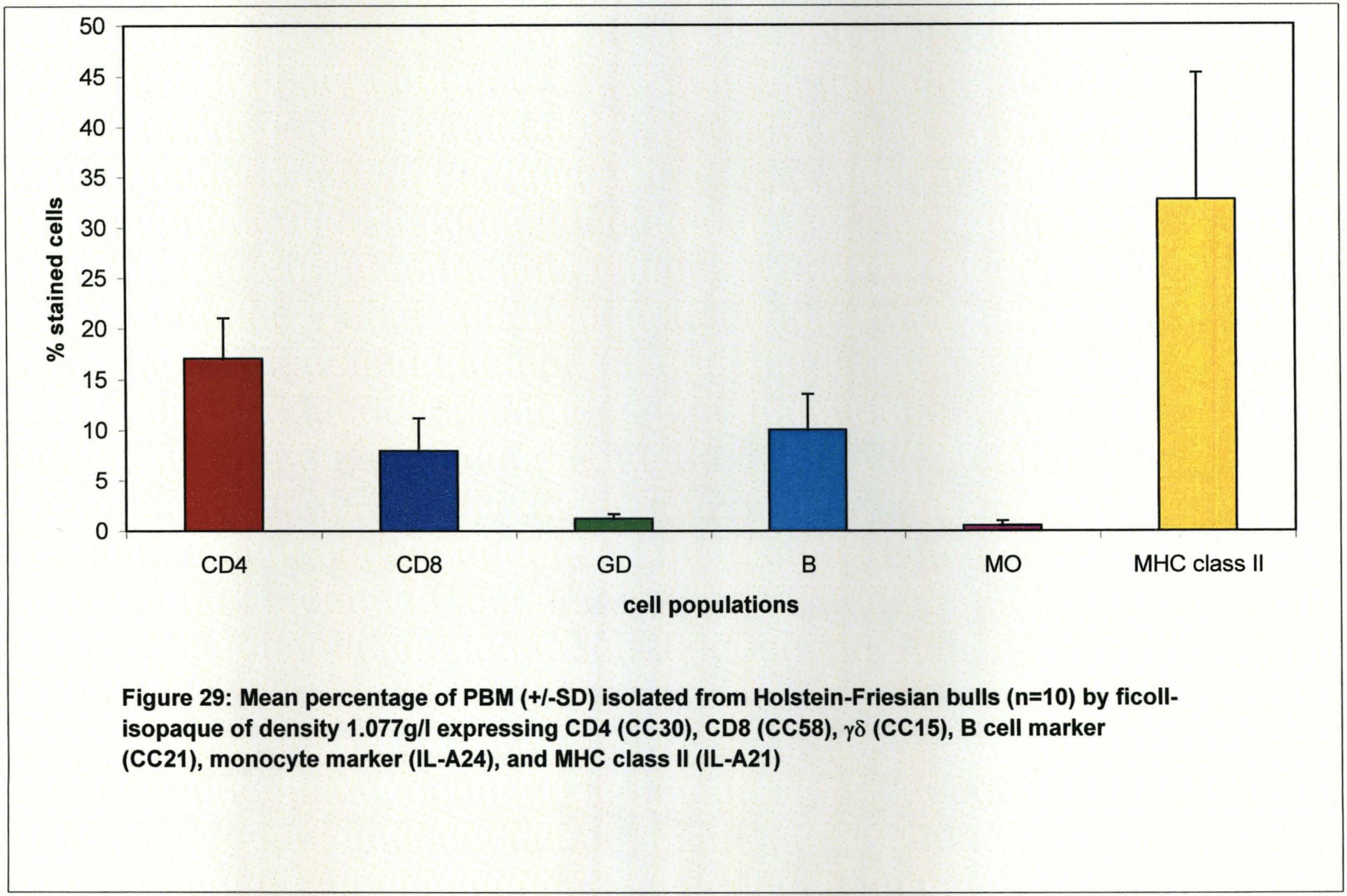


Figure 29: Mean percentage of PBM (+/-SD) isolated from Holstein-Friesian bulls (n=10) by ficoll-isopaque of density 1.077g/l expressing CD4 (CC30), CD8 (CC58), γδ (CC15), B cell marker (CC21), monocyte marker (IL-A24), and MHC class II (IL-A21)

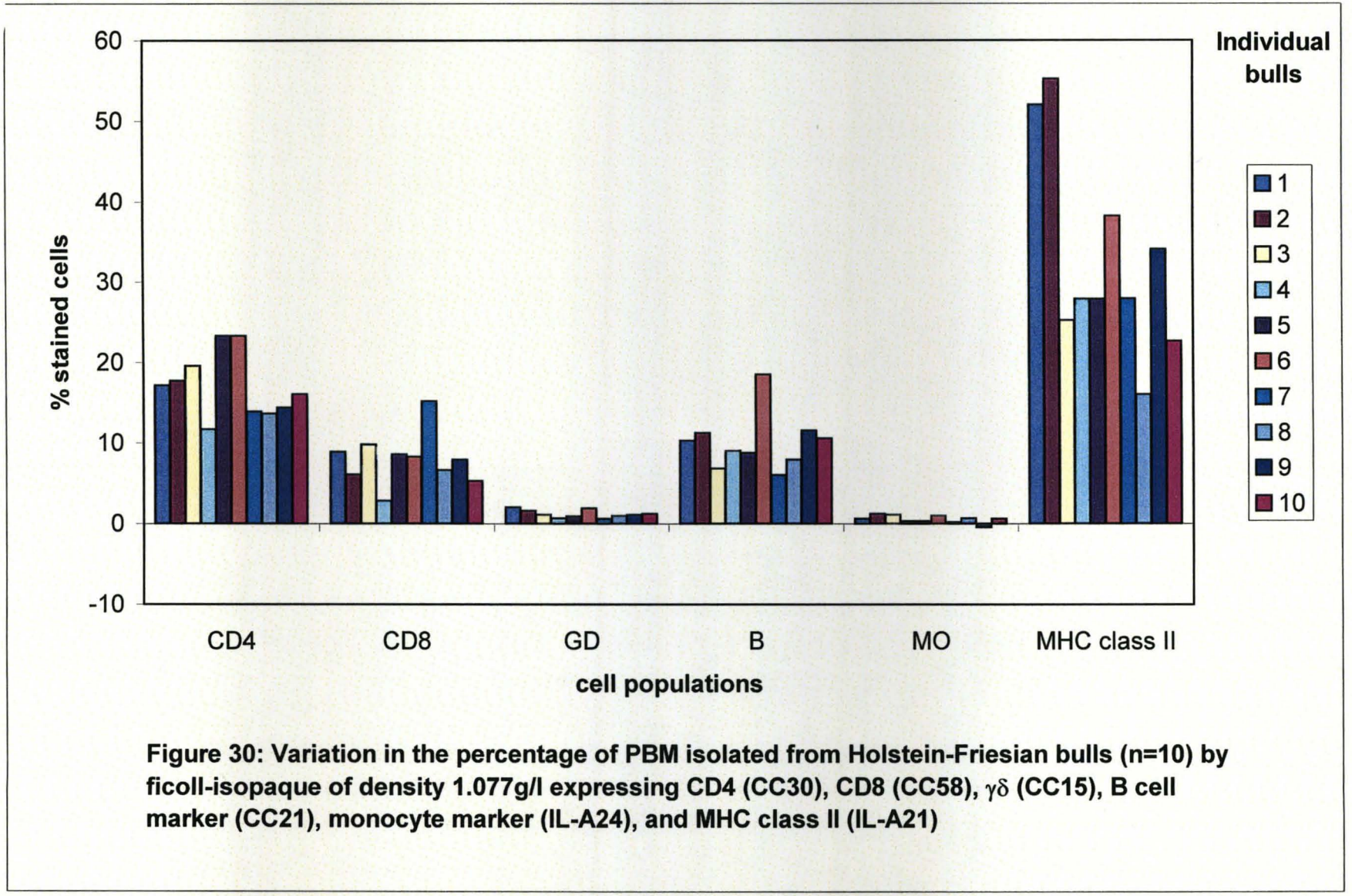


Figure 30: Variation in the percentage of PBM isolated from Holstein-Friesian bulls (n=10) by ficoll-isopaque of density 1.077g/l expressing CD4 (CC30), CD8 (CC58), γδ (CC15), B cell marker (CC21), monocyte marker (IL-A24), and MHC class II (IL-A21)

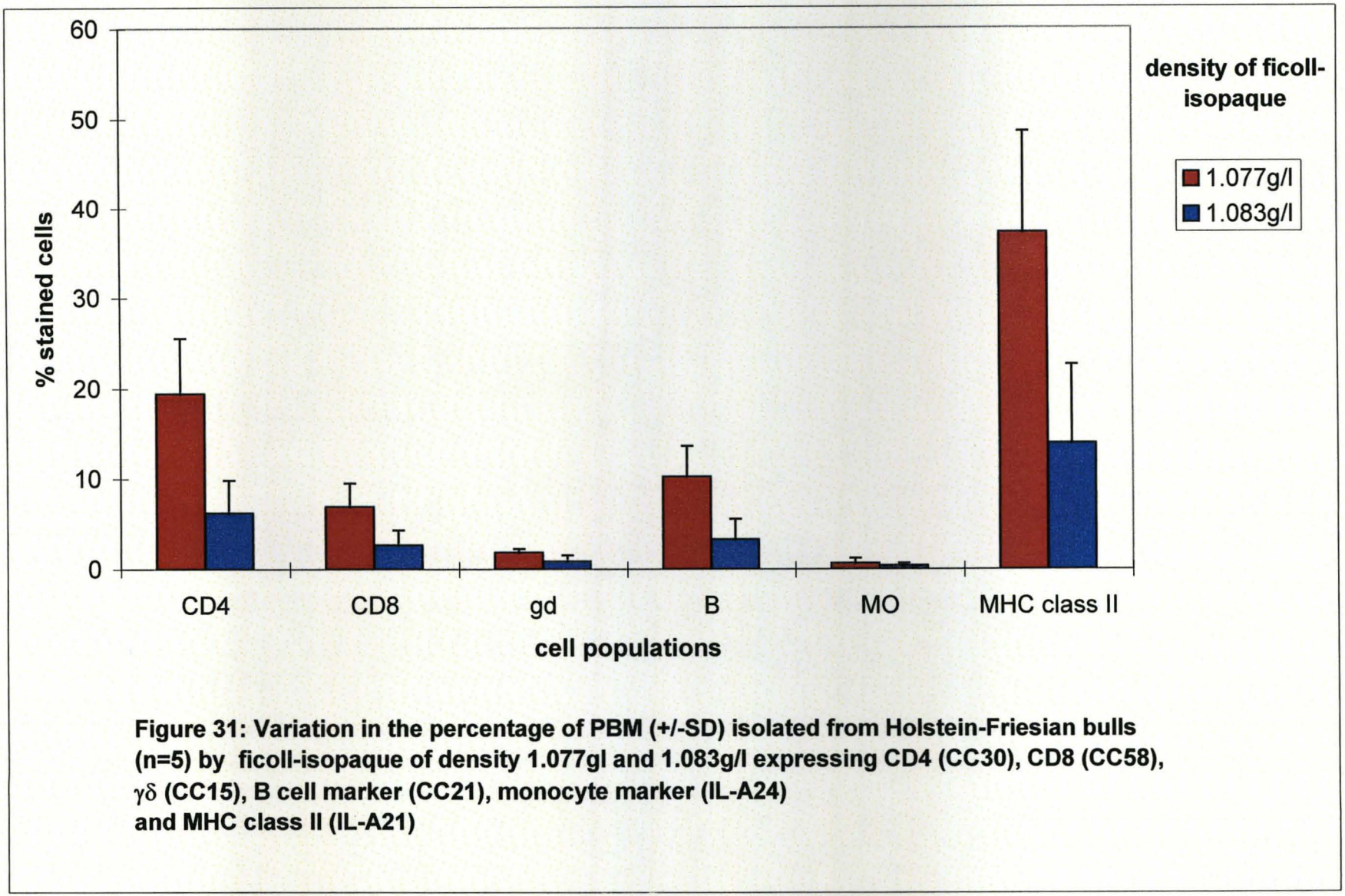


Figure 31: Variation in the percentage of PBM (+/-SD) isolated from Holstein-Friesian bulls (n=5) by ficoll-isopaque of density 1.077gl and 1.083g/l expressing CD4 (CC30), CD8 (CC58), γδ (CC15), B cell marker (CC21), monocyte marker (IL-A24) and MHC class II (IL-A21)

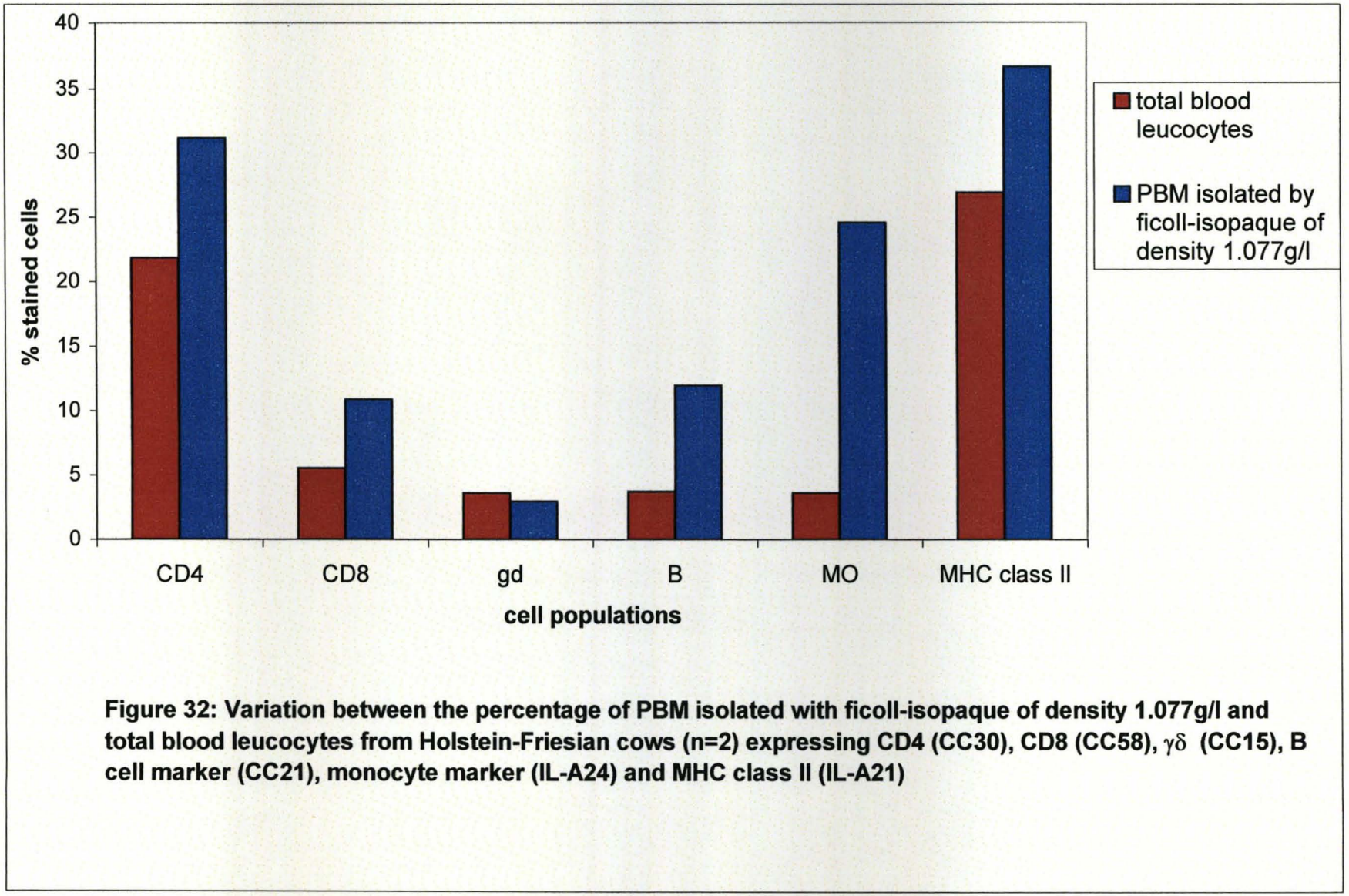


Figure 32: Variation between the percentage of PBM isolated with ficoll-isopaque of density 1.077g/l and total blood leucocytes from Holstein-Friesian cows (n=2) expressing CD4 (CC30), CD8 (CC58), γδ (CC15), B cell marker (CC21), monocyte marker (IL-A24) and MHC class II (IL-A21)

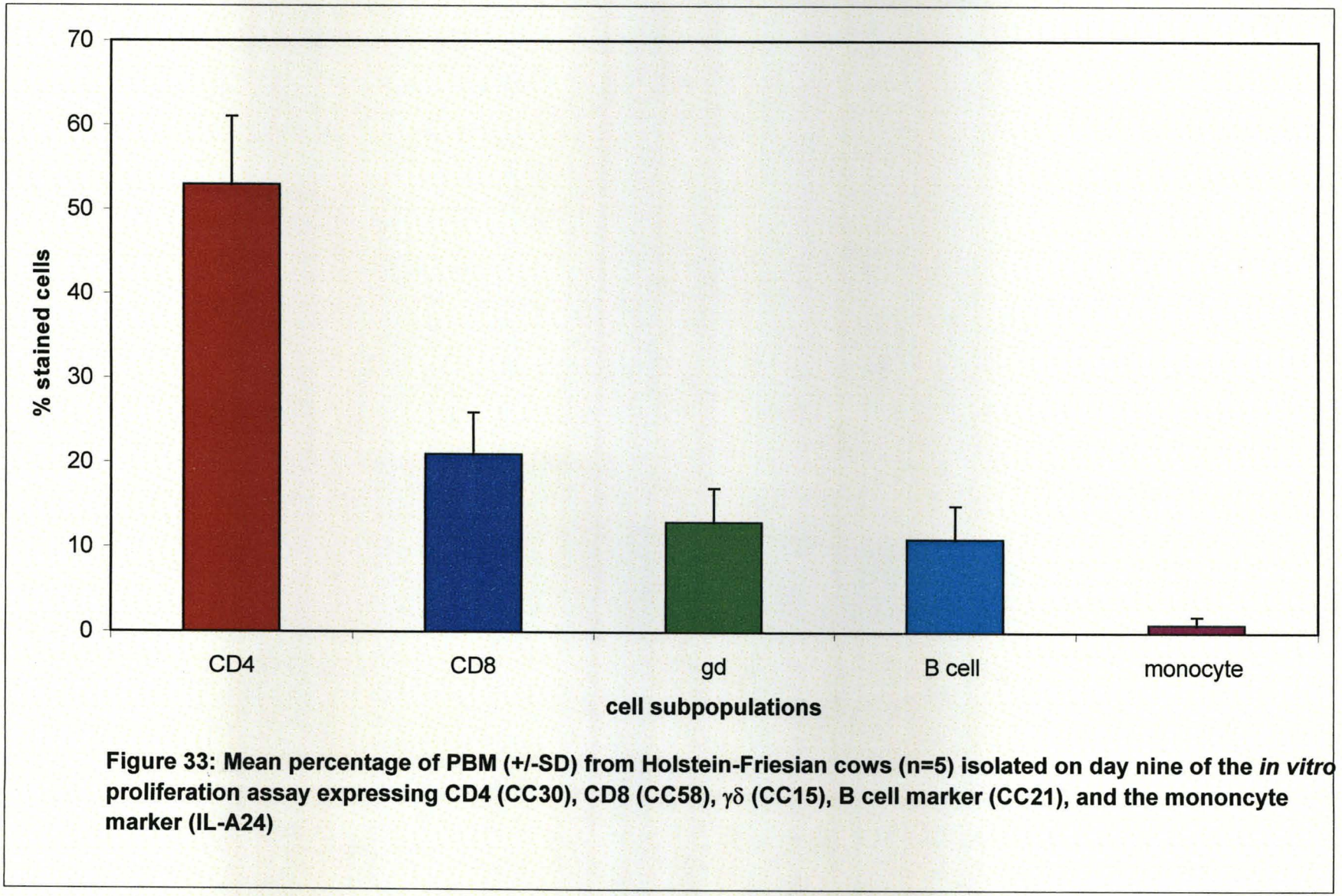


Figure 33: Mean percentage of PBM (+/-SD) from Holstein-Friesian cows (n=5) isolated on day nine of the *in vitro* proliferation assay expressing CD4 (CC30), CD8 (CC58), γδ (CC15), B cell marker (CC21), and the mononcyte marker (IL-A24)

3.5 Phenotyping of milk mononuclear cells and total milk leucocytes

The expression of MHC class II molecules on mononuclear cells isolated from milk by centrifugation with media of density 1.077g/l was investigated in Holstein-Friesian cows with either high SCC (>400,000 cells/ml milk for three consecutive months prior to sampling) or low SCC (<150,000 cells/ml milk for the month prior to sampling).

The mean percentage (+/-SD) of mononuclear cells expressing MHC class II isolated by ficoll-isopaque of density 1.077g/l from cows with high SCC was 39+/-21% (n=12). The mean percentage (+/-SD) of mononuclear cells expressing MHC class II isolated from cows with low SCC was 32+/-10% (n=9).

Considerable variation in the percentage of mononuclear cells expressing MHC class II was observed among cows with either high or low SCC. The percentage of mononuclear cells expressing MHC class II from cows with high SCC ranged from 12% to 86%. The percentage of mononuclear cells expressing MHC class II from cows with low SCC ranged from 18% to 49% (Figure: 34). Using an unpaired T test, although considerable variation in the percentage of mononuclear cells expressing MHC class II was observed among cows with either high or low SCC no significant difference (p=0.32) in expression was reported.

Isolation of milk cells by centrifugation using media of density 1.077g/l results in isolation of a mononuclear cell population, with polymorphonuclear cells being excluded by this method of isolation. Whole milk was centrifuged in the absence of density media to allow isolation of a total milk leucocyte population. The mean percentage (+/-SD) of total milk leucocytes expressing MHC class II from cows with high SCC was 13+/-10% (n=6). The mean percentage (+/-SD) of total milk leucocytes expressing MHC class II from cows with low SCC was 10+/-15% (n=6).

Considerable variation in the percentage of total milk leucocytes expressing MHC class II was observed among cows with either high or low SCC. The percentage of total milk leucocytes expressing MHC class II in cows with high SCC ranged from 2% to 29%. The percentage of total milk leucocytes expressing MHC class II in cows with low SCC

ranged from 1% to 39% (Figure: 35). Using an unpaired T test a significant difference in the expression of MHC class II in cows with a high SCC was noted between milk mononuclear cells isolated by ficoll-isopaque of density 1.077g/l and total milk leucocytes (p=0.004), (Figure: 36). Again using an unpaired T test a significant difference in the expression of MHC class II in cows with a low SCC was noted between milk mononuclear cells isolated by ficoll-isopaque of density 1.077g/l and total milk leucocytes (p=0.01), (Figure: 36).

The total milk leucocytes isolated from cows with either high SCC or low SCC were stained to examine the expression of CD4 and CD8 molecules, in addition to MHC class II molecules. The mean percentage (+/-SD) of total milk leucocytes expressing CD4 and CD8 isolated from cows with high SCC was 21+/-12% and 17+/-17% respectively (n=9). The mean percentage (+/-SD) of total milk leucocytes expressing CD4 and CD8 isolated from cows with low SCC was 16+/-13% and 8+/-9% respectively (n=6).

As in previous results, considerable variation in the percentage of total milk leucocytes expressing CD4 and CD8 was observed among cows with either high or low SCC. The percentage of leucocytes from milk expressing CD4 in cows with high SCC ranged from 6% to 47% and CD8 from 1% to 47%. The percentage of leucocytes from milk expressing CD4 in cows with low SCC ranged from 7% to 43% and CD8 from 0% to 26% (Figure: 37). From these results using an unpaired T test it was shown that there was no significant difference in the expression of CD4 (p=0.56) and CD8 (p=0.23) on total milk leucocytes from cows with high and low SCC.

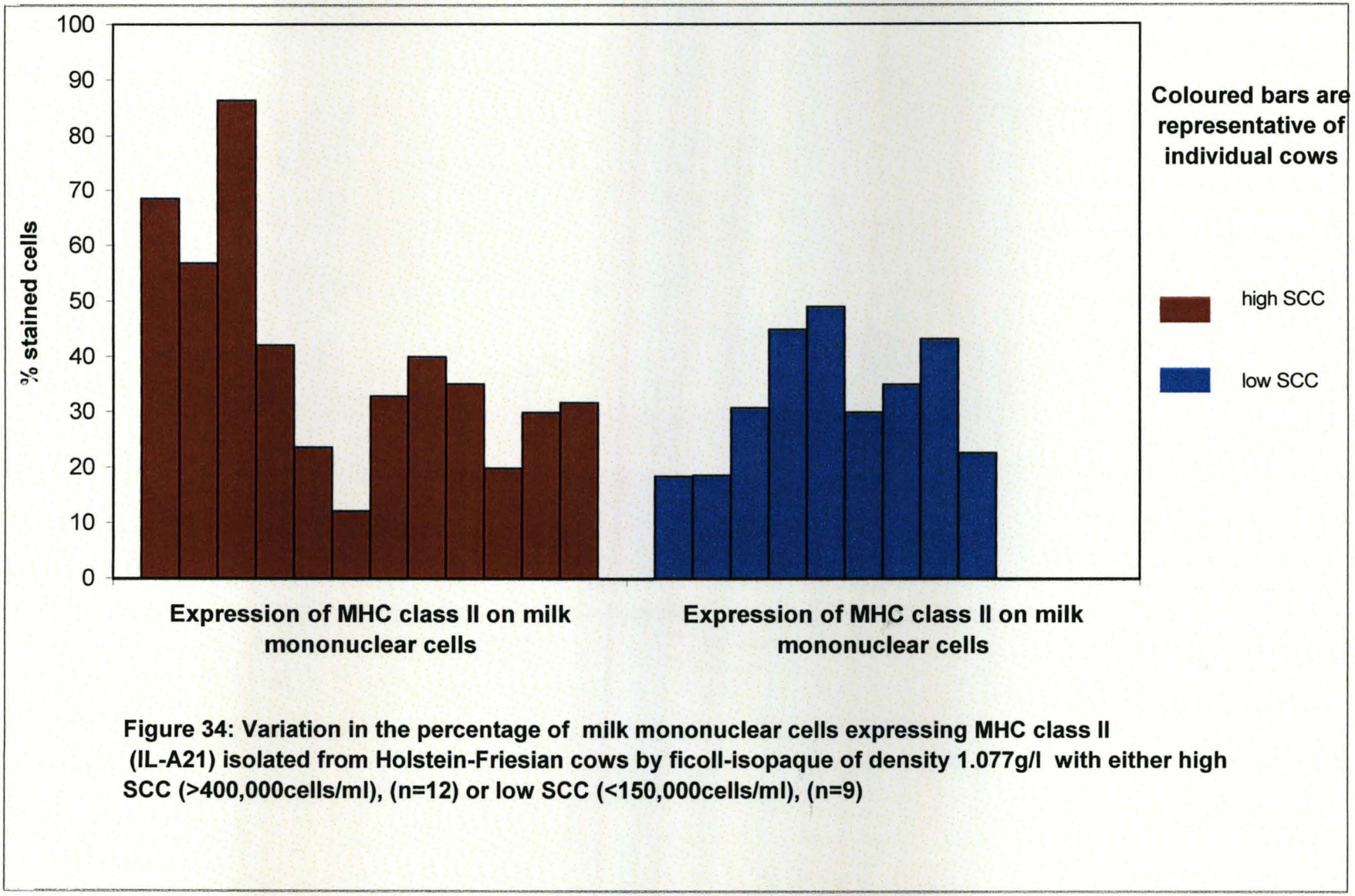


Figure 34: Variation in the percentage of milk mononuclear cells expressing MHC class II (IL-A21) isolated from Holstein-Friesian cows by ficoll-isopaque of density 1.077g/l with either high SCC (>400,000cells/ml), (n=12) or low SCC (<150,000cells/ml), (n=9)

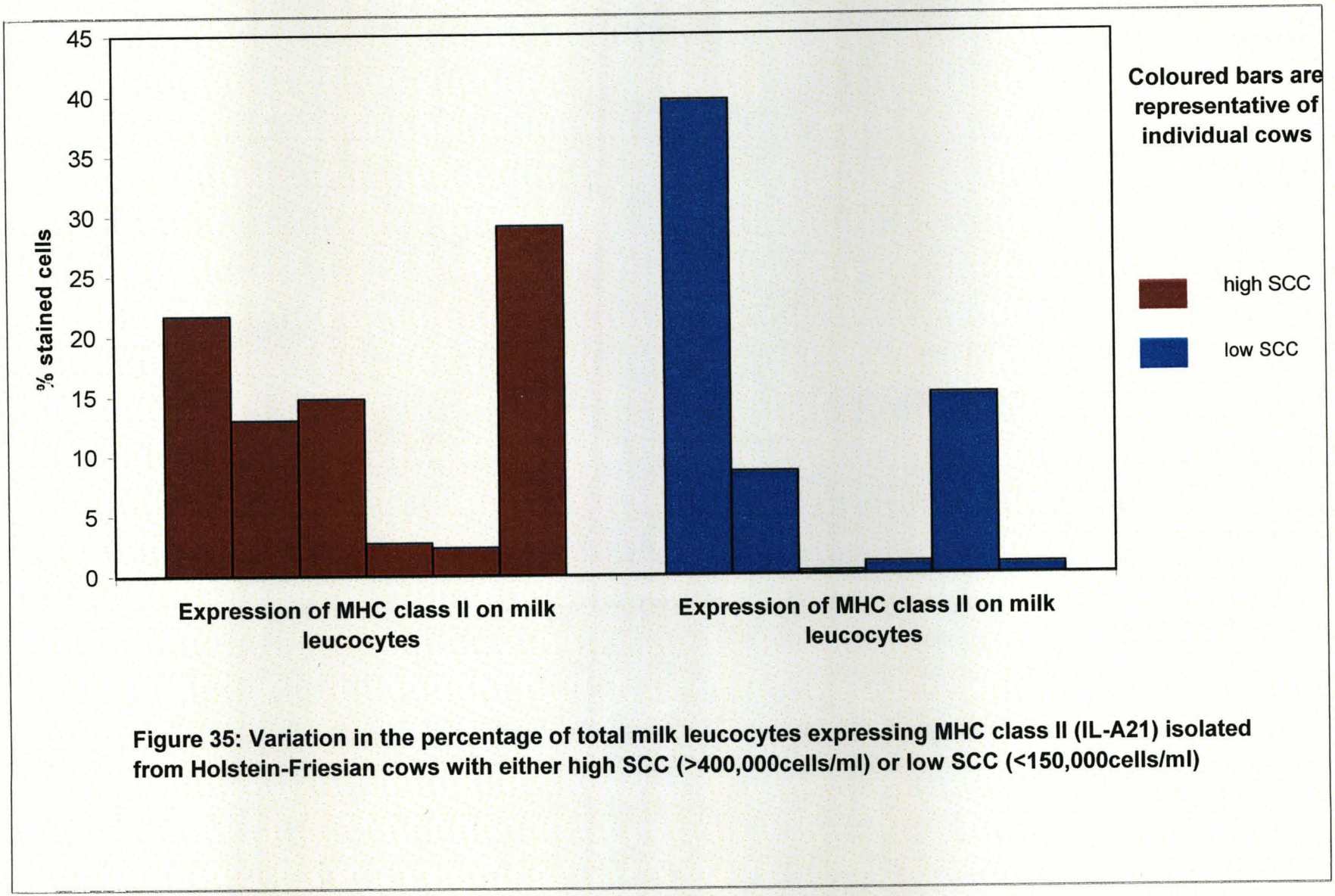


Figure 35: Variation in the percentage of total milk leucocytes expressing MHC class II (IL-A21) isolated from Holstein-Friesian cows with either high SCC (>400,000cells/ml) or low SCC (<150,000cells/ml)

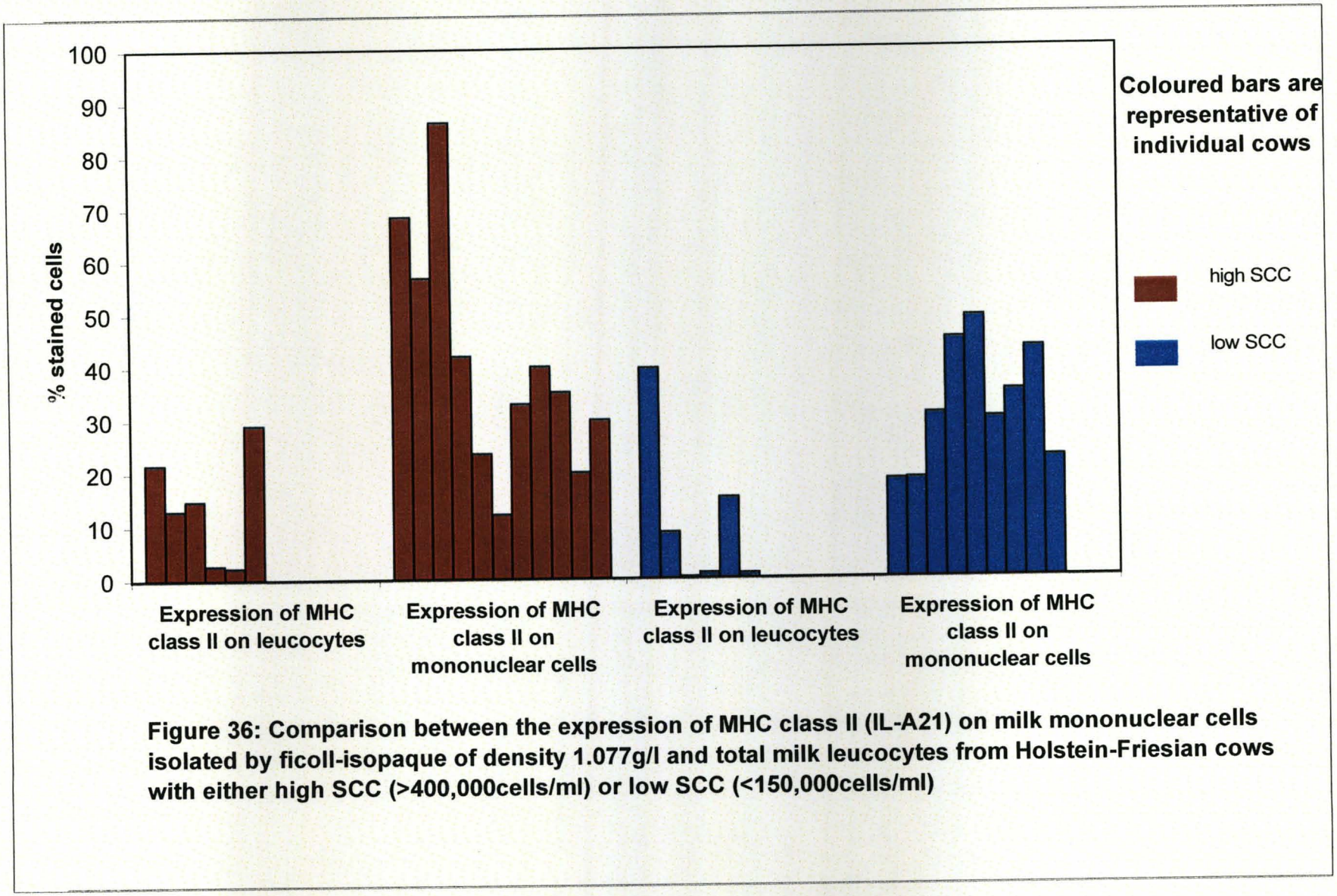


Figure 36: Comparison between the expression of MHC class II (IL-A21) on milk mononuclear cells isolated by ficoll-isopaque of density 1.077g/l and total milk leucocytes from Holstein-Friesian cows with either high SCC (>400,000cells/ml) or low SCC (<150,000cells/ml)

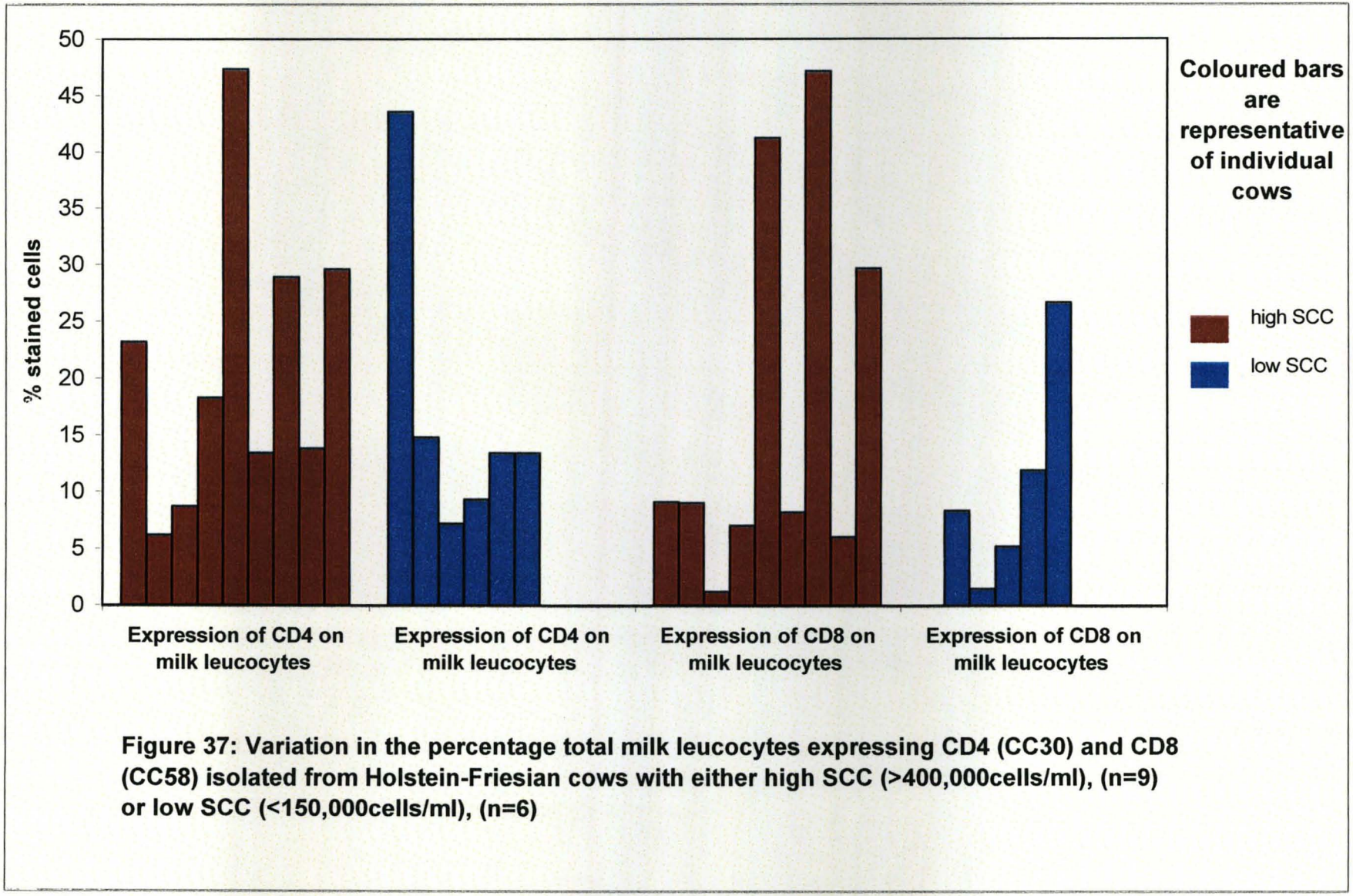
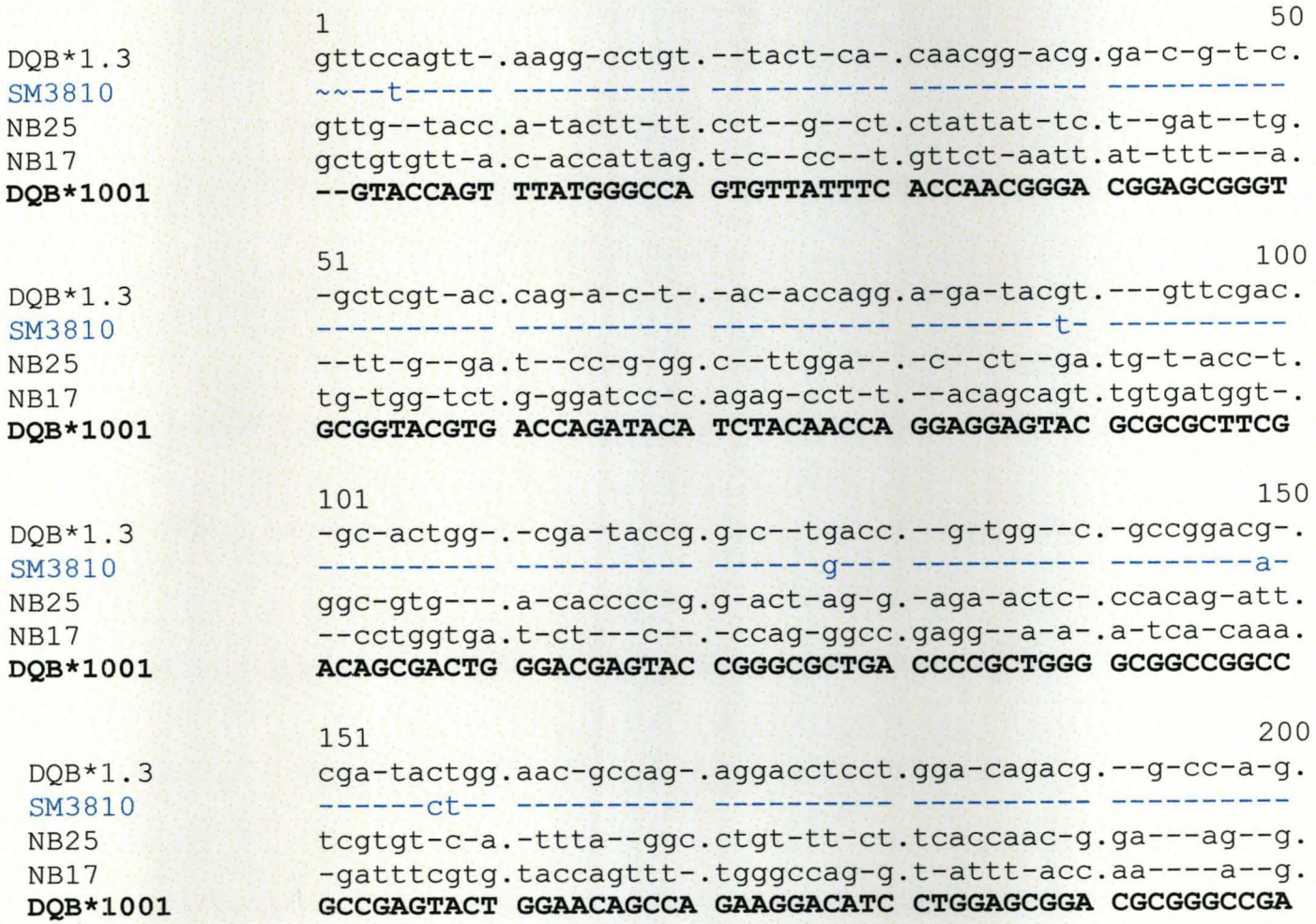


Figure 37: Variation in the percentage total milk leucocytes expressing CD4 (CC30) and CD8 (CC58) isolated from Holstein-Friesian cows with either high SCC (>400,000cells/ml), (n=9) or low SCC (<150,000cells/ml), (n=6)

3.6 Blunt End Cloning of MHC class II-DQB exon 2 of Holstein-Friesian Bulls

Polymerase Chain Reaction (PCR) amplification and sequence analysis of cloned products of the MHC DQB exon 2 loci were performed in four bulls; three of the bulls (bulls A, C, and E) were from the group with the lowest PTA for SCC, and one bull (bull F), was from the group with the highest PTA for SCC. PCR amplification using oligonucleotide primers LA40/41 (Sigurdardottir *et al.*, 1992), successfully generated an amplimer of the expected molecular size (284bp) from all four subjects. Following blunt end cloning of the PCR products, commercial sequencing was carried out; a total of 39 clones were sequenced bi-directionally; ten clones each from bulls C and E, eight clones from bull A, and 11 clones from bull F. Not all of the clones sent for DNA sequencing were generated by a single PCR amplification.

Sequence analysis of the 39 clones, identified the presence of four different sequences. Only one of the sequences identified appeared to be novel compared to all other sequences identified previously within the DQB exon 2 locus in cattle (Figure: 38). The alleles identified were compared to DQB*1001 previously identified in Holstein-Friesian cattle (Nasir *et al.*, 1996). The most commonly identified sequence was DQB*1-3 (25 of 39 clones) and all bulls examined had this sequence. All of the eight clones of Bull A sent for sequencing were identified as DQB*1-3. The clones generated by bull C resulted in identification of two sequences, DQB*1-3 and NB25, reported previously in Japanese Black cattle (Dikiniene and Aida, 1995). Bull E also generated two sequences from the clones produced, namely DQB*1-3 and a new sequence SM3810. This latter sequence was found to have 98% homology to allele DQB*1-3. Bull F, the only bull with a high PTA for SCC, generated three sequences from the clones produced, namely DQB*1-3, DQB*17 and NB25. Usually only two sequences should be identified, one from each parent, however locus duplication is a phenomenon that has been previously recorded in BoLA DQB (Sigurdardottir *et al.*, 1992; Xu *et al.*, 1994; Marello *et al.*, 1995). The frequent cloning and sequencing of the allele DQB*1.3 stimulated the development of the current study to utilise the methodology of PCR-RFLP for DQB analysis.


1 50
DQB*1.3 gttccagtt-.aagg-cctgt.--tact-ca-.caacgg-acg.ga-c-g-t-c.
SM3810 ~~--t----- ---------- ---------- ---------- ----------
NB25 gttg--tacc.a-tactt-tt.cct--g--ct.ctattat-tc.t--gat--tg.
NB17 gctgtgtt-a.c-accattag.t-c--cc--t.gttct-aatt.at-ttt---a.
DQB*1001 --GTACCAGT TTATGGGCCA GTGTTATTTC ACCAACGGGA CGGAGCGGGT
51 100
DQB*1.3 -gctcgt-ac.cag-a-c-t-.-ac-accagg.a-ga-tacgt.---gttcgac.
SM3810 ---------- ---------- ---------- --------t- ----------
NB25 --tt-g--ga.t--cc-g-gg.c--ttgga--.-c--ct--ga.tg-t-acc-t.
NB17 tg-tgg-tct.g-ggatcc-c.agag-cct-t.--acagcagt.tgtgatggt-.
DQB*1001 GCGGTACGTG ACCAGATACA TCTACAACCA GGAGGAGTAC GCGCGCTTCG
101 150
DQB*1.3 -gc-actgg-.-cga-taccg.g-c--tgacc.--g-tgg--c.-gccggacg-.
SM3810 ---------- ---------- ------g--- ---------- --------a-
NB25 ggc-gtg---.a-cacccc-g.g-act-ag-g.-aga-actc-.ccacag-att.
NB17 --cctggtga.t-ct---c--.-ccag-ggcc.gagg--a-a-.a-tca-caaa.
DQB*1001 ACAGCGACTG GGACGAGTAC CGGGCGCTGA CCCCGCTGGG GCGGCCGGCC
151 200
DQB*1.3 cga-tactgg.aac-gccag-.aggacctcct.gga-cagacg.--g-cc-a-g.
SM3810 ------ct-- ---------- ---------- ---------- ----------
NB25 tcgtgt-c-a.-ttta--ggc.ctgt-tt-ct.tcaccaac-g.ga---ag--g.
NB17 -gatttcgtg.taccagttt-.tgggccag-g.t-attt-acc.aa----a--g.
DQB*1001 GCCGAGTACT GGAACAGCCA GAAGGACATC CTGGAGCGGA CGCGGGCCGA

```
                201                                                  247
DQB*1.3          c-gac-cggt.-tgca-acac.---t--c-gg.c-ag-ctcat.-a-a.
SM3810           ------.--- ---------- ---------- -------t-- -------
NB25             -tgc-gagt-.t-accag-t-.c-tctac-a-.c--ga-g--t.a-gtgcg
NB17             a-c--gtgc-.-tacgtg-cc.-gat--at-t.-caacc-g-a.ggag-a-
DQB*1001         GGTGGA-CAG GGTGTGCAGA AACAACTACC AGGTGGAAGC CCCCTTC

                251
SM3810           -------~~~
NB25             gt-cg
NB17             g-gcg
DQB*1001         CCTTC---
```

Figure 38: Sequence analysis of a total of 39 cloned products of the MHC class II DQB exon 2.

Three previously published sequences were identified; DQB*1-3, NB25, NB17 and one novel sequence; SM3810.

Sequence data was compared to allele DRB*1001 previously identified in Holstein-Friesian cattle (Nasir *et al.*, 1997)

3.7 Restriction Fragment Length Polymorphism enzymatic digestion of MHC class II DRB3 and DQB exon 2 Polymerase Chain Reaction Products.

MHC class II DRB3

Following two-step PCR amplification, a product of 284bp was generated that corresponded to the MHC class II DRB3 exon 2. Cleavage of the PCR product with restriction endonucleases *Rsa* I, *Bst* YI and *Hae* III resulted in bands of various base pair sizes being visible. Selection of *Rsa* I, *Bst* YI and *Hae* III as the restriction enzymes was by the method of Van Eijk *et al* (1992), who showed these three enzymes could distinguish all of the BoLA-DRB3 alleles sequenced previously by Sigurdardottir *et al.* (1991). In Figure 39 the restriction enzyme digestion patterns of BoLA DRB3 exon 2 of one bull with the lowest PTA for SCC in 1997 (bull C) and three bulls with the highest PTA for SCC in 1997 (bulls F, H and J) by *Rsa* I and *Hae* III are shown. In Figure 40 the restriction enzyme digestion patterns of BoLA DRB3 exon 2 of one bull with the lowest PTA for SCC in 1997 (bull C) and three bulls with the highest PTA for SCC in 1997 (bulls F, H and J) by *Bst* YI are shown. In Figure 41 the restriction enzyme digestion patterns of BoLA DRB3 exon 2 of four bulls with the lowest PTA for SCC in 1997 (bulls A, B, D and E) and two bulls with the highest PTA for SCC in 1997 (bulls G and I) by *Rsa* I and *Hae* III are shown. In Figure 42 the restriction enzyme digestion patterns of BoLA DRB3 exon 2 of one bull with the lowest PTA for SCC in 1997 (bull C) and three bulls with the highest PTA for SCC in 1997 (bulls F, H and J) by *Bst* YI are shown. The band sizes generated by digestion with restriction endonucleases were estimated for bulls with the lowest PTA for SCC and for bulls with the highest PTA for SCC (Table: 6). Estimation of band sizes was made in correspondence with a standard digest rather than against molecular weight markers.

Restriction Enzyme	*Rsa* I	*Bst* YI	*Hae* III
Bulls with the lowest PTA for SCC			
A	180, 104, 93, 78, 63, 50	284	190, 167, 65, 52, 29
B	180, 104, 93, 78, 63, 50	284	219, 190, 65, 29
C	284, 93, 78, 63, 50	284	190, 167, 65, 48, 29
D	180, 111, 104, 54, 50, 39, 30	284	219, 190, 65
E	140, 93, 78, 63, 54, 50, 39	284, 199, 85	190, 167, 65, 52, 29
Bulls with the highest PTA for SCC			
F	180, 104, 93, 78, 63, 50	284	190, 167, 65, 52, 29
G	180, 140, 104, 39	284, 199, 112, 87, 85	167, 65, 52
H	180, 111, 104, 69	284	219, 167, 65, 52
I	180, 104	284	167, 65, 52
J	180, 141, 111, 104, 69, 54, 50, 39	284, 199, 85	167, 65, 52

Table 6: Estimated band sizes generated by restriction enzyme digestion of the PCR product of DRB exon 2 with *Rsa* I, *Bst* YI and *Hae* III in bulls with the lowest PTA for SCC (n=5) and in bulls with the highest PTA for SCC (n=5)

The combination of restriction band sizes of *Rsa* I, *Bst* YI and *Hae* III identified above can be related to a restriction digestion pattern (appendix D: Van Eijk *et al.*, 1992; Gelhaus *et al.*, 1995; www.ri.bbsrc.ac.uk, bola). The restriction digestion patterns can subsequently be related to a DRB3.2* type. The restriction digestion patterns and

corresponding DRB3.2*type for the bulls with the lowest and highest PTA for SCC are detailed in Table 7.

Bulls with the lowest PTA For SCC	**Restriction Digestion Pattern**	**DRB3.2***
A	jbd	16
	nba	23
B	jbd	16
	nbb	24
C	jbd	16
	obf	27
D	nbb	24
	bbb	03
E	jbd	16
	faa	08
Bulls with the highest PTA For SCC		
F	jbd	16
	nba	23
G	nba	23
	gea	11
H	mba	22
	nbb	24
I	nba	23
	faa	08
J	mba	22
	fba	10
	maa	32
	hba	13
	gaa	51

Table 7: The relationship between the restriction enzyme digest pattern of the PCR product of DRB exon 2 generated by *Rsa* I, *Bst* YI and *Hae* III to the DRB3.2*type in bulls with the lowest PTA for SCC (n=5) and in bulls with the highest PTA for SCC (n=5) in 1997.

The bulls A, B, C, D, E, F, G and H generated two restriction patterns indicating that they are heterozygous for DRB3*2. Bull I generated only one restriction pattern suggesting

that this particular animal is homozygous for DRB3*2. From the band sizes generated by restriction digestion bull J has generated three possible pairs of restriction patterns. Further investigation would be required to identify the expressed restriction pattern pair.

From the patterns created by restriction digest the corresponding DRB3.2*type can be related to previously sequenced and published alleles. From the ten bulls selected for their extreme PTA for SCC, 26 previously sequenced DRB3 alleles were identified. The maximum number of alleles expected from ten animals is 20. The DRB3.2*type however is not an absolute classification, some DRB3.2*types are associated with more than one sequenced allele. More detailed investigation into the characterisation of the allelic sequences is required to permit absolute classification of restriction enzyme pattern to allele. The establishment of a DRB3.2*type permits rapid typing of a large number of individuals, from which interesting animals maybe selected and investigated in greater detail. The sequenced alleles present in the bulls with the lowest and highest PTA for SCC are detailed in Table 8.

Bulls with the lowest PTA for SCC	DRB3.2* type	Sequenced DRB3* alleles
A	16	1501, 1502, 4301
	23	2702, 2703, 2705, 2706, 2707
B	16	1501, 1502, 4301
	24	0101, 0102
C	16	1501, 1502, 4301
	27	14011, 14012, 3101
D	24	0101, 0102
	03	1001, 1002
E	16	1501, 1502, 4301
	08	1201
Bulls with the highest PTA for SCC		
F	16	1501, 1502, 4301
	23	2702, 2703, 2705, 2706, 2707
G	23	2702, 2703, 2705, 2706, 2707
	11	0901, 0902, 1202
H	22	1101
	24	0101, 0102
I	23	2702, 2703, 2705, 2706, 2707
	08	1201
J	22	1101
	10	1601, 1602
	32	2401
	13	0401
	51	4201

Table 8: The relationship between the DRB3.2*type and previously sequenced DRB3 alleles in bulls with the lowest PTA for SCC (n=5) and in bulls with the highest PTA for SCC (n=5)

A difference in the frequency of each of the alleles was noticed on further examination of the data. DRB3.2*16 was identified in four of the bulls with low PTA for SCC (bulls A, B, C, and E), and one of the bulls with high PTA for SCC (bull F). DRB3.2*23 was identified in one of the bulls with low PTA for SCC (bull A), and three of the bulls with high PTA for SCC (bulls F, G and I). DRB3.2*24 was identified in two of the bulls with low PTA for SCC (bulls B and D), and one of the bulls with high PTA for SCC (bull H). DRB3.2*22 was only identified in two bulls with high PTA for SCC (bulls H and J). DRB3.2*03 was identified only in one bull which had low PTA for SCC (bull D). DRB3.2*11 was identified only in one bull which had high PTA for SCC (bull G). DRB3.2*27 was identified only in one bull which had a low PTA for SCC (bull C). Finally, DRB3.2*10, DRB3.2*32, DRB3.2*13 and DRB3.2*51 were identified only in one bull, which had a high PTA for SCC (bull J), (Table: 9).

Bulls	A	B	C	D	E	F	G	H	I	J
DRB3.2* types										
16	♦	♦	♦		♦	♣				
23	♦					♣	♣		♣	
24		♦		♦				♣		
08					♦					♣
03				•						
11							•			
22								•		•
27			•							
10										•
32										•
13										•
51										•

Table 9: Relationship between bulls with the lowest PTA for SCC and bulls with the highest PTA for SCC and previously characterised DRB3.2*types identified by restriction digestion with *Rsa* I, *Bst* YI and *Hae* III:

♦ bulls with the lowest PTA for SCC as recorded in 1997

♣ bulls with the highest PTA for SCC as recorded in 1997

• DRB3.2*types present exclusively in bulls with high or low PTA for SCC.

The relationship between bulls with the lowest PTA for SCC and bulls with the highest PTA for SCC and previously identified DRB3 alleles are presented in Table 10.

Bulls	A	B	C	D	E	F	G	H	I	J
DRB3 alleles										
*1501	♦	♦	♦		♦	♣				
*1502	♦	♦	♦		♦	♣				
*4301	♦	♦	♦		♦	♣				
*2701	♦					♣	♣		♣	
*2702	♦					♣	♣		♣	
*2703	♦					♣	♣		♣	
*2704	♦					♣	♣		♣	
*2705	♦					♣	♣		♣	
*2706	♦					♣	♣		♣	
*2707	♦					♣	♣		♣	
*0101		♦		♦				♣		
*0102		♦		♦				♣		
*14011			•							
*14012			•							
*3101			•							
*1201					♦					♣
*1001				•						
*1002				•						
*0901							•			
*0902							•			
*1202							•			
*1101								•		•
*1601										•
*1602										•
*2401										•
*0401										•
*4201										•

Table 10: Relationship between bulls with the lowest PTA for SCC and bulls with the highest PTA for SCC and previously characterised DRB3 alleles

♦ bulls with the lowest PTA for SCC as recorded in 1997

♣ bulls with the highest PTA for SCC as recorded in 1997

• alleles present exclusively in bulls with the lowest or highest PTA for SCC.

A difference in the frequency of the RFLP types associated with immunological response measured was noticed on examination of the data. DRB3.2*16 was identified in all five of the bulls with a strong immunological response induced *by S. aureus* strain A *in vitro* (bulls A, B, C, E, and F). DRB3.2*23 was identified in two of the bulls with a strong immunological response induced *by S. aureus* strain A *in vitro* (bulls A and F), and two of the bulls with a weak immunological response induced *by S. aureus* strain A *in vitro* (bulls G and I). DRB3.2*24 was identified in one of the bulls with a strong immunological response induced *by S. aureus* strain A *in vitro* (bull B), and two of the bulls with a weak immunological response induced *by S. aureus* strain A *in vitro* (bulls D and H). DRB3.2*22 was only identified in two bulls which had weak immunological responses induced *by S. aureus* strain A *in vitro* (bulls H and J). DRB3.2*03 was identified only in one bull which had a weak immunological response induced *by S. aureus* strain A *in vitro* (bull D). DRB3.2*11 was identified only in one bull which had a weak immunological response induced *by S. aureus* strain A *in vitro* (bull G). DRB3.2*27 was identified only in one bull which had a strong immunological response induced by *S. aureus* strain A *in vitro* (bull C). Finally, DRB3.2*10, DRB3.2*32, DRB3.2*51, were identified only in one bull which had a low immunological response induced by *S. aureus* strain A *in vitro* (bull J), (Table: 11).

Assuming a gene frequency (p) of 0.1 for DRB3.2*16, and using the Hardy-Weinberg equation: $p^2 + 2pq + q^2 = 1$ where $0.01 + 0.18 + 0.81 = 1$ it was estimated that the probability of a bull being either homozygous or heterozygous for DRB3.2*16 was $0.01 + 0.18 = 0.19$. The probability of five bulls being selected at random and being homozygous or heterozygous for DRB3.2*16 was estimated, therefore, as $0.19^5 = 0.00025$.

However further investigation of the ten bulls ranked on extremes of PTA for SCC showed that there was some overlap in their paternal and maternal grandsires (Table 12). The presence of common grandsires means that the association of DRB3.2*16 and proliferative response induced by *S. aureus* strain A cannot be regarded as random.

Bulls	A	B	C	D	E	F	G	H	I	J
DRB3.2* type										
16	♦	♦	♦	♦	♣	♦				
23	♦					♦	♣		♣	
24		♦		♦				♣		
08					♣					♣
03				•						
11							•			
22								•		•
27			•							
10										•
32										•
13										•
51										•

Table 11: Relationship between bulls with strong immunological response and bulls with weak immunological response and previously characterised DRB3.2*types identified by restriction digestion with *Rsa* I, *Bst* YI and *Hae* III.

♦ bulls with strong immunological response induced by *S. aureus* strain A *in vitro*

♣ bulls with weak immunological response induced by *S. aureus* strain A *in vitro*

• DRB3.2* types present exclusively in bulls with strong or weak immunological response induced by *S. aureus* strain A *in vitro*.

Grandsire	Paternal	Maternal
Bulls		
A	I	II
B	II	V
C	III	V
D	III	IV
E	III	IV
F	II	IV
G	IV	VI
H	IV	VII
I	IV	IV
J	IV	II

Table 12: Relationship between bulls with strong immunological response and bulls with weak immunological response and their paternal and maternal grandsires. There are a total of seven (I-VII) different grandsires in different combinations associated with these ten bulls

bulls with strong immunological response induced by *S. aureus* strain A *in vitro*

bulls with weak immunological response induced by *S. aureus* strain A *in vitro*

The relationship between bulls with strong immunological response induced by *S. aureus* strain A *in vitro* and bulls with weak immunological response induced by *S. aureus* strain A *in vitro* and previously identified DRB3 alleles 1 presented in Table 13.

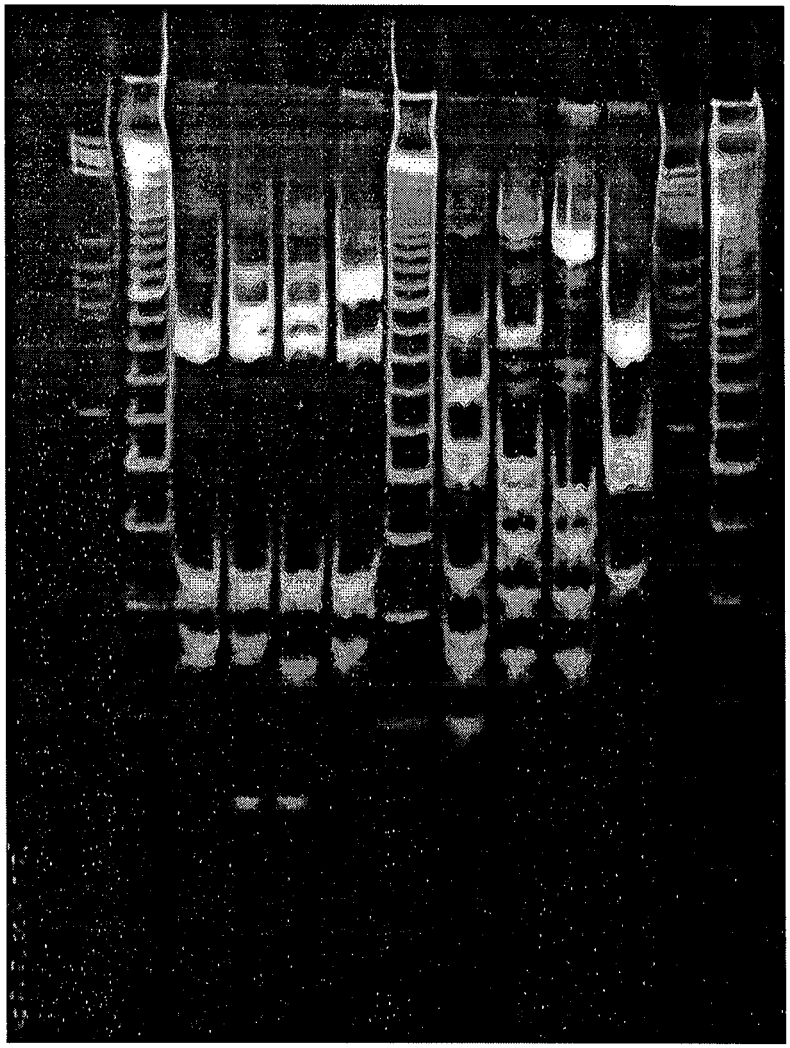
Bulls	A	B	C	D	E	F	G	H	I	J
DRB3 alleles										
***1501**	♦	♦	♦		♣	♦				
***1502**	♦	♦	♦		♣	♦				
***4301**	♦	♦	♦		♣	♦				
***2701**	♦					♦	♣		♣	
***2702**	♦					♦	♣		♣	
***2703**	♦					♦	♣		♣	
***2704**	♦					♦	♣		♣	
***2705**	♦					♦	♣		♣	
***2706**	♦					♦	♣		♣	
***2707**	♦					♦	♣		♣	
***0101**		♦		♦				♣		
***0102**		♦		♦				♣		
***14011**			•							
***14012**			•							
***3101**			•							
***1201**					♣					♣
***1001**				•						
***1002**				•						
***0901**							•			
***0902**							•			
***1202**							•			
***1101**								•		•
***1601**										•
***1602**										•
***2401**										•
***0401**										•
***4201**										•

Table 13: Relationship between bulls with strong immunological response and bulls with weak immunological response to previously characterised DRB3 alleles.

♦ bulls with strong immunological response induced by *S. aureus* strain A *in vitro*

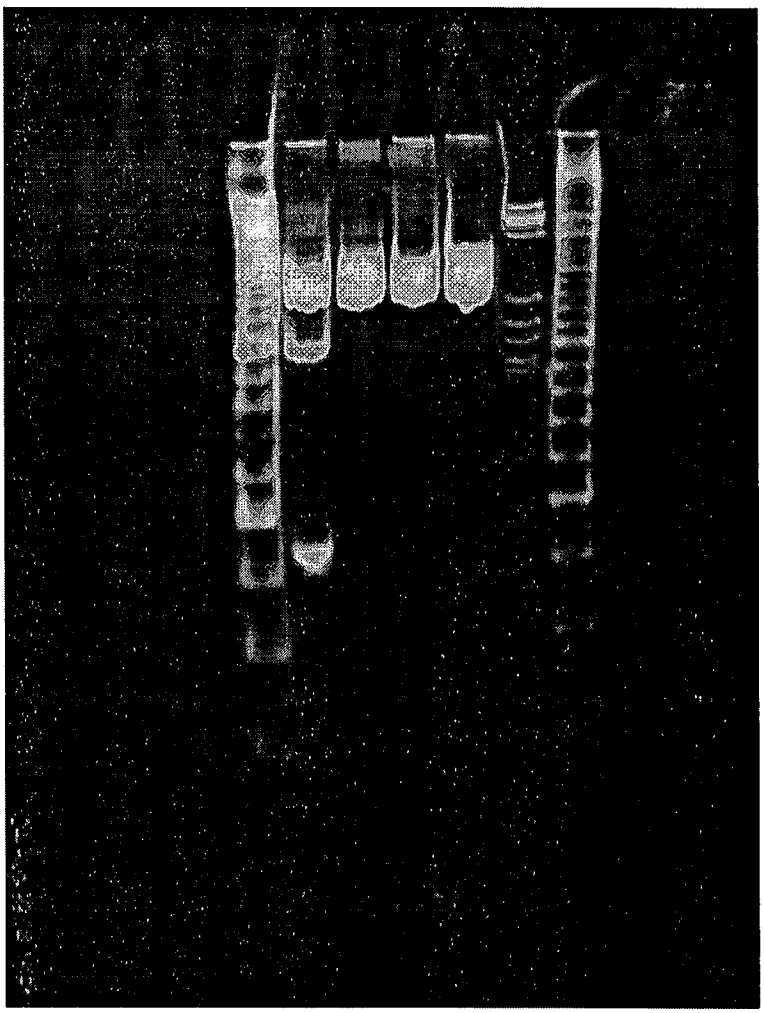
♣ bulls with weak immunological response induced by *S. aureus* strain A *in vitro*

• alleles present exclusively in bulls with strong or weak immunological response induced by *S. aureus* strain A *in vitro*.



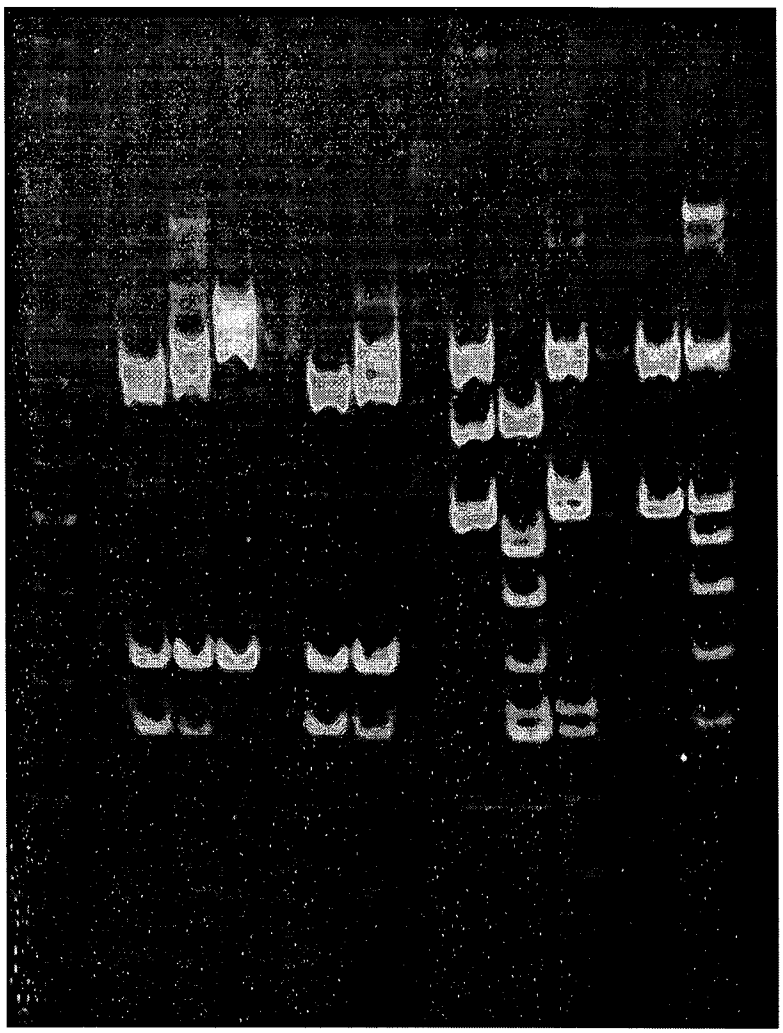
Lanes: **1 2 3 4 5 6 7 8 9 10 11 12 13**

Figure 39: Restriction digest patterns of BoLA DRB3 exon 2 of one bull with the lowest PTA for SCC in 1997 (bull C) and three bulls with the highest PTA for SCC in 1997 (bulls F, H and J) obtained by digestion with the enzymes *Rsa* I (lanes 3-6) and *Hae* III (lanes 8-11). Lanes 1 and 12 are *Hae* III control digest of pBR322 (Sigma). Lanes 2 and 13 are molecular weight markers of 20bp and 100bp. Lane 3: bull J, lane 4: bull F, lane 5: bull C, lane 6: bull H, lane 8: bull J, lane 9: bull F, lane 10: bull C, lane 11: bull H.



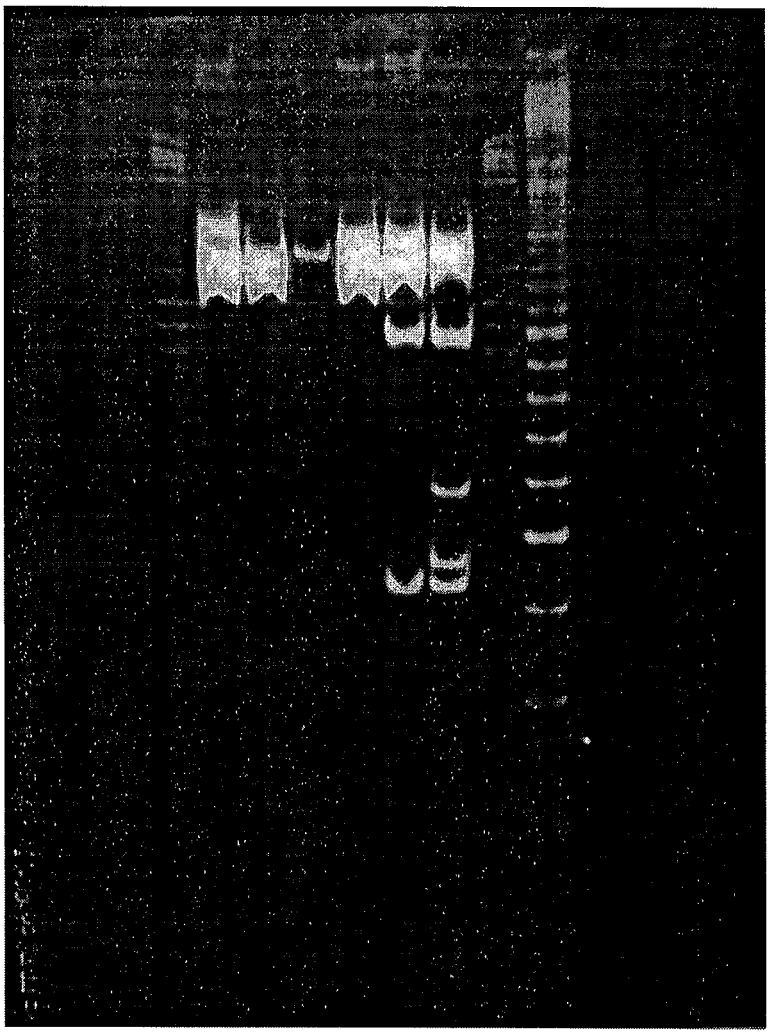
Lanes: **1 2 3 4 5 6 7**

Figure 40: Restriction digest patterns of BoLA DRB3 exon 2 of one bull with the lowest PTA for SCC in 1997 (bull C) and three bulls with the highest PTA for SCC in 1997 (bulls F, H and J) obtained by digestion with the enzymes *Bst* YI (lanes 2-5). Lane 6 is *Hae* III control digest of pBR322 (Sigma). Lanes 1 and 7 are molecular weight markers of 20bp and 100bp. Lane 2: bull J, lane 3: bull F, lane 4: bull C, lane 5: bull H.



Lanes: **1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16**

Figure 41: Restriction digest patterns of BoLA DRB3 exon 2 of four bulls with the lowest PTA for SCC in 1997 (bulls A, B, D and E) and two of the bulls recorded as having the highest PTA for SCC in 1997 (bulls G and I) obtained by digestion with the enzymes *Rsa* I (lanes 3-8) and *Hae* III (lanes 10-15). Lanes 2, 9 and 16 are *Hae* III control digest of pBR322 (Sigma). Lane 1 is molecular weight markers of 20bp and 100bp. Lane 3: bull G, lane 4: bull E, lane 5: bull D, lane 6: bull B, lane 7: bull I, lane 8: bull A, lane 10: bull G, lane 11: bull E. lane 12: bull D, lane 13: bull B, lane 14: bull I, lane 15: bull A.



Lanes: **1 2 3 4 5 6 7 8 9**

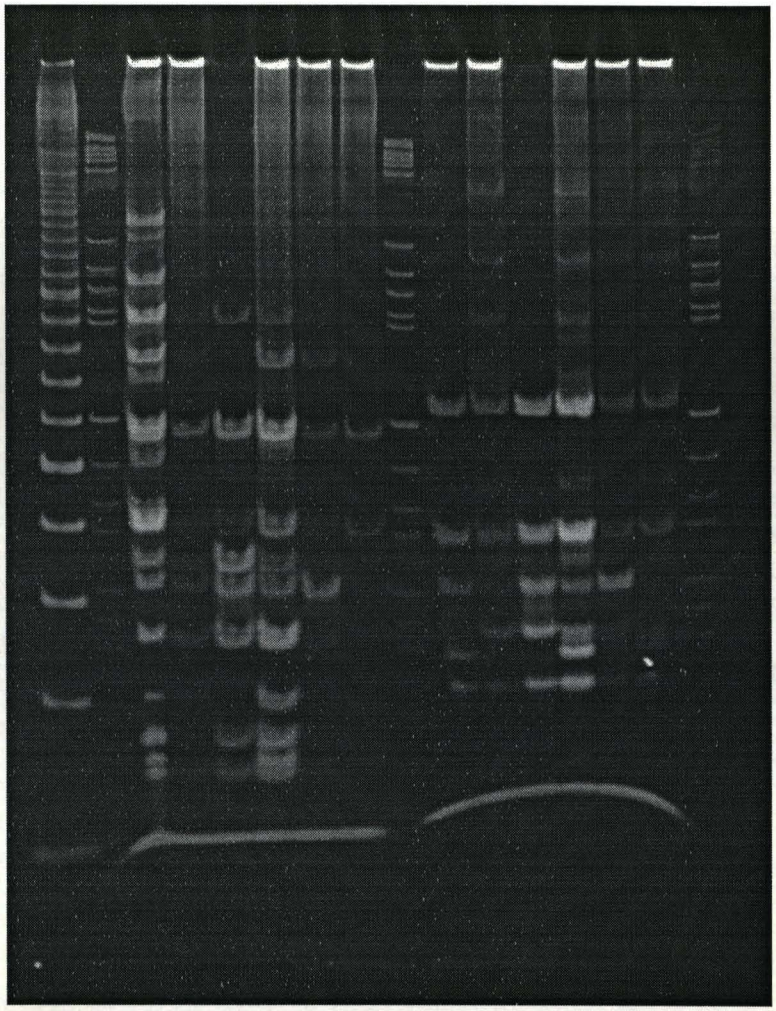
Figure 42: Restriction digest patterns of BoLA DRB3 exon 2 of four bulls with the lowest PTA for SCC in 1997 (bulls A, B, D and E) and two bulls with the highest PTA for SCC in 1997 (bulls G and I) obtained by digestion with the enzymes *Bst* YI (lanes 2-7). Lanes 1 and 8 are *Hae* III control digest of pBR322 (Sigma). Lane 9 is molecular weight markers of 20bp and 100bp. Lane 2, bull A, lane 3: bull I, lane 4: bull B, lane 5: bull D, lane 6: bull E, lane 7: bull G.

MHC class II DQB2

Following PCR amplification a 214bp product was generated which corresponded to the MHC class II DQB exon 2 region. After enzymatic digestion of the PCR product with *Rsa* I, *Brs* I and *Hae* III, RFLP banding patterns were visible. In Figure 43 the restriction enzyme digestion patterns of BoLA DQB exon 2 of four bulls with the lowest PTA for SCC in 1997 (bulls A, B, D and E) and two bulls with the highest PTA for SCC in 1997 (bulls G and I) by *Rsa* I and *Hae* III are shown. In Figure 44 the restriction enzyme digestion patterns of BoLA DQB exon 2 of four bulls with the lowest PTA for SCC in 1997 (bulls A, B, D and E) and two bulls with the highest PTA for SCC in 1997 (bulls G and I) by *Bst* YI are shown. These patterns, although relatively clean, were generally too complex to be resolved unambiguously as allelic patterns. Due to the clean nature of the bands, the results cannot simply be dismissed as being caused by partial digestion or non-specific PCR. The number of bands present may be created by the presence of multiple alleles. The presence of multiple alleles was detected in most animals and suggests the phenomenon of gene duplication, which can be problematic for analytical purposes.

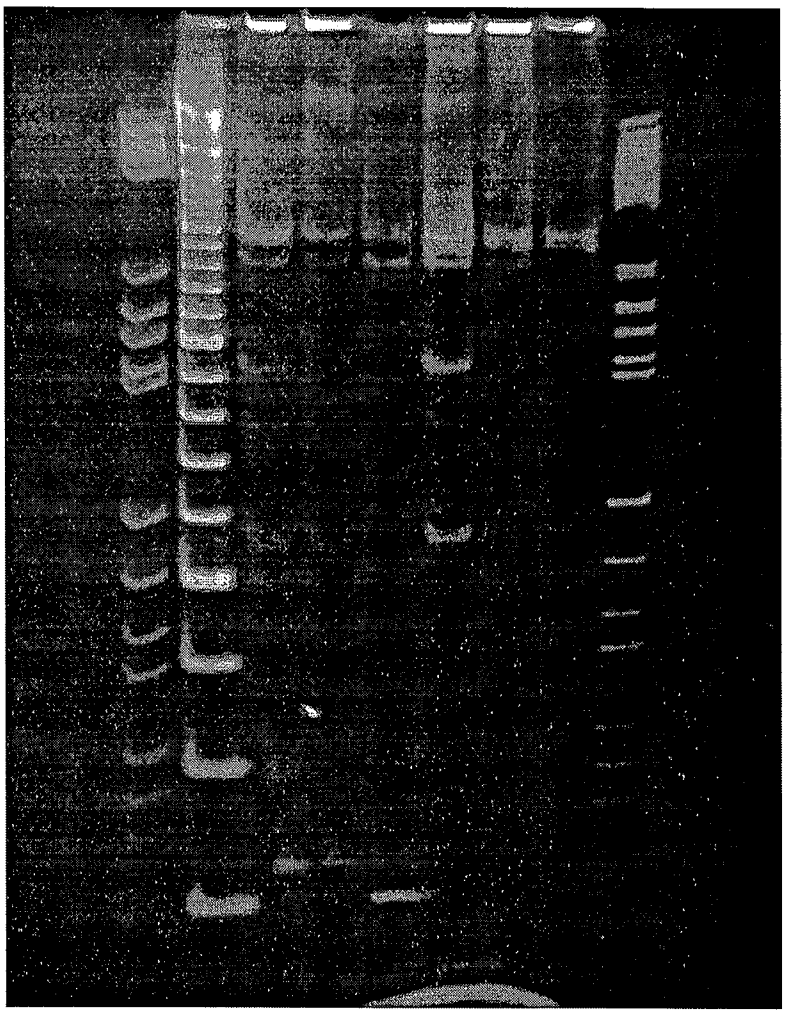
Rather than dismiss the restriction banding patterns of the DQB2 as too complex for analysis, the current study reported similarities in banding patterns among animals. Bulls A and D, both with low PTA for SCC, and bulls E and I, one with low PTA for SCC and the other with high PTA for SCC respectively, showed similarities in their *Rsa* I patterns. Two bulls with low PTA for SCC (bulls D and E) and one bull with high PTA for SCC (bulls I), showed similarities in their *Hae* III patterns, bulls A and D, both with low PTA for SCC, showed similarities in their *Brs* I patterns. Similarities were also noted in the banding patterns of the MHC class II DRB3 among animals. Bulls A and B, both with low PTA for SCC, bulls D and I, one with low PTA for SCC and the other with high PTA for SCC respectively, and bulls C and F, one with low PTA for SCC and the other with high PTA for SCC respectively showed similarities in their *Rsa* I patterns. Bulls A and I, one with low PTA for SCC and the other with high PTA for SCC respectively, bulls B and D, both with low PTA for SCC, and bulls C and F, one with low PTA for SCC and the other with high PTA for SCC respectively, showed similarities in their *Hae* III patterns.

Again with respect to immunological response, similarity in banding patterns of MHC class II DQB2 was seen among animals. Bulls A and D, both with a strong immunological response induced by *S. aureus* strain A *in vitro*, and bulls E and I one with a strong immunological response induced by *S. aureus* strain A *in vitro* and the other with a weak immunological response induced by *S. aureus* strain A *in vitro*, showed similarities in their *Rsa* I patterns. Bulls D, with a weak immunological response induced by *S. aureus* strain A *in vitro* and bulls E and I, which had strong immunological response induced by *S. aureus* strain A *in vitro*, showed similarities in their *Hae* III patterns. Bulls A and D, both with a strong immunological response induced by *S. aureus* strain A *in vitro*, showed similarities in their *Brs* I patterns.



Lanes: **1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16**

Figure 43: Restriction digest patterns of BoLA DQB exon 2 of four bulls with the lowest PTA for SCC in 1997 (bulls A, B, D and E) and two bull with the highest PTA for SCC in 1997 (bulls G and I) obtained by digestion with the enzymes *Rsa* I (lanes 3-8) and *Hae* III (lanes 10-15). Lanes 2, 9 and 16 are *Hae* III control digest of pBR322 (Sigma). Lane 1 is molecular weight markers of 20bp and 100bp. Lane 3: bull A, lane 4: bull I, lane 5: bull B, lane 6: bull D, lane 7: bull E, lane 8: bull G, lane 10: bull A, lane 11: bull I, lane 12: bull B, lane 13: bull D, lane 14: bull E, lane 15: bull G.



Lanes: **1 2 3 4 5 6 7 8 9**

Figure 44: Restriction digest patterns of BoLA DQB exon 2 of four bulls with the lowest PTA for SCC in 1997 (bulls A, B, D and E) and two bull with the highest PTA for SCC in 1997 (bulls G and I) obtained by digestion with the enzymes *Bsr* I (lanes 3-8). Lanes 1 and 9 are *Hae* III control digest of pBR322 (Sigma). Lane 2 is molecular weight markers of 20bp and 100bp. Lane 3: bull A, lane 4: bull I, lane 5: bull B, lane 6: bull D, lane 7: bull E, lane 8: bull G.

Chapter IV

Discussion

4.1 Proliferation of Bovine Peripheral Blood Mononuclear Cells

The method of *in vitro* antigenic stimulation of PBM employed was established during the course of the current study. The method established was a modification of an antigen presentation assay, used previously by Fitzpatrick (1992), developed to study the proliferative response of separated populations of T cells and APC isolated from Holstein-Friesian cows to the mastitis pathogen *S. uberis*. The previous study required withdrawal of large volumes of blood followed by defibrination, to prevent clotting. Defibrination was achieved by either mixing the blood with glass beads when dealing with large volumes or shaking by hand with smaller volumes. The sample was then mechanically defibrinated using a large plastic beaker containing the glass beads and a motor-driven rotating paddle. The method of defibrination was initially described by Glass and Spooner (1989) and further modified by Fitzpatrick (1992). It was decided to refine the initial isolation technique to reduce the volume of blood required, the time involved in isolation of PBM, and to minimize the mechanical stress exerted on the PBM by the isolation technique. A pure population of peripheral blood T cells isolated by the miniMACS method cultured *in vitro* with *S. aureus* strain A and fresh irradiated APC failed to proliferate. Isolation of T cells by positive miniMACS selection, as mentioned previously may have damaged the cells altering their functional capabilities, however, when T cells were isolated by negative selection, they also to failed to proliferate when cultured *in vitro* with *S. aureus* strain A and freshly isolated APC. For the current study, peripheral blood was collected by vacutainer directly into lithium heparin coated tubes. This eliminated the requirement for defibrination, therefore, reducing the time, the volume of blood and the stress exerted on the PBM. The PBM population was isolated by centrifugation using media of density 1.077g/l, that used most frequently for isolation of these cells in cattle by others (Boyum, 1968; Wilson *et al.*, 1986; Taylor *et al.*, 1994; Schuberth *et al.*, 1996; Shafer-Weaver and Sordillo, 1997; McCole *et al.*, 1998; Asai *et al.*, 1998).

Cell numbers in healthy lactating mammary glands tend to be relatively low, requiring large volumes of milk for isolation of sufficient numbers of cells for *in vitro* studies (Nonnecke and Kehrli, 1985). The use of PBM as the responding cell population to a mammary gland pathogen was regarded as logical for during an intramammary infection cells are recruited from the peripheral blood of cattle to the mammary gland (Sordillo *et al.*, 1997). Previously published results suggest that a large proportion of intramammary lymphocytes are derived from the peripheral system (Yamaguchi *et al.*, 1999). This migration of cells from the peripheral blood to the mammary gland has also been reported in rats (Seelig, 1980). Migration of cells from the peripheral blood of cattle to the mammary gland can be measured by the rise in the number of somatic cells recorded in milk (Heald, 1979; Gudding *et al.*, 1984; Nickerson, 1985). Migration of cells from the peripheral system involves rolling of leucocytes onto the vascular wall followed by tight adhesion (Zimmerman *et al.*, 1992; Lawrence and Springer, 1993). For migration of leucocytes across the endothelium to be successful, opening of the tight junctions present in the endothelium must be accomplished (Milks *et al.*, 1986). Recruitment of leucocytes and the exudation of plasma from peripheral blood through the mammary parenchyma (Schlam *et al.*, 1971), across the epithelium to the lactiferous ducts and glandular alveoli (Anderson, 1983), was reported due to intramammary infection of cows caused by *S. aureus* (Nickerson and Heald, 1981). In addition to the migration of cells from peripheral blood to the mammary gland, IgG, the major immunoglobulin class in milk (Norcross, 1977), is not produced locally within the mammary gland but undergoes selective transfer from serum (Murphy *et al.*, 1964; Pierce and Fienstein, 1965).

The uptake and distribution of *S. uberis* in cows was followed from the site of inoculation in the mammary gland via the teat to the subepithelial connective tissue of the mammary gland. The bacteria were then transported to the lymph vessels and the supramammary lymph node, from where they entered into the peripheral circulation (Nashar *et al.*, 1990). Cellular responses induced by the intramammary inoculation of *S. uberis* resulted in an accumulation of neutrophils in the secretory acini (Thomas *et al.*, 1994), and up-regulation of the adhesion molecule, CD18, a β_2 integrin subunit, which combines with CD11a to form LFA-1, on neutrophils in the mammary gland and on neutrophils in the peripheral blood of cattle (Persson *et al.*, 1996). Demonstration of the transfer of cells

between the mammary gland and peripheral blood was reported when cattle were inoculated via the intramammary route with bovine rotavirus. Intramammary inoculation of heifers and cows with bovine rotavirus in a water-in-oil emulsion was capable of inducing both systemic and local antigen specific transformation of lymphocytes and the production of neutralising antibodies (Castrucci *et al.*, 1984; Archambault *et al.*, 1988). The results of this study suggested that primed lymphocytes migrate to the mammary gland, which has been published previously (Seelig, 1980). However, the development of local immunity in the mammary gland after intramammary inoculation cannot be ruled out (Archambault *et al.*, 1988). Antigen specific lymphocytes were also detected in the peripheral blood of cows inoculated via the intramammary route with killed *Mycobacterium bovis* (Nonnecke *et al.*, 1986), suggesting migration of these antigen specific lymphocytes from the mammary gland to the peripheral circulation.

In non-ruminant species, a functional link between mammary and gut associated lymphoid tissue has been described (Evans, 1980; Kortbeek-Jacobs and Van der Donk, 1981). In ruminant species, a link between mammary and peripheral lymph nodes has been reported (Harp and Moon, 1987; Harp *et al.*, 1988). Gastrointestinal priming with antigen has been shown to elicit a mammary immune response in non-ruminants (Goldblum *et al.*, 1975; Parmely and Beer, 1977; Evans, 1980). This common mucosal immune system, known as the entero-mammary link, where specific immunity at distant mucosal sites following intestinal exposure to antigen has been described in non-ruminants, but has not been established as fully functional in ruminants (Chang *et al.*, 1981; Moon and MacDonald, 1983). It has been reported that localisation of mammary gland lymphocytes in intestinal mesenteric lymph nodes is poor and that accumulation occurs preferentially in mammary and prescapular nodes as shown previously in sheep (Harp and Moon, 1987), and cows (Harp *et al.*, 1988).

Development of the *in vitro* assay for the current study required optimisation of PBM and *S. aureus* concentrations to determine which combination induced an optimal proliferative response. The kinetics of the proliferative response were followed over an eleven day culture period to determine the day of peak proliferative response, measured by ^{3}H-thymidine incorporation in the responding PBM population, as used routinely by others (Schore *et al.*, 1981; Concha *et al.*, 1996; Shafer-Weaver and Sordillo, 1997). The

degree of stimulation was measured as counts per minute of the test well divided by the counts per minute of the control well and represented as a SI. Others have reported using Δ cpm, where the control cpm is simply subtracted from the test well cpm (Lan *et al.*, 1995). In the current study the concentration of *S. aureus* and PBM that induced the optimal proliferative response was $0.5x10^6$ bacteria/ml and $2x10^6$ cells/ml, respectively. The day of peak proliferation of PBM induced by *S. aureus* strain A occurred on day seven to nine of culture. Others have reported that when T lymphocytes and APC in an *in vitro* antigen presentation assay were mixed at a ratio of 10:1, *S. uberis* induced a proliferative response which peaked on day six (Fitzpatrick *et al.*, 1995). In a separate study by Miller-Edge and Splitter (1986), PBM challenged with UV-inactivated Bovine Herpes Virus-1 (BHV-1) induced a peak proliferative response on day 5-6. The kinetics of the response induced by antigen in the current study is, therefore, similar to those reported previously. Proliferation assays are widely used to study *in vitro* immunological responses to a variety of antigenic, mitogenic, parasitic and viral stimuli. Mitogens have been used to evaluate lymphocyte function (Concha *et al.*, 1980), and in cattle, to elucidate immunological problems that may arise due to defects in regulatory pathways (Lan *et al.*, 1995). Detection of cytokine production by PBM induced by *Brucella abortus* in cattle was measured during vaccine trials through collection of proliferation assay culture supernatant (Stevens and Olsen, 1994). The proliferative response of bovine PBM induced by UV-inactivated BHV-1 was used as a measure of T cell mediated immunological response (Rouse and Babiuk, 1974). *In vitro* proliferative studies permit investigation of responses to parasites such as *Theileria annulata*, a protozoan parasite of cattle, which often results in death 2-3 weeks post *in vivo* experimental challenge (Samantary *et al.*, 1980; Preston *et al.*, 1992; Campbell *et al.*, 1997).

In the current study, the SI measured on the day of peak proliferation was reported to range from 0 to 164. The variation in the magnitude of the proliferative response induced by antigenic challenge was also reported by others (Fitzpatrick *et al.*, 1995; Miller-Edge and Splitter, 1986; Nonnecke and Harp, 1985; McCole *et al.*, 1998). The variability among animals in the intensity of the immune response triggered, reflects the efficiency of antigenic processing, association between antigenic peptides and MHC class II, and ultimately presentation to T helper cells (Buus *et al.*, 1987). Individual variability in the

intensity of an immune response induced by a specific antigen has been reported as common in an outbred population, such as cattle (Lutje and Black, 1991). Marked variation in the inflammatory response among cows was reported after intramammary infusion with whole killed staphylococci or staphylococcal cell walls (Targowski and Berman, 1975). The use of inbred strains of mice in studying immunological responses to various antigens has the advantage over outbred populations, in that variation due to an individual is minimized as parental genetic contribution and environmental affects are controlled producing animals which are genetically identical or nearly identical (Hooper *et al.*, 1987).

The number and type of cells present in peripheral blood capable of recognizing and responding to the pathogenic stimulus will affect the intensity of the immune response. Previous exposure to the pathogenic stimulus may result in the presence of $CD4^+$ T cells expressing a memory phenotype (CD45RO). The presence of $CD4^+$ memory T cells alters the kinetics of the proliferative response, requiring a much lower does of antigen presented by co-stimulatory molecules to induce a proliferative response (Rogers *et al.*, 2000). When T cells, expressing memory phenotype, are induced to proliferate by a recall antigen, they display rapid kinetics associated with their proliferative response, while naïve T cells exhibit a lag period prior to induction of a proliferative response (Rogers *et al.*, 2000). The accumulation of $CD8^+$ T cells near the epithelium suggests a potential role in maintaining the integrity of the bovine mammary gland through the removal of damaged or infected cells (Taylor *et al.*, 1984). Conversely, $CD8^+$ T cells have also been shown to mediate hypo-responsiveness of lymphocytes from bovine mammary glands infected with *S. aureus* to mitogens or bacterial antigen (Park *et al.*, 1992). Murine $CD8^+$ splenic T cells inhibit antigen positive anti-TCR induced proliferation of $CD4^+$ T cells (Hisatsune *et al.*, 1990), and immunoglobulin production (Huston, 1991).

The results of the current study reported that the within assay repeatability of the *in vitro* proliferative assay was good, indicating that methodology and operator error were unlikely to explain the observed variation among animals in the proliferative response induced by *S. aureus* strain A. The between assay repeatability in the current study was, however, poorer. Following the kinetics of the response, it was noted that the repeatability was reduced during the period when the PBM were undergoing clonal

expansion in response to antigenic stimulus compared with the period of initial culture or the end phase of the response. Activated T cells have been shown to activate resting T cells *in vitro*, in a non-MHC restricted manner (Mokhtarian *et al*, 1997). This activation does not require viable cells and occurs when initial stimulation is induced by mitogen or antigen (Brod *et al.*, 1990; Bouchonnet, 1994). Non-specific activation of the T cell population through the presence of an unknown protein present in the culture media was eliminated by the lack of a detectable proliferative response in the absence of *S. aureus* strain A. Far less is known regarding cell proliferation induced by Gram positive whole bacteria, for example when compared with the knowledge of cell proliferation induced by bacterial lipopolysaccharide, a product of Gram negative bacteria (Cuzzola *et al.*, 2000).

The variation in proliferative response of PBM induced by *S. aureus* strain A noted among cows, was also noted among strains of *S. aureus*. These strains of *S. aureus*, four mammary gland isolates and one vaginal isolate, were characterized as genetically different by REFP analysis during the current study. Characterisation of strain C and strain D, isolated from the mammary gland of the same cow, by REFP, resulted in two identical enzymatic digestion patterns. The diversity of *S. aureus* genotypes within a herd has been reported as limited (Lam *et al.*, 1996) due to the contagious nature of transmission of *S. aureus* between cows and between quarters during milking. The majority of recurrent cases of *S. aureus* mastitis within a herd were due to isolates of the same genotype, suggesting either intermittent shedding of bacteria from a chronically infected quarter or repeated infection with the same pathogen (Lam *et al.*, 1996). Restriction Enzyme Fragmentation Pattern analysis identified several different *S. aureus* genotypes within a herd, but also within individual cows, and within individual quarters, suggesting that mixed infections do occur within the mammary gland (Young, 1997). The other strains of *S. aureus*, A, B and F used in the current study were identified as genetically distinct from each other by REFP analysis. Characterisation of *S. aureus* strain A and strain B by REFP was supported by previously published patterns (Young, 1997), however, *S. aureus* strains C, D and F in the current study had not been characterized previously. The strongest proliferative response induced by a mammary gland isolate was by strain A. The mammary gland isolate, strain B induced a very weak proliferative response in some animals and failed to induce a proliferative response in others. The mammary gland isolates, strain C and strain D induced a proliferative

response that was greater than strain B but less than strain A. The proliferative response induced by the vaginal isolate, strain F, was far greater than that to strains isolated from the mammary gland. This suggests that this vaginal isolate may represent a common strain of *S. aureus*, possibly causing widespread exposure to cows, which may result in a greater number of circulating memory cells primed to respond to this pathogen. Variation in the virulence factors produced by individual strains may affect the proliferative response mounted against them. Cell-mediated reactions *in vivo* have been induced by living and killed, whole staphylococci, non-fractionated staphylococcal lysates, purified cell wall, and defined peptidoglycan sub-units of the cell wall (Derbyshire and Berman, 1968; Baughn and Bonventre, 1975). Secreted toxins also contribute to the pathogenesis of *S. aureus* and those isolated from cases of bovine mastitis have been shown to express α, β, γ and δ toxins, enterotoxin and coagulase (Bramley *et al.*, 1989; Matsunga *et al.*, 1993). The variation in virulence factors among strains of *S. aureus* may help to explain the variation in proliferative response, not only among cows but also variation within an individual cow, to various different strains of the same bacteria. Variation among animals in response to immunological stimuli has been reported after vaccination, where the antigen to be administered has to be prepared in line with strict protocols (Reinhardt *et al.*, 1999).

The variation in proliferative response and kinetics associated with new batches of *S. aureus* strain A may have been due to difference in the initial preparation of the bacteria between laboratories. To ensure that adequate numbers of bacteria were available, subsequent batches of *S. aureus* strain A were cultured as bigger batches. One explanation for the variation among batches is that differences in preparation may have resulted in an alteration in the immunological epitopes expressed on the surface of the bacteria. Enzymatic digestion of *S. aureus* strain A and REFP analysis carried out in the current study showed that, genotypically, the original strain A, used to optimise the proliferative assay and test all of the bulls sampled, was identical to the strain A cultured subsequently. Alteration in the kinetics of the proliferative response induced by *S. aureus* strain A with the day of peak proliferation occurring later than with the original batch may be due to differences in concentration of bacteria between the original batch and subsequent batches. This could limit the number of bacteria available for APC processing and presentation to T cells.

Rapid induction of an effective immune response is essential in the prevention of colonization by invading pathogens (Jensen and Eberhart, 1975; Sordillo *et al.*, 1997). The variation among animals in eliciting a proliferative response *in vitro* may reflect their ability to present an effective defence against invading pathogens *in vivo*. Failure to recognize different strains of *S. aureus* may be due to an inability to initially process antigen and subsequently present antigenic peptides in association with MHC class II molecules to $CD4^+$ T cells (Nonnecke and Harp, 1989; Sordillo *et al.*, 1997).

In the results of the current study it was noted that when a strong proliferative response was induced by *S. aureus* strain A in a particular animal, in general the proliferative response induced by the other *S. aureus* strains, C, D and F was also strong. Selection for improved resistance to one pathogen may potentially result in susceptibility to another. Mastitis is a multi-factoral disease with an extremely complex aetiology, therefore, improved resistance to one pathogen may not be true for other mastitis causing pathogens (Shook, 1993).

The large variation induced by antigenic stimuli among animals reported in the current study has also been reported when PBM were challenged with mitogens (Schore *et al.*, 1981). This study reported that the variation in proliferative response among animals was far greater in the presence of mitogen when compared to antigen. Marked variation in lymphocyte blastogenesis was also noted with day-to-day sampling (Smith and Schultz, 1977). Many of the previously published studies relating to the immunological responses of cattle have been carried out using mitogenic stimuli rather than antigenic stimuli (Concha *et al.*, 1980; Lutje and Black, 1992).

The kinetics of the proliferative response induced by *S. aureus* in the current study could be suggestive of a primary response to antigen. The kinetics of an immune response may be used as an indicator of an animal's previous exposure to antigen. The kinetics associated with a primary immune response, where the responding cell population is naïve, are different to those where the responding cell population has encountered the antigen previously (Mackay *et al.*, 1990). The day of peak proliferation induced by antigenic stimuli is earlier in a secondary immune response when compared to that in a

primary response. Peripheral blood mononuclear cells isolated from cats when challenged with human serum albumin (HSA) produced a proliferative response that peaked at day 8-9. When rechallenged with HSA the proliferative response peaked on day 2 (Bishop *et al.*, 1992). The kinetics of this study were similar when peripheral blood leucocytes from healthy humans were challenged then rechallenged with antigen (Plebanski *et al.*, 1992). Expression of the different forms of the cell surface marker CD45 can distinguish naïve from memory T cells (Smith *et al.*, 1986). Naïve T cells express CD45RA, and require recognition of high densities of antigen presented by APC (Ingulli *et al.*, 1997; Cella *et al.*, 1997). The kinetics of $CD45RA^+$ T cells is suggestive of a primary proliferative response induced by antigenic stimuli (Mackay *et al.*, 1990). Memory T cells express CD45RO and proliferate in response to a recall antigen presented by APC at a lower concentration than that presented primarily (Rogers *et al.*, 2000). The kinetics of $CD45RO^+$ T cells are suggestive of a secondary proliferative response (Pilling *et al.*, 1996), and as would be expected, T cells expressing a memory phenotype have been shown to exhibit more rapid effector functions when compared to naïve T cells (Swain *et al.*, 1996).

In the current study, *S. aureus* strains A, C, D, and F produced enterotoxins, and strains C and D also produced TSST-1. Production of SE was detected by RPLA, which has been described as sensitive and rapid, and requiring no specialized equipment (Park and Szabo, 1986; Wieneke and Gilbert, 1987; Matsunaga *et al.*, 1993; Takeuchi *et al.*, 1998). Of the four *S. aureus* strains tested, the two mammary isolates recovered from the same cow but different quarters: strain C and strain D, both produced SEC and TSST-1, which was expected, as they were genotypically identical as determined by REFP analysis. *Staphylococcus aureus* strain A, the other mammary gland isolate, produced SED only and strain F, the vaginal isolate, produced SEA and SEB. Others studies have reported SEC and TSST-1 as the most frequently isolated SE from sheep, goats and cattle (Orden *et al.*, 1992; Matsunaga *et al.*, 1993; Kenny *et al.*, 1993). Bovine *S. aureus* isolates are reported to frequently produce more than one toxin, especially SEC, SED and TSST-1 (Ferens *et al.*, 1998). Staphylococcal enterotoxin A and TSST-1 have been reported as more potent inducers of the characteristic mitogenic polyclonal T cell response when compared to SEB and SEC (Yokomizo *et al.*, 1995). Staphylococcal enterotoxin B has been reported by others to induce an *in vivo* state of immunosupression that affects

humoral and cell mediated immunity when challenged with antigen complexes such as sheep red blood cells and allo-MHC molecules in mice (Pinto *et al.*, 1978). The induction of an immune response by SEB is characterized by early activation and clonal expansion of the responding cell population.

To ensure that the proliferative response being measured was due to induction by *S. aureus* and not enterotoxin, PBM were cultured in the presence of SEB and the kinetics were followed. In the current study PBM incubated with SEB *in vitro*, induced a proliferative response that peaked on day three to four of culture. Others have reported similar findings when the proliferative response of PBM induced by SEA, SEB and SED was studied in humans (Fleischer and Schrezenmeirer, 1988), and cattle (Yokomizo *et al.*, 1994). The difference in the day of peak proliferative response induced by *S. aureus* compared with SEB, highlights the difference in type of immune response generated by SE compared to formalin-fixed whole *S. aureus*. Staphylococcal enterotoxins are bi-functional molecules capable of recognising and binding to both the MHC molecule and the TCR (Fleisher, 1989; White *et al.*, 1989). The requirement for MHC class II molecules is not due to immunological recognition of processed SE, as SE act directly on the T cells, but rather due to cross linking of variable parts of their antigen receptors with MHC class II molecules on accessory or target cells (Fleischer and Schrezenmeier, 1988). Staphylococcal enterotoxin stimulation of T cells according to the composition of the TCR β chain alone (Choi *et al.*, 1989), rather than the precise antigenic specificity of their TCR (Wood *et al.*, 1991). As a result of SE being able to stimulate a variety of Vβ T cell types they are regarded as polyclonal stimulants rather than monoclonal stimulants. This polyclonal ability to stimulate a range of Vβ T cell types has earned SE the name superantigens (Choi *et al.*, 1989).

T lymphocytes have been shown to be the predominant cell type that proliferates in response to SE (Yokomizo *et al.*, 1994), with equal proportions of $CD4^+$ T cells and $CD8^+$ T cells induced in human and murine studies (Uchiyama *et al.*, 1990). Further studies have reported activation of T cells lacking both the CD4 and CD8 cell surface marker by SE, indicating that CD4 and CD8 markers are not essential for induction of a SE response (Quarantino, 1991). Staphylococcal enterotoxin B has been shown in humans to downregulate the expression of mucosal T cell homing receptor on human

activated T cells (Hernandez-Caselles *et al.*, 1996), as well as exacerbate the inflammatory response by the secretion of cytokines. In cattle, the activation of $CD8^+$ T cells induced by staphylococcal superantigens has been reported to result in a down-regulation of the $CD4^+$ T cell immune response (Park *et al.*, 1993). Elevated levels of $CD8^+$ T cells have been reported in *S. aureus*-contaminated milk (Ferens *et al.*, 1998). The down regulation of $CD4^+$ T cells by activation of $CD8^+$ T cells suggests a potential role of SE in the pathogenesis of intramammary infection. T cell unresponsiveness has been reported as mediated by peripheral cell apoptosis (Rellahan *et al.*, 1990) and induction of anergy (Migita and Ochi, 1993). It has been suggested that the phenomenon of T cell unresponsiveness may not affect the host's antibacterial response but rather represent an efficient mechanism by which Gram positive bacteria subvert the hosts immune response (Muraille *et al.*, 1997).

In the current study, PBM isolated from cows cultured in the presence of 14% normal milk and SEB resulted in a reduction in the proliferative response induced by SEB. A concentration of 7% normal milk also reduced the proliferative response induced by SEB but to a lesser extent than the higher concentration. Soluble components of milk may inhibit lymphocyte proliferation by masking cell surface receptors (Brock and Mainou-Fowler, 1983). Mammary gland secretions have been reported previously to non-specifically depress the activity of mammary gland lymphocytes (Schore *et al.*, 1981). The presence of colostral skim has also been shown to inhibit the proliferative response of PBM isolated from cattle induced by mitogenic stimulus *in vitro* (Collins and Oldham, 1986; Torre and Oliver, 1988). Bovine peripheral blood lymphocytes cultured in the presence of cell free milk for 30 minutes, which were then washed prior to incubation with PHA resulted in a reduction in proliferative response induced by the mitogenic stimulus (Parmely *et al.*, 1976). Human peripheral blood leucocytes cultured *in vitro* in milk demonstrated an inhibition of phagocytosis due to the presence of the milk (Russell *et al.*, 1976), as well as a reduction in proliferative response induced by mitogenic stimulus (Diaz-Jouanen and Williams, 1974). Other studies have reported that the reduction in proliferative response can be attributed to the fat and casein present in the milk, which penetrate the monocytes and macrophages, resulting in a reduction in the ability to process pathogens (Lee and Outteridge, 1976).

In the current study, the presence of an anti-MHC class II antibody resulted in a significant reduction the proliferative response of PBM induced by *S. aureus* strain A. The expression of MHC class II molecules on the surface of antigen presenting cells is necessary for effective processing of antigen and subsequent presentation to T cells (Babbitt *et al.*, 1985; Sette *et al.*, 1987). Addition of the anti-MHC class II MAb blocked the site for presentation of the antigenic peptide, which is presented to the responding T cell population as an antigenic peptide-MHC class II complex. Inhibition of the proliferative response of PBM induced by an antigenic stimulus was reported after depletion of MHC class II expressing cells. Reduction in proliferative response induced by ovalbumin was reported in the presence of an anti-MHC class II MAb (Hooper *et al.*, 1987; Glass and Spooner, 1989; Fitzpatrick *et al.*, 1995). The proliferative response in the current study, although reduced, was not abrogated as reported by others in mice (Hooper and Taylor, 1987) and cattle (Fitzpatrick, 1992). Incomplete blocking of the available MHC class II binding sites may have arisen due to insufficient anti-MHC class II MAb being present. The addition of PBM, *S. aureus* strain A and anti-MHC class II MAb simultaneously may have permitted a degree of antigenic processing to occur before the anti-MHC class II MAb could block all the MHC class II sites on present on the APC present. The culture of PBM and anti-MHC class II MAb prior to the addition of *S. aureus* strain A, may have resulted in complete inhibition of the proliferative response as reported previously by others.

Variation in proliferative response among individuals can be influenced by age, sex, breed, stress, health status and genetic background (Schalm *et al.*, 1976; Kristensen *et al.*, 1982). The variation in the proliferative response observed among progeny groups in the current study suggested that genetic control plays an important role in the proliferative response induced by *S. aureus*. The proliferative response of PBM isolated from Holstein-Friesian cows sired by two different bulls, induced by *S. aureus* strain A showed statistically significant differences ($p=0.03$) in proliferative responses when measured on the day of peak proliferation, day nine. The significant difference in proliferative response induced by *S. aureus* strain A between the two progeny groups suggests that genetics associated with immune function can influence the magnitude of the proliferative response induced. The immune system is the most important factor affecting disease resistance and susceptibility (Nonnecke and Harp, 1989). Expression of MHC molecules

on cells which form part of the adaptive immune system have been shown to have a pronounced effect on resistance to infectious agents. This has been reported in mice in relation to antibody production (Biozzi *et al.*, 1974). Five, bi-directional, selective breedings were carried out to characterise antibody production (Biozzi *et al.*, 1979). After several generations of selection, high and low responder lines, which differed in antibody production by 80-200 fold, were obtained. The genetic control demonstrated in these lines of selected mice was shown to span unrelated antigens, not just those used in the experimental design (Biozzi *et al.*, 1974; Siqueira *et al.*, 1977; Heumann and Stiffel, 1978). The variation in antibody production between the two lines of mice was estimated to be a result of ten independently segregated loci with additional additive effects (Feingold *et al.*, 1976). A genome-wide mapping search (Puel *et al.*, 1995), confirmed the involvement of *Igh* and MHC linked genes (Lieberman *et al.*, 1972; Vidard *et al.*, 1990) located on chromosomes 12 and 17 respectively. The results also highlighted the presence of immunomodulatory genes. These immunomodulatory genes were located on five distinct chromosomes (2, 4, 6, 8 and 10), (Puel *et al.*, 1995). Biozzi mice with high antibody production after H-2 typing were suggested to express K^d, D^q with the class II MHC derived from an unknown haplotype (Colombani *et al.*, 1979). This has also been reported in chickens in relation to Marek's disease, where a specific haplotype, B^{21}, seems to convey resistance to Marek's disease in chickens of many different genetic backgrounds (Lamont, 1998). As described in Biozzi mice, the contribution of non-MHC alleles, linked to antibody responses, with MHC alleles, linked to resistance to Marek's disease may affect susceptibility/resistance of lines of chickens to Marek's disease (Pinard and Vanderzijpp, 1993). An association between the MHC and resistance/susceptibility to mastitis has also been reported in cattle (Solbu, 1983; Larsen *et al.*, 1985). Susceptibility to clinical mastitis has been reported previously in cattle with respect to BoLA class II DQ^{1A} haplotype (Lunden *et al.*, 1990). This DQ haplotype was measured in sires and susceptibility to clinical mastitis was recorded in their daughters. Previously published studies in heifers have shown that those animals carrying the class I combination W8/W20 had lower SCC than those animals without this combination (Meyer *et al.*, 1989). Aarestrup *et al.* (1995) reported that allele A19 (A6) was associated with a decreased likelihood of isolation of *S. aureus*. Conversely allele A31(A30) was associated with an increased likelihood of isolation of *S. aureus*.

The initial results from the progeny groups led to the hypothesis that the proliferative response induced by *S. aureus* in cows may also be measurable in bulls. Initially, ten bulls were selected on the basis of extreme PTA for SCC, five bulls with the highest PTA for SCC (mean 13.71+/-3.45) and five bulls with the lowest PTA for SCC (-9.33+/-3.06). It was anticipated that selection of extreme bulls would provide the greatest chance of detecting a difference in proliferative response induced by *S. aureus*. The kinetics of the response induced by *S. aureus* in bulls was similar to that previously reported in cows. The proliferative response of PBM isolated from bulls induced by *S. aureus* strain A, could suggest the kinetics of a primary response. Kinetics of a primary response would be reasonable as recognition of *S. aureus* via the same route as the cows i.e., the mammary gland, would not be possible in bulls. Bulls may have had previous exposure to *S. aureus* via a number of anatomical sites, such as the skin and, therefore, secondary immune responses would also be possible. The restriction digest patterns of *S. aureus* strains isolated from different sites in cows has not been investigated to date and no work has been performed in bulls. It is, therefore, not possible to state if the *S. aureus* strain A used in the current study is solely an intramammary gland isolate, or whether it is widespread on other sites on cattle and throughout the country. Variation in the magnitude of the proliferative response induced by *S. aureus* was noted in bulls, with some animals generating a stronger proliferative response of PBM induced by *S. aureus* than others. Bulls with a low PTA for SCC generally had a stronger proliferative response induced by *S. aureus* than bulls with a high PTA for SCC. The results showed that the responses of the two bull groups were not dichotomous but bimodal and overlapping, in that one of the five bulls with low PTA for SCC had a weak proliferative response induced by *S. aureus* strain A, while one of the five bulls with high PTA for SCC had a strong proliferative response induced by *S. aureus* strain A. The results also indicated that there was a highly significant negative correlation between the log SI and PTA for SCC. A negative correlation means that as the PTA for SCC reduces, the proliferative response induced by *S. aureus* strain A increases. These results suggest that the measurement of the proliferative response of PBM may be used as a possible marker for identifying bulls that have an improved response to mastitis pathogens, in the case of the current study, *S. aureus*. This may allow identification of bulls that could be selected for breeding programs based on the potential to improve their progeny's ability to respond to mastitis pathogens. The rationale for this method of selection is that it has been shown

that the incidence of clinical mastitis was higher during the first and second lactations among daughters of sires that transmitted higher somatic cell score (SCS), (Nash *et al.*, 2000). While daughters of sires that transmitted lower SCS had fewer incidences of mastitis (Nash *et al.*, 2000). The increased incidence of mastitis in first lactation cows was reflected throughout the herd life of those cows (Grootenhuis, 1981). It has been suggested that selection of cows with very low SCS may result in cows that were unable to respond to infection (Schukken *et al.*, 1988; Kremer *et al.*, 1993). Very low SCC translate to very low levels of leucocytes present in the mammary gland. These cells are present in the mammary gland to combat bacterial pathogens, which have managed to evaded the innate immune system, therefore, removal of these cell types may predispose cows to a higher risk of clinical mastitis (Suriyasathaporn *et al.*, 2000). Others have reported a linear association between clinical mastitis and SCS which does not support the theory that selection of cows for very low SCS will produce cows that are unable to respond to infection (Philipsson *et al.*, 1995; Rogers *et al.*, 1998; Nash *et al.*, 2000).

The association shown between the PTA for SCC and the immune response induced by *S. aureus* in the extreme bulls led to a more extensive study to examine if this association was maintained among bulls across the range of PTA for SCC. A total of 103 bulls were chosen at random for the current study. The mean PTA for SCC in 1998 was 4.8+/-6.0, and the mean proliferative response (+/-SD) induced by *S. aureus* strain A was 9.1+/-16.0 when tested on the day of peak proliferation. Results from the current study showed no correlation between PTA for SCC and immune response in the 103 bulls chosen from the population at random. During the course of the current study, the PTA for SCC changed considerably for the 103 Holstein-Friesian bulls. As young bulls generated more daughters through progeny testing, the reliability of the estimate of PTA for SCC increased, as more lactational records were included. For daughter's lactational records to be included, a minimum of six individual monthly SCC for a single lactation must be available (Mrode and Swanson, 1996). The estimate of PTA for SCC does not, however, take into account daughters that have been culled or excluded from monthly SCC measurements possibly due to chronic infection or persistently high SCC. Exclusion of those daughters culled as a result of persistently high SCC or chronic infection will effect the estimate of PTA for SCC for an individual bull resulting in an underestimation of PTA for SCC. As the estimate for PTA for SCC is based on monthly lactational SCC,

elevated recordings are more likely to represent infection due to contagious pathogens rather than environmental pathogens, as the latter usually result in transient increases in SCC rather than chronic elevation. The data in the current study was re-analysed on a number of occasions to account for the PTA for SCC being updated twice yearly by the Animal Data Centre. With the implementation of EC legislation regarding the levels of somatic cells in milk and the heavy financial penalties imposed by milk purchasers, farmers are eager to remove cows that have persistently elevated SCC and those with chronic IMI as a result of contagious pathogens from their herds. Bull breeding companies will also be eager to remove bulls that are likely to pass on an increase in SCC to their daughters from their breeding programs. The removal of bulls with the highest PTA for SCC should narrow and reduce the range of PTA for SCC in bulls. Even when the changing PTA for SCC data was taken into account, there was still no correlation between PTA for SCC and log SI. It is possible, therefore, that the *in vitro* proliferative assay developed in the current study might be useful in potentially detecting the very good or very poor bulls, rather than being able to rank all potential breeding bulls in terms of PTA for SCC. Another possibility for the failure to correlate immune response and PTA for SCC in the randomly selected bulls is that PTA for SCC will reflect infection with organisms other than *S. aureus*. Alternatively, the trait being measured may be so complex and reflect infection with such a wide range of organisms that the power and design of the current study is insufficient to detect any association, even if one is present. The ability to eliminate extremely poor bulls from progeny testing programs at an early stage would be of considerable benefit to the breeding industry. Early culling of bulls, which are undesirable due to increased prevalence of disease in their progeny would create considerable economic saving on progeny testing in addition to improving the overall health of the future dairy herd in the UK.

4.2 Phenotyping of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were isolated from healthy lactating Holstein-Friesian dairy cows by centrifugation using a medium of density 1.077g/l, that used most frequently for isolation of these cells in cattle by others (Boyum, 1968; Wilson *et al.*, 1986; Schuberth *et al.*, 1996; Shafer-Weaver and Sordillo, 1997; McCole *et al.*, 1998; Asai *et al.*, 1998). Although many studies have utilised flow cytometry to analyse differences in cell subpopulations by identification of cell surface markers, there is little information available in the available literature that defines a time period for fixation and sample analysis. The time period should provide a result where the cells identified represent accurately the cells present *in vivo*. For the purpose of the current study, initial fixation of the PBM was carried out as soon as possible, and no later than ten hours after the blood sample was collected. Results from the current study (not reported) showed that storage of blood for a 24 hour period prior to cell labelling reduced the level of expression of cell surface markers and reflects those findings previously reported in humans (Weiblen *et al.*, 1983; Ashmore *et al.*, 1989). There are, however, published studies that have found differently, reporting that overnight storage of human whole blood did not affect lymphocyte surface marker expression (Shield *et al.*, 1983; Nicholson *et al.*, 1984; Prince and Arens, 1986). Lloyd *et al.* (1995) reported that sheep whole blood could be stored for up to 48 hours without significantly affecting the cell surface expression of CD4, CD5, CD8 or the B cell marker. For the purpose of the current study, flow cytometry was carried out on stained, fixed, PBM as soon as possible, and no later than two days post fixation. Previously published work in sheep reported that stained, fixed cells stored at 4°C, showed no significant reduction in cell surface marker expression after 24 hours. However, the same study reported a significant reduction in cell surface marker expression of CD5, CD8, B cell marker, and γδ T cell markers after a period of seven days (Lloyd *et al.*, 1995).

From the phenotyping studies carried out on PBM of cows, the mean percentage (+/-SD) of cells expressing CD4 was 26+/-8%, CD8 was 16+/-10%, γδ was 7+/-4%, the B cell marker was 17+/-8%, the monocyte marker was 38+/-17% and the MHC class II marker was 34+/-9% of the PBM population, which are similar to those reported previously in cattle (Taylor *et al.*, 1994).

The mean percentage (+/-SD) of $CD4^+$ T cells in peripheral blood was found to be 26+/-8% of the PBM population in the current study. The percentage of $CD4^+$ T cells has been reported previously to range from 15-35% of the PBM population depending on the age of the animal (Bensaid and Hadam, 1991). Others have reported the percentage of $CD4^+$ T cells to range from 23-42% of the PBM population (Harp *et al.*, 1991; Shafer-Weaver *et al.*, 1999; Soder and Holden, 1999).

The mean percentage (+/-SD) of $CD8^+$ T cells in peripheral blood was found to be 17+/-8% of the PBM population in the current study. The percentage of $CD8^+$ T cells in adult cattle has been reported previously as ranging from 11-22% of the PBM population (Harp *et al.*, 1991; Taylor *et al.*, 1994; Shafer-Weaver *et al.*, 1999). The percentage of $CD8^+$ T cells in the current study varied considerably among cows with one cow expressing CD8 on 41% of the stained PBM population. The elevated levels of $CD8^+$ T cells may have simply reflected an extreme variation in this cow. None of the cows at the time of sampling showed any visible signs of illness or were suffering from an intramammary infection, however, the elevated levels of $CD8^+$ T cells may have been due to infection that was not detectable clinically in this individual animal.

The mean percentage (+/-SD) of T cells expressing the γδ marker in peripheral blood was found to be 7+/-4% of the PBM population in the current study. The percentage of T cells expressing the γδ marker has been reported previously to range from 5-15% of PBM (Davis *et al.*, 1996). Other authors reported that 10+/-2% (Soder and Holden, 1999), or 8+/-1% (Taylor *et al.*, 1994) of PBM expressed the γδ T cell marker, supporting the results of the current study. All of the cows used in the current study were in at least their second lactation. In calves aged 1-2 weeks of age, γδ T cells have been reported to comprise 40-80% of PBM (Hein and Mackay, 1991; Wyatt *et al.*, 1994). The percentage of $\gamma\delta^+$ T cells in cattle has been reported previously to decline with age, with $\gamma\delta^+$ T cells accounting for 5-10% of PBM in adult animals of 5-8 years of age (Clevers *et al.*, 1990).

The mean percentage (+/-SD) of lymphocytes expressing the B cell marker was found to be 17+/-8% of the PBM population in the current study. The percentage of cells

expressing the B cell marker has been reported previously in cattle as 21% (Frunchtmann *et al.*, 1977), 31+/-4% (Shafer-Weaver *et al.*, 1999) and 35+/-2% (Taylor *et al.*, 1994), or ranging from 10-30% (Reeves and Renshaw, 1978; Yang, 1981) of the PBM population.

The mean percentage of peripheral blood cells expressing the monocyte marker was found to be 38+/-17% of the PBM population in the current study. The percentage of cells expressing the monocyte marker has been reported by others as 16+/-1% (Fitzpatrick *et al.*, 1995) or 20+/-2% (Shafer-Weaver *et al.*, 1999) of the PBM population. These published results are significantly different from those found in the current study. The primary MAb used in the current study was IL-A24, which has been reported to stain 94% of cells identified by morphology as peripheral blood monocytes (Ellis *et al.*, 1987). Of the ten cows in the current study, six of the animals were less than four years of age, while four animals were older than four years of age. In the older cows, the mean percentage (+/-SD) of cells expressing the monocyte marker was 19+/-8%, and in younger cows the mean percentage (+/-SD) of cells expressing the monocyte marker was 50+/-7%. These results suggest that the age of the cow may have an effect on the percentage of PBM expressing the monocyte marker.

The mean percentage of cells expressing the MHC class II marker was found to be 34+/-9% of the PBM population in the current study. The percentage cells expressing MHC class II has been reported previously in cattle as 22+/-2% (Soder and Holden, 1999), 43+/-3% (Fitzpatrick *et al.*, 1995) and 32+/-3% (Taylor *et al.*, 1994) of the PBM population, supporting the current work. Expression of MHC class II molecules on unstimulated PBM is present on monocytes, dendritic cells and B cells (Wiman *et al.*, 1976).

The stage of lactation has been reported previously to effect cell subpopulations expressing certain surface markers in the peripheral blood and mammary gland of cattle (Taylor *et al.*, 1994; Asai *et al.*, 1998). All of the cows sampled during the current study were lactating. The stage of lactation did vary among cows, ranging from just after parturition to a few days prior to drying off. None of the cows were sampled during the dry period. Variation in the percentage of peripheral blood lymphocytes during lactation has been reported as minimal (Taylor *et al.*, 1994). The percentages of $CD4^+$ and $CD8^+$ T

cells in peripheral blood however has been reported previously to be higher prior to calving compared to the period prior to drying off (Park *et al.*, 1992; Taylor *et al.*, 1994; Asai *et al.*, 1998). A reduction in the percentage of peripheral blood cells expressing CD3, CD4, CD8, and the γδ T cell marker has also been reported prior to calving with levels returning to normal after calving (Kimura *et al.*, 1999). The reduction in the percentage of cells expressing the γδ T cell marker in peripheral blood prior to calving was supported by Shafer-Weaver and Sordillo (1996), who reported levels of cells expressing the γδ T cell marker to be higher during lactation compared to at calving. Expression of MHC class II molecules on PBM was shown to be similar during lactation and prior to parturition (Shafer-Weaver and Sordillo, 1996). No obvious differences in the proportions of cell subpopulations identified in cows were seen in the current study, although the numbers of cows at various stages of lactation was too small for statistical analysis.

It was not possible, due to time constraints and low cell numbers, to examine all cell subpopulations in milk isolated from the cows in the current study. Cell isolation from milk requires large volumes of milk due to low cell concentrations, especially in normal, uninfected, milk. Cell isolation from infected milk can also be extremely difficult due to the presence of clots or pus, which trap the cells. During the current study, expression of CD4 on milk leucocytes was shown to be similar between cows with high SCC (>400,000 cells/ml of milk over a period of three consecutive months) and cows with low SCC (<150,000 cells/ml of milk at the time of sampling). Expression of CD8 on milk leucocytes was shown to be higher in cows with persistently high SCC compared to cows with low SCC. Variation in the percentage of cells expressing certain cell surface markers has been reported with stage of lactation in the bovine mammary gland (Asai *et al.*, 1998). The ratio of $CD4^+$ T cells to $CD8^+$ T cells has been shown to vary with the stage of lactation (Yamaguchi *et al.*, 1999). There are greater numbers of $CD8^+$ T cells than $CD4^+$ T cells during lactation (Park *et al.*, 1992), a situation which is reversed late on during the dry period when $CD4^+$ T cells predominate (Asai *et al.*, 1998). The overall percentage of $CD8^+$ T cells has been reported to remain constant throughout lactation, unlike the $CD4^+$ T cell population (Taylor *et al.*, 1994). Milk mononuclear cells from cows with persistently high SCC expressing MHC class II was reported in the current study as 39+/-21% and from cows with low SCC as 32+/-10%. Cows with consecutively

high monthly SCC records were chosen as persistently elevated SCC are characteristic of chronic intramammary infection, often due to infection with *S. aureus*. The proportion of cells expressing the MHC class II marker was lower in milk leucocytes compared to milk mononuclear cells in both groups of cows. The lower proportion of cells expressing MHC class II in the leucocyte population is not unexpected as this population contains neutrophils, which do not express MHC class II. The results of the current study do not show up-regulation of MHC class II expression in cows with high SCC compared to cows with low SCC. The increased percentage of $CD8^+$ T cells in cows with persistently high SCC may be one possible explanation for the apparent lack of up-regulation, with the increase in $CD8^+$ T cells suppressing the MHC class II/$CD4^+$ T cell response. Differences between the percentages of MHC class II expressing cells isolated from peripheral blood and the mammary gland has also been reported. The mean percentage of cells expressing the MHC class II marker in peripheral blood has been reported as 22+/-2% of PBM compared to 13+/-4% in the mammary gland (Soder and Holden, 1999). This value has also been reported as 32+/-3% of PBM and 2+/-0.17% in the mammary gland (Taylor *et al.*, 1994). The expression of MHC class II on milk leucocytes reported in the current study is higher than those published previously. The secretion of IFN-γ by activated T cells has been reported to up-regulate the expression of MHC class II on the surface of macrophages (Steeg *et al.*, 1982). Up-regulation of MHC class II was also reported on mammary epithelial cells after inoculation of formalin-fixed *S. uberis* into the gland (Fitzpatrick *et al.*, 1992). The expression of MHC class II described in the previously published studies was in non-infected glands, while in the current study, the cows were known to be suffering from an intramammary infection. The high level of expression of MHC class II reported in the cows with low SCC was shown to be due to one animal. This group was chosen based on the month prior to sampling lactational record and one possibility is that the cow with a high proportion of cells expressing MHC class II was suffering from a subclinical intramammary infection, which had not been detected clinically at the time of sampling. Immunological indicators, such as expression of surface markers on cell subpopulations, have been reported in a recent study to reflect intramammary infection and health status to the same degree or better than bacteriology or somatic cell counting (Rivas *et al.*, 2000). Although not investigated in the current study, cells expressing the $\gamma\delta$ T cell marker in cattle have been reported as higher in PBM (8+/-1%) when compared to the mammary gland (2+/-0.3%), (Taylor *et al.*, 1994). The

situation in cattle is in contrast to that of humans where human breast milk is relatively rich in cells expressing the γδ T cell marker compared to peripheral blood (Bertotto *et al.*, 1990).

The proportion of the various cell subpopulations identified in peripheral blood of cows during the current study suggested that these animals were capable potentially of generating an immune response if challenged. During the current study, only the proliferative response of PBM induced by antigenic stimuli was investigated rather than the effector functions that are responsible for removal of the pathogen, such as opsonisation, phagocytosis, or cell cytotoxicity. The proliferating PBM population induced by *S. aureus* strain A in cows in the current study was shown to comprise T cells expressing CD4 (53+/-8%), CD8 (21.3+/-5%) and γδ (13.3+/-4%) molecules. T cells expressing the CD4 cell surface marker are required for the development of humoral and cell mediated immunity, as well as the recruitment of neutrophils and non-specific phagocytic cells to the site of inflammation (Kimura *et al.*, 1999). T cells expressing the CD8 marker protect and elicit recovery from viral, certain bacterial and parasitic infections (Kimura *et al.*, 1999). Previously published studies on the functional properties of ruminant γδ T cells have been limited and inconclusive. γδ T cells have been identified as a predominant cell type that proliferates during autologous mixed leucocyte reactions (Goddeeris *et al.*, 1997), suggesting that they may recognise self-determinants expressed on the surface of damaged or stressed cells. γδ T cells may be involved in the defence against intracellular microbial infections, as well as the production of cytokines in response to antigenic challenge (Haas, 1993; Bluestone *et al.*, 1988). The protective role of γδ T cells in cattle associated with cytokine production has been supported by the positive correlation between the percentage of γδ T cells present and the secretion of IgM and IFN-γ (Ferrick *et al.*, 1995; Nonnecke *et al.*, 1997). These findings suggest that γδ T cells contribute to cellular and humoral immune responses of cattle. $\gamma\delta^+$ T cells in cattle that lack expression of WC1 represent a small proportion of PBM, but are more abundant in the spleen, mammary gland and mammary gland secretions (Wyatt *et al.*, 1994 & 1995; MacHugh, 1997). γδ T cells that express and lack the WC1 marker have been shown to be able to modulate an immune response directed against *S. aureus* and *Mycobacterium paratuberculosis* (Chiodini and Davis, 1992;

Brown *et al.*, 1994). γδ T cells are also found in large numbers in the skin, suggesting a role in the protection of epithelial surfaces (Mackay and Hein, 1989; Clevers *et al.*, 1990).

B cells are one cell type capable of acting as APCs. On receiving a second signal from the T cell, after effective presentation of antigen, B cells proliferate and differentiate into plasma cells (Butler, 1998). The maturation of B cells into antibody producing cells leads to the development of the humoral immune response (Morafo *et al.*, 1999). The percentage of B cells present in the peripheral blood of cattle has been reported as 20% of the lymphocyte population and similar to the numbers reported in mammary gland (Hellstrom *et al.*, 1976; Concha *et al.*, 1978). Reduced numbers of B cells in the mammary gland was reported in cows suffering from an intramammary infection (Yang *et al.*, 1987), suggesting that during an intramammary infection the development of an antibody mediated response may be impaired.

Monocytes present in peripheral blood are the precursors for tissue macrophages and participate in the development of a secondary immune response (Filion *et al.*, 1984; Glass and Spooner, 1989). T cell recognition of antigen requires interaction between the TCR, MHC and peptide fragments of antigen, which has been processed by the APC (Ashwell and Schwartz, 1986; Glass and Spooner, 1989). For development of specific immune responses, the expression of MHC class II cell surface markers on antigen presenting cells is of key importance (Petroff *et al.*, 1997). Expression of MHC class II is a requirement for T cells to recognise antigenic peptide presented on the surface of the APC.

The presence of a cell population, lacking all identifiable cell surface markers, has been reported in the peripheral blood of different species. These cells, termed null cells, have been reported to range from 20-40% of the PBM population of cattle (Wilson *et al.*, 1986). They have also been reported to represent 5-10% of mouse spleen cells and human peripheral blood lymphocytes and up to 50% of peripheral blood lymphocytes of sheep (Kristensen *et al.*, 1982). Functionally, these null cells have been ascribed the same activity as NK cells (Kiessling and Haller, 1978; Roder *et al.*, 1980). Natural killer cells are known to produce cytokines that regulate the immune response, and are themselves regulated by the cytokines produced during an immune response (Fearon and Locksley, 1996). Natural killer cells contribute to early regulatory signals as well as effector

functions during an immune response (Raymond and Wilkie, 1998). The NK population isolated from Yorkshire pigs have been described as non-granular, non–adherent cells (Pinto and Ferguson, 1988) expressing CD2 as well as in some cases CD8 (Pescovitz *et al.*, 1988; Saalmuller and Reddenase, 1988). It has been suggested that null cells may participate in a cytotoxic mechanism, Spontaneous Cell Mediated Cytotoxicity (SCMC), (Leibold *et al.*, 1979), which has been reported previously in pigs, cattle and horses (Leibold *et al.*, 1980).

Migration of cells from peripheral blood to the mammary gland can be measured by the rise in the number of somatic cells recorded in milk (Nickerson, 1985). The link between peripheral blood and the mammary gland can also be demonstrated by the presence of IgG in milk. Immunoglobulin G, the major Ig class in milk (Norcross, 1977), is not produced locally within the mammary gland but undergoes selective transfer from serum (Murphy *et al.*, 1964; Pierce and Feinstein, 1965). Further demonstration of the transfer of cells between the mammary gland and peripheral blood was reported when cattle inoculated via the intramammary route with bovine rotavirus showed detectable levels of neutralising antibodies and antigen specific lymphocytes in the peripheral blood (Castrucci *et al.*, 1984; Archambault *et al.*, 1988). Migration of antigen-specific lymphocytes from peripheral blood to the mammary gland has also been reported in rats (Seelig, 1980). In non-ruminant species a functional link between mammary and gut associated lymphoid tissue has been described (Evans, 1980; Kortbeek-Jacobs and Van der Donk, 1981). In cattle species mammary gland lymph node lymphocytes were shown to localise to the mammary gland lymph node, the node of origin, and to the prescapular node, the peripheral node. However, only a very small proportion of mammary gland lymph node lymphocytes that were radiolabelled were detected in the intestinal mesenteric node (Harp *et al.*, 1988). The localisation of mammary gland lymph node lymphocytes to the node of origin and the prescapular node but not to the intestinal mesenteric node has also been reported in sheep (Harp and Moon, 1987). These results indicate that the entero-mammary link described in non-ruminants is not as functional in ruminants (Chang *et al.*, 1981; Moon and McDonald, 1983).

The isolation of PBM by density centrifugation was used again in the current study to identify cells expressing CD4, CD8, the γδ T cell marker, the B cell marker, the monocyte

marker and the MHC class II marker in Holstein-Friesian bulls. The results from the phenotyping studies showed the mean percentage (+/-SD) of peripheral blood cells expressing CD4 was 17+/-3%, CD8 was 7+/-3%, the γδ T cell marker was 1+/-0.5%, the B cell marker was 10+/-3%, the monocyte marker was 0.5+/-0.5%, and the MHC class II was 32+/-12% of the PBM population of bulls. Results from the current study showed that the percentage of peripheral blood cells expressing the various cell surface markers was not significantly lower in the PBM population of bulls than those previously reported in cows, with the exception of expression of the monocyte marker. However, the difference in the proportion of cells expressing CD4 and the γδ marker approached significance. Unfortunately there have been very few published studies on the expression of cell surface markers of PBM of Holstein-Friesian bulls with which to compare the results obtained in the present study. A study by Nonnecke *et al.* (1997) was carried out on PBM of young Holstein bulls that had reached sexual maturity. The MAbs used in this study to identify PBM for various cell surface markers were different from those used in the current study. In the study by Nonnecke *et al.* (1997) the mean percentage (+/-SD) of PBM expressing the B cell marker was 22+/-3%; expressing the MHC class II marker was 48+/-3% and expressing the γδ T cell marker was 16+/-3%, which were all higher than those of the current study. A possible explanation for the difference in the level of γδ T cells may be due to the age of the bulls studied. The age of the bulls is of relevance as γδ T cells have been shown to diminish in number in the peripheral blood of cattle as they mature (Wyatt *et al.*, 1994; Davis *et al.*, 1996; Soder and Holden, 1999). The bulls sampled during the current study were mature bulls all over the age of four years. The bulls sampled by Nonnecke *et al.* (1997) were described as sexually mature. Bulls are reported to be physiologically mature in terms of reproduction as early as nine months of age. The MAb used in the current study to stain PBM expressing the γδ T cell marker in cattle has been used previously by others, although not in bulls (Clevers *et al.*, 1990; Morrison and Davis 1991; MacHugh *et al.*, 1997). No comparison of the percentage of PBM expressing the monocyte marker can be made, as expression of this cell surface marker was not examined by Nonnecke *et al.* (1997). The results of the percentage of PBM expressing CD4 and CD8 were not published by Nonnecke *et al.* (1997). The expression of CD3, a cell surface marker present on all T cells, was shown to be 48+/-5% of PBM. The CD3 molecule is required for T cell development as well as the assembly

and efficient expression of the TCR (Exley *et al.*, 1991; Tanaka *et al.*, 1995). Cells expressing CD3 would include $CD4^+$ T cells and $CD8^+$ T cells. The combined percentage of $CD4^+$ and $CD8^+$ T cells in the current study accounted for 24+/-6% of PBM, which is below that reported previously by Nonnecke *et al.* (1997). As no MAb was used to detect the expression of the cell surface marker CD3 in the current study, differences between CD3 and CD4/CD8 expression on PBM cannot be compared. $CD4^+$ and $CD8^+$ T cells both express an αβ TCR, while CD3 expression has been associated with both αβ and γδ TCR (Bank *et al.*, 1986; Van Neerven *et al.*, 1990).

On comparison of the percentage of cell subpopulations present in PBM of cows and bulls, the mean percentage (+/-SD) of PBM expressing CD4 was noted to be lower in bulls (17+/-3%) than in cows (26+/-8%), as mentioned previously this result was not statistically significant. A reduction in the mean percentage (+/-SD) of PBM expressing CD8 was also noted in bulls (7+/-3%) compared to cows (16+/-10%). Furthermore, a significant reduction in the mean percentage (+/-SD) of PBM expressing the monocyte marker was noted in the current study in bulls (0.5+/-0.5%) compared to cows (38+/-17%). Differences in MAb used to identify cell surface markers are apparent among the previously published studies. The MAb IL-A24, used in the present study, has been shown to stain 94% of cells identified by morphology as peripheral blood monocytes (Ellis *et al.*, 1987). For consistency, all the MAb used to study the different cell subpopulations of PBM of cows were the same as those used to study the different cell subpopulations of PBM of bulls. The differences in the percentage of cell expressing the various cell surface markers reported by the current study may represent differences in biological factors including, age, sex or genetics rather than the methodology used to isolate and stain the cells. One possible explanation for the lower proportion of cells expressing the various cell surface markers in bulls compared to cows is the presence of null cells, which lack cell surface markers to identify cell subpopulations by flow cytometry. These null cells may form a greater proportion of PBM in bulls than in cows.

To try and explain the differences in the percentage of stained cells reported in the current study for PBM of bulls, media of different densities were used to isolate PBM, to investigate if cells were being lost during density centrifugation. Other studies have reported using ficoll-isopaque of density 1.083g/l (Naessens *et al.*, 1990; Asai *et al.*,

1998). The use of whole blood (Naverro *et al.*, 1996), or whole blood with erythrocyte lysis (Davis *et al.*, 1996; Sopp and Howard, 1996) or centrifuged whole blood with the layer of mononuclear cells removed without the use of a density medium (Kimura *et al.*, 1999) have all been reported in flow cytometry studies.

Results from this study indicated that the mean percentage (+/-SD) of all cell subpopulations studied were lower in the PBM isolated by the media of greater density when compared to the density media routinely used, indicating that mononuclear cells were not being lost into the pellet on centrifugation.

To investigate the possible benefits of the whole blood isolation technique, as described by others (Naverro *et al.*, 1996; Davis *et al.*, 1996; Sopp and Howard, 1996), a comparison was made between the percentage of stained cells isolated from bulls by centrifugation with media density 1.077g/l, as used routinely, and the percentage of stained cells isolated from whole blood treated to remove the erythrocyte population. The results of the current study showed that cells isolated by density centrifugation had a higher percentage of cells expressing CD4, CD8, the B cell marker and the MHC class II marker when compared to those isolated using the whole blood with erythrocyte lysis technique. The population of cells isolated from whole blood following erythrocyte lysis still contains neutrophils, which would proportionally reduce the percentage of cells expressing the various cell surface markers when compared to PBM. Interestingly the percentage of cells expressing the γδ T cell marker was unaffected by the centrifugation media used. A possible explanation for this may be that some cells expressing γδ are lost due to density centrifugation with media 1.077g/l and, therefore, can only be identified when whole blood analysis is carried out.

4.3 Restriction Fragment Length Polymorphism enzymatic digestion of MHC class II DQB and DRB exon 2 Polymerase Chain Reaction products

Development of an immune response induced by pathogens is a complex mechanism. One essential step in this pathway involves recognition of processed peptides by T cells. Recognition of antigenic peptides expressed on the surface of APC by T cells must be in association with an appropriate MHC molecule (Klein, 1986).

The MHC of vertebrates comprises class I and class II genes, which encode cell surface proteins with a central function in the immune system (Klein, 1975; Zinkernagal and Doherty, 1979; Nagy *et al.*, 1981). Experiments performed in mice, which were designed to study antibody production induced by natural immunogens, demonstrated that after several generations, mice could be segregated based on high or low antibody production (Biozzi *et al.*, 1979). The trait of antibody production was shown to be under polygenic control, and that the genetic control exhibited in differing levels of antibody production was shown not to be restricted to the original immunogens tested. The difference between high and low antibody production in these selectively bred mice was shown to involve MHC linked genes (Stiffel *et al.*, 1977; Vidard *et al.*, 1990).

Definition of the MHC class II region is important for studying T cell responses induced by antigen. In vaccine development, knowledge of the candidate species MHC genes is needed for successful vaccine development (Glass *et al.*, 1991; van Lierop *et al.*, 1995). The recognition of foreign antigen or a subunit of a vaccine in association with self MHC class II molecules on the surface of APC is a known requirement for T helper cell activation (Schwartz *et al.*, 1986). The effect of MHC class II polymorphisms can be used to study the T-cell response to peptides after vaccination in an outbred population (van Lierop *et al.*, 1995). Definition of the MHC class II region has also been reported as important in the progression of MHC disease association studies, where resistance and susceptibility to persistent lymphocytosis has been reported previously to be associated with BoLA MHC class II genes (Xu *et al.*, 1993). In particular the association with DRB-linked resistance has been shown to be dominant, whereas susceptibility is inherited as a complex recessive trait (Wu *et al.*, 1989). The ability of an MHC class II molecule to bind peptide and subsequently form a complex with an appropriate T cell is determined

by the structural variation of the MHC molecule (Buus *et al.*, 1987). These structural variations, termed polymorphisms, are characterised by a large number of alleles at each locus and a large number of amino acid substitutions between alleles (Ellegren *et al.*, 1993). The antigen-binding site is encoded within exon 2 of the respective MHC class II α and β genes (Brown *et al.*, 1993). Polymorphisms in exon 2 have been reported to affect the range of antigenic peptides that can be bound and presented, making exon 2 particularly appropriate to study the presence and function of immune response genes.

Major Histocompatibility Complex mediated susceptibility to infectious disease has been associated with a low responder status to immunodominant pathogen derived peptides (Braciale *et al.*, 1989). The low responder status may correlate to holes in the T cell repertoire created during T cell education within the thymus (Lewin *et al.*, 1999). Major Histocompatibility Complex associations in outbred species, while although well documented, few are understood clearly at the physiological level (Lewin *et al.*, 1999). Inter-haplotype interactions may contribute to sources of functional polymorphism in MHC molecules, especially in the DQ isotype. The α and β regions of the DQ isotype are extremely polymorphic. This may complicate the analysis of MHC function or disease association and explain the complex patterns of reactivity observed for some T cell clones (Glass and Millar, 1994). The genomic arrangement of the MHC class II α and β chains encoding genes consists of five and six exons respectively. Exon 2 of DRB3 and DQB represents the extracellular domain of the MHC class II genes (Lewin *et al.*, 1999). The α1 and β1 domains comprise the antigen-binding site and are encoded within the extracellular region. In contrast to MHC class I antigen binding sites, the MHC class II sites have conserved residues distributed throughout the antigen binding site (Rudensky *et al.*, 1991; Hunt *et al.*, 1992). Amino acid substitution, which results in sequence variability, occurs almost exclusively within exon 2 of the MHC class II genes (Rask *et al.*, 1991). Amino acids possess an electrical charge, which is either positive or negative; thus, an amino acid substitution may alter the polarity of the antigen-binding site. Alteration in polarity may result in peptides with like charges to the antigen-binding site being repelled. Changes in amino acid sequence in the region of the antigen-binding site have been linked to resistance and susceptibility towards Bovine Leukaemia Virus (BLV), (Stern *et al.*, 1994).

At present, a bull's potential as a sire is measured by his daughters' phenotype. This method of progeny testing which is routinely carried out by breeding companies is both time consuming and expensive, as well as potentially producing many daughters that may express undesirable traits including susceptibility to disease. In comparison to progeny testing, MHC alleles expressed in cattle can be measured accurately and relatively cheaply by a variety of established laboratory techniques early in life (Bernoco *et al.*, 1991; Davies *et al.*, 1993). A genetic marker, which may predict animals that are resistant/susceptible to disease, would be of benefit in the control of IMI in dairy cattle (Aarestrup *et al.*, 1995). As the husbandry and management control measures currently available on farms are insufficient to reduce adequately the incidence of IMI, the effects of selection of superior sires has been suggested as a desirable control method (Nonnecke and Harp, 1989; Schukken *et al.*, 1994). To date, genetic selection for improved resistance to disease has concentrated on those diseases for which vaccine development has been unsuccessful or where the development of a vaccine is not economically viable, rather than in the direction of improved udder health (Lewin, 1996). Further development of the current study involved investigation of the locus encoding the MHC class II DRB and DQB genes.

Typing of BoLA class II has been successfully performed using various techniques (Bernoco *et al.*, 1991) including mixed lymphocyte cultures (Davis and Splitter, 1991), serology (Emery *et al.*, 1987), immunoprecipitation with isoelectric focusing (Watkins *et al.*, 1989; Glass *et al.*, 1992), DNA probes (MuggliCockett and Stone, 1988), PCR-RFLP (Van Eijk *et al.*, 1992) and ultimately DNA sequencing (MuggliCockett and Stone, 1989; Sigurdardottir, 1991). DNA sequencing is the gold standard in defining genomic alleles (Aldridge *et al.*, 1998), however the technique is laborious and not practical for studying whole populations (Groenen *et al.*, 1989; Sigurdardottir, 1991). Even with the development of new techniques for the typing of BoLA-DRB3 exon 2, the technique of PCR-RFLP is still used routinely (Dietz *et al.*, 1997a; Sharif *et al.*, 1998a; Gilliespie *et al.*, 1999; Aravindakshan *et al.*, 2000). Polymerase Chain Reaction-RFLP is a powerful and sensitive technique, permitting a high level of allele identification with the additional benefits of being non-radioactive and having high throughput efficiency (Van Eijk *et al.*, 1992).

In the current study, PCR amplification of BoLA DQB exon 2, and blunt end cloning of three bulls with low PTA for SCC and one bull with a high PTA for SCC, generated a total of four allelic sequences, three of which had been reported previously (Nasir *et al.*, 1997), and one novel allelic sequence. Blunt end cloning of DQB exon 2 demonstrated frequent isolation of allele DQB2*1-3. The frequent isolation of this allele may have been due to a low degree of polymorphism within one locus of the BoLA DQB region (Nasir *et al.*, 1997), as DQB polymorphisms have been reported to be restricted to the second DQB locus in humans (Berdoz *et al.*, 1989). Polymerase Chain Reaction-RFLP was used subsequently as a rapid typing method to improve detection of DQB alleles. The results of the current study demonstrated that following enzymatic digestion of DQB exon 2 by PCR-RFLP, the patterns although relatively clean, were generally too complex to be resolved unambiguously as previously recognised allelic patterns. The lack of definition of polymorphisms at the DQB loci has been reported previously (Davies *et al.*, 1994). The number of bands generated by enzymatic degradation of DQB exon 2 suggested the presence of multiple alleles in most animals studied, characteristic of gene duplication (Russell, 2000, personal communication). The suggestion of gene duplication in the current study was supported by further cloning and sequencing, which showed the presence of three distinct alleles in one individual bull. Gene duplication has been reported previously to effect approximately half of the common class II haplotypes in European cattle (Andersson and Rask, 1988; Sigurdardottir *et al.*, 1992). It is believed that the DQB1 gene is present in all haplotypes, whereas DQB2, DQB3 and DQB4 are present only in duplicated haplotypes (Sigurdardottir *et al.*, 1992). The DQB3 and DQB4 genes are the most divergent, with DQB3 exhibiting multiple substitutions at the conserved positions in the β-chain genes (Lewin *et al.*, 1999). It has been reported previously that a high level of linkage disequilibrium exists between DQ and DR polymorphisms, which is a non-random association often linked with selection (Sigurdardottir *et al.*, 1991).

Development of the current study resulted in the DRB locus exon 2 being investigated. Of the three DRB genes only DRB3 is expressed at high levels on cells in peripheral blood (Burke *et al.*, 1991). The DRB3 locus in cattle, unlike humans, is well-conserved (Sigurdardottir *et al.*, 1991). The level of conservation exhibited makes the DRB3 locus in cattle possibly the most important in terms of function (Ellegren *et al.*, 1993). The

rationale behind the investigation of DRB3 alleles in the current study was that this locus may potentially affect many traits that are linked to immunity, including SCC and mastitis incidence (Dietz *et al.*, 1997b). The alleles present in DRB3 exon 2, which contains the antigen binding site, may therefore be of importance when selecting for improved udder health (Starkenburg *et al.*, 1997).

A recent study reported a total of 63 alleles identified by PCR-RFLP in the DRB3 locus (Davies *et al.*, 1997). Results from the current study based on PCR-RFLP of DRB3 exon 2 of ten Holstein-Friesian dairy bulls identified 12 DRB3.2*types, namely DRB3.2*16, 23, 24, 8, 22, 3, 11, 27, 10, 32, 13 and 51. The results of the current study showed that DRB3.2*16 was present in five of the ten bulls, DRB3.2*23 was present in four of the ten bulls, DRB3.2*24 was present in three of the ten bulls, DRB3.2*8 was present in two of the ten bulls, DRB3.2*22 was present in two of the ten bulls, and DRB3.2*3, 11, 27, 10, 32, 13, and 51 were each only present in one of the ten bulls. On further consideration of the four DRB3.2*types that were present in more than one of the bulls, DRB3.2*16 was present in four of the five bulls with the lowest PTA for SCC and one of the five bulls with the highest PTA for SCC. DRB3.2*23 was present in one of the five bulls with the lowest PTA for SCC and was present in three of the five bulls with the highest PTA for SCC. DRB3.2*24 was present in two the five bulls with the lowest PTA for SCC and was present in one of the five bulls with the highest PTA for SCC. DRB3.2*8 was present in one of the five bulls with the lowest PTA for SCC and was present in one of the five bulls with the highest PTA for SCC. DRB3.2*22 was present in two of the five bulls with the highest PTA for SCC only. These five alleles were, therefore, the most frequent alleles recognised in the current study (Table 11).

The frequency of DRB3.2*16 has been reported in 835 Holstein cows as 0.09, DRB3.2*23 as 0.06, DRB3.2*24 as 0.19, DRB3.2*8 as 0.20 and DRB3.2*22 as 0.13 (Sharif *et al.*, 1998b). Others have reported the frequencies of DRB3.2*16 as 0.10, DRB3.2*23 as 0.09, DRB3.2*24 as 0.14, DRB3.2*8 as 0.14 and DRB3.2*22 as 0.14 (Dietz *et al.*, 1997a), or DRB3.2*16 as 0.06, DRB3.2*23 as 0.08, DRB3.2*24 as 0.08, DRB3.2*8 as 0.21 and DRB3.2*22 as 0.08 of DRB alleles identified (Kelm *et al.*, 1997).

A study of Holstein-Friesian cows in at least their second lactation, reported that DRB3.2*16 was associated with a significant reduction in SCS (Sharif *et al.*, 1998b). The data analysed in this study was collected from cows in herds across Ontario, where cases of mastitis were graded as either mild or severe. Mild cases were characterised by abnormal milk with no clinical signs. Severe cases were characterised by abnormal milk and systemic illness such as fever or anorexia. Bacteriological analysis of the isolates from mild and severe cases showed that the two groups presented different pathogen profiles. The causative agents of mild cases were identified as coagulase negative *Staphylococcus* species (24.1%), *Streptococcus* species (16.8%), *S. aureus* (9.5%) and coliforms (9.5%). In the severe cases the causative agents were identified as predominately coliforms (25.8%), *S. aureus* (17.2%) and coagulase negative *Staphylococcus* species (Scott *et al.*, 1996). Identification of the bacterial isolates from cases of mastitis is important as the influence of the BoLA alleles on the immune response may differ significantly among pathogens (Dietz *et al.*, 1997a). For example when analysing the affect of BoLA alleles on SCC, the differences between pathogens such as *S. aureus*, that results in chronically elevated SCC, and *E. coli* that results in transiently elevated SCC (Dietz *et al.*, 1997a) are important factors which must be considered. The results of the previously published study indicated that BoLA alleles had no statistically significant association with the incidence of mild cases of mastitis, however, a certain DRB3 allele (DRB3.2*23) was shown to significantly increase the incidence of severe cases of mastitis, as defined previously (Sharif *et al.*, 1998b).

There have been conflicting reports as to the effect of DRB3.2*16 on SCC used as an indicator of udder health. Dietz *et al* (1997a) reported that DRB3.2*16 was associated with an increase in the risk of elevated SCC in the second lactation, in addition to an increase in the susceptibility to intramammary infection by major pathogens in those animals suffering from acutely elevated SCC. The results of this study classified SCC as chronically elevated when SCC for three consecutive months was >300,000 cells/ml or if SCC for two consecutive months was >500,000 cells/ml. *Staphylococcus aureus* and *S. agalactiae* were named as causative agents for chronically elevated SCC, and *E. coli* as the causative agent of acutely elevated SCC. Dietz *et al.* (1997a) did, however, state that pathogens that induce a chronic elevation of SCC in some cows may result in acute elevation of SCC in others. Cases and controls in this study by Dietz *et al.* (1997a) were

matched to eliminate the effects of breed, herd, lactation number and days in lactation, all factors that affect SCC. In a study designed to measure the genetic association between measures of mastitis prevalence and genotypes for the MHC class II DRB3 locus, DRB3.2*16 was reported to increase the Estimated Breeding Value (EBV) associated with mastitis in cows in the first lactation (Kelm *et al.*, 1997). The increase in the EBV associated with DRB3.2*16 was undesirable as it resulted in an increase in the incidence of mastitis. An association between DRB3.2*16 and reduced numbers of circulating mononuclear cells has been reported previously (Dietz *et al.*, 1997b). Interestingly during the same study, DRB3.2*16 was reported to be associated with an increase in the levels of IgM and serum complement (Dietz *et al.*, 1997b). Interpretation of the results from this study suggests that expression of allele DRB3.2*16 lowers the number of mononuclear cells, which may be important for the development an effective immune response.

Thus, the findings of Kelm *et al.* (1997) and Dietz *et al.* (1997a) suggested that DRB3.2*16 was associated with an increase in SCS and SCC respectively, in contradiction to those of the current study, where it was found that four of the five bulls with low PTA for SCC generated a RFLP pattern associated with DRB3.2*16. However the findings of Starkenburg *et al.* (1998), and Sharif *et al.* (1998a, 1998b) support the results of the current study.

DRB3.2*23, present in one of the five bulls with low PTA for SCC and three of the five bulls with high PTA for SCC, was the second most frequent allele identified in the ten bulls investigated in the current study. DRB3.2*23 has been reported previously to be expressed at a relatively high frequency in Holstein cattle (Dietz *et al.*, 1997a). These researchers also reported within this study an association between DRB3.2*23 and an increase in SCC in cows in their second or greater lactation, this supports the results of the current study where three of the five bulls with the highest PTA for SCC expressed DRB3.2*23. Another published study in Holstein cows supported the results of Dietz *et al.* (1997b) in that DRB3.2*23 was associated with an increase in SCS and with an increase in the number of cases of mastitis (Sharif *et al.*, 1998b). Again, the results of this published study support those reported in the current study. The increase in the number of cases of mastitis was based on pooled samples that were not differentiated by

severity. With respect to DRB3.2*23, Dietz *et al.* (1997b) and Sharif *et al.* (1998b) agree that when DRB3.2*23 was detected, those cows had higher SCC during the second lactation. A possible association between DRB3.2*23 and an increase in placenta retention post calving has also been reported (Dietz *et al.*, 1997a), supporting the hypothesis that MHC alleles may be important in a number of diseases or disorders in cattle.

DRB3.2*24, present in two of the five bulls with the low PTA for SCC and one of the five bulls with the high PTA for SCC, was reported previously to be associated with susceptibility to clinical mastitis (Lunden *et al.*, 1990). It has been published previously that DRB3.2*24 was associated with a reduction in the EBV for SCS. It was also noted within the same study that DRB3.2*24 was associated with a significant increase in the EBV for IMI caused by major mastitis pathogens. It was suggested that DRB3.2*24 may result in susceptibility of the mammary gland to initial establishment of an IMI but conversely may result in resistance to the bacteria and its virulence factors once established within the mammary gland (Kelm *et al.*, 1997). As well as the effect on SCC and the incidence of clinical mastitis, DRB3.2*24 has also been reported to increase fat yield in cows during the first lactation (Starkenburg *et al.*, 1997). The effect on fat yield was not reproduced in any of the other yield traits examined, nor was any adverse effect on a yield trait reported. The association between DRB3.2*24 and clinical mastitis reported by Kelm *et al.* (1997) was not supported by other published studies (Starkenburg *et al.*, 1997; Sharif *et al.*, 1998b).

DRB3.2*8 was present in one bull with a low PTA for SCC and one bull with a high PTA for SCC. Within the Holstein cattle population DRB3.2*8 has been reported previously as the most frequently isolated allele (Sharif *et al.*, 1998b). DRB3.2*8 has been reported to be associated with an increase in SCC in first lactation Holstein cows (Dietz *et al.*, 1997a). DRB3.2*8 has also been associated with susceptibility to mastitis in Holsteins as a result of infection with the contagious pathogen *S. aureus* (Berryere *et al.*, 1994). Kelm *et al.* (1997) reported an association between DRB3.2*8 and an increase in the number or cases of clinical mastitis, a reduction in milk production and a reduction in protein yield. The results of Kelm *et al.* (1997) identifying traits associated with DRB3.2*8 were also supported by Starkenburg *et al.* (1997). DRB3.2*8 has been positively correlated with

serum IgM and *in vitro* IgM production, as well as serum complement (Dietz *et al.*, 1997b). DRB3.2*8 has also been reported to be negatively associated with circulating levels of mononuclear cells and antibody dependant neutrophil cytotoxicity but positively associated with blastogenesis induced by Concavalin A *in vitro* (Dietz *et al.*, 1997b). The positive association with blastogenesis was not reported with any of the other alleles examined in this study. As DRB3.2*8 was present in only one bull from each of the groups based on PTA for SCC in the current study further conclusions on its potential role in intramammary immunity can not be made. No alleles studied by Kelm *et al.* (1997) demonstrated a consistent directional effect on all measures of mastitis. The lack of consistency is indicative of the complexity of both the immune system and intramammary infections.

DRB3.2*22, present in one of the five bulls with the low PTA for SCC and one of the five bulls with the high PTA for SCC has been suggested previously to reduce the risk of elevated SCC during the second lactation (Dietz *et al.*, 1997a). As with other alleles mentioned conflicting results have been reported. DRB3.2*22 has also been associated with an increase in the number of cases of cows suffering from chronically elevated SCS during the first lactation (Starkenburg *et al.*, 1997). As DRB3.2*22 was present in only one bull from each of the groups based on PTA for SCC in the current study further conclusions on its potential role in intramammary immunity cannot be made.

Reasons for the reported differences in published studies have been linked to differences in experimental design i.e. selective breeding of cows to known sires compared to a natural selection based within commercial dairy herds (Sharif *et al.*, 1998b). Differences in the pathogens isolated from the cows sampled during the individual studies and the linkage of DRB3.2 to other BoLA loci may contribute to the differences between the published studies. The DQ and DR regions appear to be in strong linkage disequilibrium, with greater than 30 DQ-DR haplotypes identified (Sigurdardottir *et al.*, 1988). Genotyping of the BoLA DRB3 locus represents the influence of other closely linked loci. An association between component four of complement and BoLA has been suggested (Skow *et al.*, 1988). A polymorphism in component four may result in linkage disequilibrium with the DRB3 allelic state, which may alter serum complement levels. In mice, the genes controlling TNF-α and heat shock protein residing within other loci have

been linked to the MHC class II DRB locus (Grosz *et al.*, 1992). Further studies with large populations of cattle are required to determine the links between BoLA and the traits of innate and adaptive immunity (Dietz *et al.*, 1997b). The strong effects of the environment may influence the moderate to low heritability of the traits studied whereby temporal effects such as changes in the environment and pathogens within the mammary gland may have a substantial contribution to variation measured (Starkenburg *et al.*, 1997). Another possibility for the reported differences between published studies may be due to incomplete lactation records, which were used to calculate the EBV (Kelm *et al.*, 1997). One possibility for incomplete lactation records would be that cows had been selectively culled from the herd. Although selective culling of persistently infected or sick cows is regular practice within a commercial herd, it may bias the results presented in the current study. Selective culling would normally result in elimination of those cows suffering from chronic IMI usually as a result of contagious pathogens such as *S. aureus* and would thus means that PTA for SCC would be underestimated by the methods currently for calculation.

The complex aetiology of a multi-factorial disease such as mastitis may explain the reported inconsistency between published studies and the effects of certain BoLA alleles. Variation in the causative agent, genetic background and the criteria used for defining mastitis have been suggested as other possible reasons for inconsistencies in published studies (Sharif *et al.*, 1998b). Linkage between regions of the MHC may also be a possible explanation for inconsistencies in published studies, where the affect of alleles in a different region of the MHC will contribute to the effect of the allele being examined. Alleles within the DR and DQ region of the MHC class II are known to be linked and this association has been reported between DRB3.2*24 and the DQ^{1A} haplotype in cattle (Davies *et al.*, 1994).

Variation in allelic frequency has been shown to differ between breeds (Sharif *et al.*, 1998a). For example, DRB3.2*8, 3, 11, 16, 22 and 23 have been reported as representing 89% of the DRB3 allelic frequency in Holsteins (Dietz *et al.*, 1997a; Sharif *et al.*, 1998b). However in Jerseys, with the exception of DRB3.2*23, DRB3.2*3, 8, 11, 16 and 22 have not been identified (Sharif *et al.*, 1998b; Gillespie *et al.*, 1999). The difference in expression of DRB3.2*16 in Holstein-Friesian bulls with a strong proliferative response,

and the absence of DRB3.2*16 in bulls with a weak proliferative response induced by *S. aureus* strain A in the current study was shown to be statistically significant (p=0.00025.). The association with allele DRB3.2*16 and the proliferative response induced by *S. aureus* strain A cannot be classified as random, as a result of the paternal and maternal grandsire data reported in Table 12. From this information it can be seen that there is a crossover of gransires with a total of seven different grandsires resulting in the ten bulls chosen for this study based on their PTA for SCC.

Genetic selection of dairy cows rests heavily on improvement of production traits, for example milk yield, which is positively correlated with mastitis (Emanuelson *et al.*, 1988; Shook, 1989; Rogers *et al.*, 1998). Investigation into the possible association between DRB3 alleles and production traits (Sharif *et al.*, 1998b), suggested that in this particular study there was no association between DRB3.2*16 and production traits. The previously published studies where DRB3.2*16 has been reported to lower SCS indicates that the lack of association between DRB3.2*16 and production traits is beneficial. The lack of association in these previously published studies between DRB3 alleles and production traits means that selecting animals for improved udder health through identification of MHC alleles should not compromise milk production. During the current study the PTA for the production traits, milk yield, fat and protein percentage for the ten extreme bulls were also available for 1997. The results of the current study showed no statistically significant association between PTA for SCC and the production traits, milk yield, fat and protein percentage. The results of the current study also indicated that there was no association found between the proliferative response induced by *S. aureus* strain A or the presence of DRB3.2*16 and the PTA for production traits. Interestingly bull D, recorded as having a low PTA for SCC but a weak proliferative response induced by *S. aureus* strain A had the daughters with the lowest PTA for milk yield, fat and protein percentage. Unlike PTA for SCC, low PTA for production traits is undesirable, indicating the daughters have lower milk yields, fat and protein percentages. By comparison, only bull J, recorded as having the highest PTA for SCC of the ten bulls and a weak proliferative response induced by *S. aureus* strain A had daughters with PTA for production traits as low as bull D.

One bull with a high PTA for SCC, bull J, generated three pairs of restriction digest DRB pattern combinations. It was considered that double restriction digestion of the product created by PCR might help to clarify the RFLP patterns of this bull. The initial digest was carried out with *Bst* YI, which can on some occasions leave the PCR product undigested. The undigested product was cut from the gel and digested further with *Rsa* I. This potentially reduced the possible pattern combinations by half, creating a unique *Rsa* I pattern (Russell, 2000, personal communication). Of the possible combinations generated by bull J, DRB3.2*8 and DRB3.2*22 are the most likely candidates for these two alleles are the most frequently identified in American and Canadian Holstein-Friesian populations (Sharif *et al.*, 1998b; Dietz *et al.*, 1997a).

Improving disease resistance based on genetic selection does have potential pitfalls. The complex aetiology of diseases such as mastitis as well as the polygenic nature of the host's immune response, makes absolute resistance to all pathogens impossible to achieve (Lewin *et al.*, 1999). The combination of MHC alleles of an individual on a chromosome results in a haplotype. Haplotypes are derived from both parents, one paternal and one maternal, the combination of these two haplotypes creates a heterozygous genotype. During selection of genetically controlled traits it is important to maintain heterozygosity to promote long-term herd health (Lewin *et al.*, 1999). Breeding animals that are homozygous at the MHC loci would compromise the peptide binding repertoire of the immune system (Lewin, 1989). Genes out with the MHC region have also been reported to contribute to mastitis resistance and susceptibility (Teale, 1999), for example, genes controlling morphological features such as udder and teat conformation (Seykora and Daniels, 1991). What still remains unclear, however, is the precise nature of the important genes within, or linked, to the MHC region. This type of detailed research requires the entire haplotype associated with the resistance/susceptibility to be identified. A reduction in the ability of the host's immune system to recognise antigenic peptides may result in resistance to one particular disease but susceptibility to another (Starkenburg *et al.*, 1997). While in some studies alleles DRB3.2*16 and 22 have been associated with undesirable udder health they have also been associated with a statistically significant reduction in the risk of cystic ovarian disease (Sharif *et al.*, 1998b). No other alleles identified in this previously published study were reported to have a significant effect on the occurrence of cystic ovarian disease.

The effect of MHC genes on disease resistance and susceptibility has not only been associated with infectious bacterial pathogens. The association of BLV with BoLA genes was initially detected by the use of serology (Lewin and Bernoco, 1986; Palmer *et al.*, 1987). Persistent lymphocytosis (PL) as a result of BLV is characterised by a chronic increase in the absolute number of $CD5^+$ B cells in peripheral blood (Mirsky *et al.*, 1996). Persistent lymphocytosis develops in 15-30% of cases of BLV over a period of 1-3 years. The development of PL has been reported to be controlled by BoLA in different herds and breeds throughout the world (Lewin and Bernoco, 1986; Palmer *et al.*, 1987; Bernoco and Lewin, 1989; Xu *et al.*, 1993). Alleles of the DRB3 are most closely associated with resistance and susceptibility to PL in BLV infected cattle, with the haplotype A14-DRB3*11 strongly associated with resistance (Xu *et al.*, 1993). The position of glutamic acid and arginine at position 70-71 is one common feature that all the resistant phenotypes share whereas those phenotypes associated with susceptibility share a valine-aspartic acid-threonine-tyrosine repeat at position 75-78. These regions detailed above are part of the peptide-binding groove and crucial in the recognition of antigenic peptides by cells of the immune system (Stern *et al.*, 1994). As well as amino acid sequence the DRB3.2*16, 8, 22 and 24 have been reported previously to be associated with susceptibility to PL in BLV infected cattle (Lewin *et al.*, 1994). In contrast to those alleles listed above, DRB3.2*23 has been associated with resistance to PL as manifested by BLV (Xu *et al.*, 1993). The association of these alleles with BLV has been confirmed by others (Lewin *et al.*, 1988; Sulimova *et al.*, 1995; Zanotti *et al.*, 1996).

During the current study the antigen presentation assay described previously by Fitzpatrick (1992) was further developed to measure the proliferative response of PBM isolated from Holstein-Friesian cattle to the contagious mastitis pathogen *S. aureus*. *Staphylococcus aureus* is a predominant cause of subclinical mastitis in dairy herds (Wilson and Richards, 1980), and often results in chronic IMI. Dairy cows suffering from chronic IMI are usually removed from the herd, improving the herd's health status but reducing longevity and increasing the cost to the dairy farmer.

Breeding of dairy cattle worldwide is based mainly on profitability. Since 1998 the Animal Data Centre has published Predicted Transmitting Abilities for SCC, which are

estimates of sires' potential to transmit a reduction or increase in SCC measured in their daughters. The PTA for SCC were chosen to investigate a possible association with the proliferative response induced by *S. aureus*, as SCC in milk are used as an indicator of infection that can often be attributed to *S. aureus* (Holdaway *et al.*, 1996). Progeny testing carried out by commercial breeding companies to generate PTA for SCC and production traits is both labour and cost intensive, therefore, the rationale of the current study was to investigate a possible association between PTA for SCC and the proliferative response induced by *S. aureus in vitro* of PBM isolated from Holstein-Friesian bulls. An association between PTA for SCC and the proliferative response of PBM induced by *S. aureus* would indicate the potential to use the *in vitro* immunological assay to select bulls for breeding whose progeny may be relatively resistant to *S. aureus* mastitis. This would reduce the time and cost involved in the selection of superior bulls.

The hypothesis was tested first in cows sired by two different bulls. A statistically significant difference between two progeny groups was reported during the current study, indicated the potential sire's genes to contribute to immunological responses measured in his progeny. The significant result reported between the two progeny groups led to the ten bulls, held at Genus Ltd. breeding establishments, being selected for measurement of the proliferative response induced by *S. aureus* strain A. The bulls chosen represented the two extremes of the spectrum for PTA for SCC.

The results of the proliferation assay in the ten bulls showed a statistically significant difference between the two groups of bulls ($p=0.05$). As discussed previously the results were bimodal and overlapping, in that one bull with a low PTA for SCC showed a weak proliferative response and one bull with a high PTA for SCC showed a strong proliferative response. The results showed a strong negative correlation ($r=-0.7$) between logSI and PTA for SCC, indicating that as the PTA for SCC increased, the proliferative response induced by *S. aureus* strain A decreased. The study was extended to study bulls selected at random from the breeding population. The negative correlation, however, was not reported between the proliferative response of the randomly selected bulls and PTA for SCC. The difference in the correlation results between the two groups of bulls suggests that the *in vitro* immunological assay developed during the current study requires

further work to determine the assays potential for testing bulls reliably for their immunological response to *S. aureus* prior to the established progeny testing schemes.

The BoLA DRB locus has been reported previously to affect many traits related to immunity including SCC and mastitis incidence (Dietz *et al.*, 1997b). Results from the current study identified 12 different DRB3.2* types that had been previously published (Davies *et al.*, 1997). Frequency of these DRB3.2* types varied between those bulls selected for low PTA for SCC and those selected for high PTA for SCC. Allele DRB3.2*16 was identified in four of the bulls with low PTA for SCC and one of the bulls with high PTA for SCC in the current study. When the proliferative response induced by *S. aureus* strain A was measured, the five bulls with the strongest proliferation, i.e. the four bulls with the low PTA for SCC and one bull with high PTA for SCC, were all shown to express allele DRB3.2*16, suggesting a possible association between ability to respond to *S. aureus* antigen and expression of this allele. In contrast, none of the five bulls with the weakest proliferative response induced by *S. aureus* strain A expressed allele DRB3.2*16. The association of DRB3.2*16 and a strong proliferative response induced by *S. aureus* suggest that this allele may be used as a potential marker for improved induced immunological response to *S. aureus*.

Complex traits are associated with continuous underlying distribution of measurement and the genes responsible for the genetic variation are known as Quantitative trait loci (QTL) (Geldermann, 1975). Disease resistance is a complex trait, which is under the control of many genes each with different phenotypic contributions (Heyen *et al.*, 1999). At present the genetic linkage map of cattle is made up of more than 1500 genetic markers, known as microsatellites. These microsatellites have been used to identify possible QTL affecting milk production, health traits (Ashwell *et al.*, 1997; Zhang *et al.*, 1998) and body conformation traits (Ashwell *et al.*, 1998). Further studies with larger populations of cattle are necessary to determine QTL that may be linked with disease resistance and enhance genetic improvement using marker-assisted selection in cattle worldwide.

Chapter V

5.1 Future Work

While the current project focused on measuring immune response to the important contagious mastitis pathogen, *S. aureus*, it is recognised that other mastitis pathogens are increasing in prevalence, especially *E. coli* and *S. uberis*. It is known that the pathogenesis and clinical signs associated with an IMI due to contagious and environmental pathogens are often very different. The clinical signs as a result of infection with a contagious pathogen are often mild and of long duration. In contrast, the signs often associated with an IMI caused by environmental pathogens tend to be more clinically obvious but transient in nature. Differences in cellular immune responses to various pathogens might be expected in a similar way to those shown in the current study using different strains of *S. aureus*. Investigation of the differences in proliferative response induced by different pathogens is important as it is possible that by selecting animals for resistance to a particular pathogen that this may result in susceptibility to another.

T cell epitopes to foot and mouth disease virus (FMDV) have been identified previously by peptide scanning of cattle immunised with whole-virus vaccine (Collen *et al.*, 1991; van Lierop *et al.*, 1994). Further studies have shown that MHC class I and class II haplotypes defined the selection of T cell epitopes (Glass *et al.*, 1991). Using similar approaches to those used for FMDV, the interactions among MHC, T cells and epitopes could be explored for mastitis. The current study used whole bacterial antigen to induce a proliferative response of PBM *in vitro*. Identification of T cell epitopes would refine the current *in vitro* proliferation assay and allow investigation of the important epitopes involved in induction of cellular proliferative response and possibly contributing to defence of the mammary gland against infection.

Future development of the *in vitro* proliferation assay could involve measurement of cytokine production induced by antigenic stimuli as an alternative to, or in conjunction with, measurement of cell proliferation. The use of a well-established method such as

ELISA could be used to identify a range of cytokines. Possible candidates for investigation would include IL-2 and IFN-γ, which are cytokines that are characteristic of a Th1-type response, and IL-4 and IL-10, which are cytokines that are characteristic of a Th2-type response. In addition to detection of secreted cytokines, identification of cytokine messenger RNA using reverse transcription-PCR could be undertaken. This requires extraction of total cellular RNA, which is then reverse transcribed. The cytokine specific cDNAs corresponding to each cytokine sequence created can then be amplified by PCR (Ito and Kodama, 1996). Cytokine detection could permit characterisation of the type of Th response, as well as reducing the time for *in vitro* culture, removing the requirement for use of radioisotopes and allowing quantification of the immune response.

During the current study, phenotyping of PBM isolated from cows and bulls showed that bulls had a significantly lower proportion of cells expressing the various cell surface markers than cows, and cattle, in previously published studies. In the case of γδ T cells, their function of is still being investigated, however, they are thought to be of importance in the ruminant immune system (Hein and Mackay, 1991). Further investigation of the reported lower levels of all cell subpopulations in bulls is required.

The proportion of cells expressing the surface markers associated with neutrophils and dendritic cells should be studied. Identification of the proportion of cells expressing neutrophil markers would allow the use of whole blood techniques thus eliminating the requirement for density centrifugation and the possible differential loss of cell types. Dendritic cells are known to be the most potent of the APC and are essential for the initiation of immune responses in naïve animals (Steinman, 1991; Banchereau and Steinman, 1998). Although dendritic cells usually form a very small proportion of cells within the immune system, their presence and function in the bovine immune system is essential in the modulation of an effective immune response. Future studies of antigen presentation in the local mammary gland environment would benefit from studies on the dendritic cell population.

Future studies should investigate the expression of CD45RO on the surface of cells isolated from peripheral blood and tissue and local mucosal sites in cattle. Expression of CD45RO is indicative of a memory phenotype and T cells deficient in surface expression of CD45RO have been reported previously to fail to proliferate in response to antigen or TCR stimuli (Pingel and Thomas, 1989; Weaver *et al.*, 1991). It is likely that previous exposure to various antigenic stimuli would result in expression of a memory phenotype on cells isolated from cattle. It would be of interest to investigate the naïve/memory phenotype of the cells involved in peripheral and mammary gland immunity as lack of expression of CD45RO on cells isolated from cattle after *in vitro* stimulation with antigen could indicate inadequate T cell development and function (Kong *et al.*, 1995).

Clarification of the DRB3 types identified by PCR-RFLP is required to establish the expressed alleles in the individual bulls used during the current study. This would require improvement in cloning and sequencing techniques through the creation of new primer sets to eliminate the recurrent isolation of an individual sequence. Further work would be required to identify the ambiguous PCR-RFLP patterns generated by DQB exon 2. Improvements in the methods used routinely for rapid identification of BoLA genes are continuing. The use of single strand conformational polymorphisms (SSCP) or density gradient gel electrophoresis improves the reliability and discriminatory powers of PCR-RFLP (Mikko and Andersson, 1995; Aldridge *et al.*, 1998). In a recent study with HLA class I alleles, reference strand conformation analysis (RSCA), an electrophoretic typing method, has been suggested to supersede current non-sequenced based analytical approaches (Arguello *et al.*, 1998).

A comparison between the immune responses and phenotype of peripheral blood and milk cells could be part of a future study. While the proportion of cells present in peripheral blood and in the mammary gland of cows has been well documented (Concha *et al.*, 1977; Hageltorn and Saad, 1986; Park *et al.*, 1993), the use of the *in vitro* proliferation assay developed during the current study and ELISAs for the detection of cytokines could be employed to investigate the previously reported hyporesponsiveness to antigen by cells present in the mammary gland. It has been described previously that local immunity in the normal, healthy mammary glands is inactive or suppressed (Newby and Bourne, 1977). Investigation of the migration, location, presence and function of

various cell populations involved in local mucosal mammary gland immunity induced by *S. aureus* and other antigenic stimuli may elucidate possible mechanisms resulting in hyporesponsiveness within the mammary gland and provide methods whereby mammary gland immunity may be enhanced.

Appendix A

Data from 104 bulls

Columns are left to right are as follows:

1. bull name
2. stimulation index 1
3. stimulation index 2
4. stimulation index 3
5. stimulation index 4
6. counts per minute in the presence of *S. aureus* strain A
7. counts per minute in the absence of *S. aureus* stain A
8. PTA for SCC in 1998
9. PTA for SCC in November 1999

```
data one;
input @30  SI1 @38 SI2 @45 SI3 @53 SI4  @60 CPMT1 @69 CPMC1  @76 SCC1 @86 SCC2;
 simean = mean(si1,si2,si3,si4);
 logsim = log10(simean+1);
cards;
amsweer captain                33       0        .        .     7262      214        .        8
amsweer skip leader             0       .        .        .      477      301      9.5        9
beechpike genesis              12       .        .        .      738       57        .        .
bromham norton                  0       7        .        .      500      303      0.4       -2
catlane corsair                 0       0        .        .      206      247        .       -9
cradenhill j marauder           0       .        .        .       66       89        .        .
cradenhill prelude free         4       .        .        .      388       88       -1        7
cradenhill superson             8       8        .        .     6267      775        .        1
ernespie mascot                17       .        .        .     3021      177      7.1        5
fielders exchequer             27      45       33        5     9016      335        .       -8
frankenhof bodygaurd            5       3        .        .     1977      385        .        5
frankenhof capsule             19       .        .        .     5681      291        .        6
frankenhof dacey               16       6        .        .     6059      369        .        5
g.aeromon                       0       .        .        .       71       61     -2.3        2
g.ashton                        5       .        .        .     1519      303     -1.7       -3
g.barometer                     0       0        .        .      193      136       -2       -4
g.bonanza                      14       .        .        .     5209      373     -0.8       -9
g.brandon                       4      10        4       14     1776      367        .        1
g.canby                         0       0        .        .      154      221      4.8       14
g.carolina                     13       0        .        .     2273      171      3.9        4
g.carter                       17      10        .        .     5311      301        .        0
g.derry                         6       2        .        .     2535      384       10       13
g.diamond                       7       .        .        .      520       73        .       -6
g.dieppe                        0       .        .        .       75       63      7.8       26
g.domaine                       4       .        .        .     1875      405      7.3       -1
g.dordogne                      2       .        .        .      638      232        .       14
g.emperor                      39       0        .        .    19241      486     -7.5       -2
g.flame                         0       .        .        .      293      224     11.7       18
g.fortune                       3       .        .        .     1227      409        .        5
g.frontier                     14       4        .        .     6976      496        .       15
g.garcon                        0       5        .        .      318      248        .        9
g.goodwin                       0       2        .        .      215      307        .        .
g.hollywood                     0       .        .        .      243      206        .       -3
g.kennedy                      17      16        .        .     7850      441       -6       -8
g.lakewood                      3      21        .        .     4195     1399     10.2        3
```

g.lincroft	3	.	.	.	1382	428	-5	-12
g.lomas	21	.	.	.	1722	82	.	.
g.longview	0	0	.	.	153	176	0.9	-1
g.melbourne	49	.	.	.	3585	73	.	9
g.pendleton	17	.	.	.	9117	511	1.9	-2
g.penland	5	0	.	.	1192	206	4.5	-4
g.perkins	12	.	.	.	647	52	.	4
g.roscoe	9	10	.	.	3656	378	.	7
g.townley	0	4	.	.	455	317	.	3
g.trenton	6	.	.	.	4749	696	0.3	-3
holywell maine	25	0	.	.	19144	741	-0.2	1
holywell milwaukee	0	6	.	.	278	332	-0.1	-1
hunday lightning	3	0	0	.	960	263	-0.1	-12
kolhorner comet	2	2	.	.	1185	441	9.7	10
kolhorner crusader	0	.	.	.	236	305	.	11
kynarton fruiton	3	48	.	.	734	192	.	27
llandanglws mascot rebel	27	.	.	.	14164	522	3.7	2
m.alicia edderton	15	.	.	.	7566	479	2.7	4
m.alicia frontier	131	.	.	.	9894	75	10.4	1
m.cadet fortran	2	0	4	.	386	159	6.6	5
m.cadet foxton	12	0	.	.	5373	424	.	18
m.deb farnley	0	.	.	.	248	191	1.1	-9
m.deb fountain	3	9	.	.	1897	643	0.3	-3
m.deirdre dependable	0	0	.	.	92	226	-1.6	0
m.diamond felton	3	29	.	.	1221	349	9.4	16
m.diamond finson	0	.	.	.	273	206	8.9	-4
m.dream gallery	0	14	.	.	537	476	.	1
m.elsa fountain	8	3	30	2	2321	285	.	9
m.elsas contest	0	0	.	.	216	155	-0.7	8
m.exalt	0	11	.	.	813	430	9.3	8
m.extol	2	.	.	.	490	239	13.8	16
m.federal	4	.	.	.	2791	621	6	4
m.flirt eastheath	12	.	.	.	5587	456	22.6	23
m.flirt francis	5	3	.	.	1040	204	7.9	8
m.flirt freeman	9	0	.	.	1564	163	9.5	13
m.flirt fusion	20	12	.	.	4523	220	5	5
m.flirt guesswork et	21	.	.	.	2261	107	.	12
m.genie finstock	0	.	.	.	474	322	15.6	18
m.genie fitment	0	.	.	.	331	184	.	13
m.genie florin	0	.	.	.	159	192	11.9	.

m.genies double	0	0	.	.	210	164	3.7	7
m.holly endive	0	.	.	.	133	82	19.6	23
m.jenny ensignet	2	.	.	.	295	119	8	7
m.joy easming	0	.	.	.	531	297	-3.4	-9
m.leilani fixation	0	0	0	.	341	193	.	10
m.lemon folio	7	0	.	.	22929	3133	.	-4
m.lill formula	7	13	15	5	2883	388	.	6
m.mandy favour	15	32	2	10	8758	565	.	-6
m.mandy fern	38	.	.	.	20472	538	2	11
m.mandy functional	4	.	.	.	977	233	-0.7	5
m.marissa glamour	5	.	.	.	357	71	.	.
m.martha felton	0	0	0	.	311	401	.	-3
m.martha franchise	0	.	.	.	281	185	.	5
m.rozana firth	3	5	0	0	1307	436	.	-6
m.rozana fullerton	5	.	.	.	374	67	8.4	2
m.secure gunner	8	.	.	.	.	.	.	2
m.vale eastwell	7	.	.	.	2432	245	7	8
mars chieftain	2	0	.	.	485	229	.	14
olympian muscat	8	0	.	.	2557	304	6.4	8
olympian musketeer	11	.	.	.	5215	448	1	0
skalsumer lucky boy	2	7	.	.	697	325	.	17
star ebony	2	.	.	.	415	197	11.4	12
tallent vivaldi	22	.	.	.	1990	90	3.5	3
vero elton boy	25	45	.	.	11299	446	.	12
weetondale hotshot	2	.	.	.	597	247	8.4	21
woeudhoeve criteriun	48	.	.	.	17346	355	.	9
woudhoeve commander	0	0	.	.	289	264	3.3	7
woudhoeve criteriun	5	.	.	.	906	169	.	9
zandenburg wayne	0	.	.	.	544	386	.	-2

;

```
proc print;
 run;

proc corr;
 run;

proc gplot;
 plot SCC2*logsim;
 run;
```

Appendix B

Data from 104 bulls

Columns from left to right are as follows:

1 bull code number
2 stimulation index 1
3 stimulation index 2
4 stimulation index 3
5 stimulation index 4
6 counts per minute in the presence of *S. aureus* stain A
7 counts per minute in the absence of *S. aureus* strain A
8 PTA for SCC in 1998
9 PTA for SCC in November 1999
10 Mean of the stumulation indices
11 Logged stimulation indices

OBS	SI1	SI2	SI3	SI4	CPMT1	CPMC1	SCC1	SCC2	SIMEAN	LOGSIM
1	33	0	.	.	7262	214	.	8	16.5	1.24304
2	0	.	.	.	477	301	9.5	9	0.0	0.00000
3	12	.	.	.	738	57	.	.	12.0	1.11394
4	0	7	.	.	500	303	0.4	-2	3.5	0.65321
5	0	0	.	.	206	247	.	-9	0.0	0.00000
6	0	.	.	.	66	89	.	.	0.0	0.00000
7	4	.	.	.	388	88	-1.0	7	4.0	0.69897
8	8	8	.	.	6267	775	.	1	8.0	0.95424
9	17	.	.	.	3021	177	7.1	5	17.0	1.25527
10	27	45	33	5	9016	335	.	-8	27.5	1.45484
11	5	3	.	.	1977	385	.	5	4.0	0.69897
12	19	.	.	.	5681	291	.	6	19.0	1.30103
13	16	6	.	.	6059	369	.	5	11.0	1.07918
14	0	.	.	.	71	61	-2.3	2	0.0	0.00000
15	5	.	.	.	1519	303	-1.7	-3	5.0	0.77815
16	0	0	.	.	193	136	-2.0	-4	0.0	0.00000
17	14	.	.	.	5209	373	-0.8	-9	14.0	1.17609
18	4	10	4	14	1776	367	.	1	8.0	0.95424
19	0	0	.	.	154	221	4.8	14	0.0	0.00000
20	13	0	.	.	2273	171	3.9	4	6.5	0.87506
21	17	10	.	.	5311	301	.	0	13.5	1.16137
22	6	2	.	.	2535	384	10.0	13	4.0	0.69897
23	7	.	.	.	520	73	.	-6	7.0	0.90309
24	0	.	.	.	75	63	7.8	26	0.0	0.00000
25	4	.	.	.	1875	405	7.3	-1	4.0	0.69897
26	2	.	.	.	638	232	.	14	2.0	0.47712
27	39	0	.	.	19241	486	-7.5	-2	19.5	1.31175
28	0	.	.	.	293	224	11.7	18	0.0	0.00000
29	3	.	.	.	1227	409	.	5	3.0	0.60206
30	14	4	.	.	6976	496	.	15	9.0	1.00000
31	0	5	.	.	318	248	.	9	2.5	0.54407
32	0	2	.	.	215	307	.	.	1.0	0.30103
33	0	.	.	.	243	206	.	-3	0.0	0.00000
34	17	16	.	.	7850	441	-6.0	-8	16.5	1.24304
35	3	21	.	.	4195	1399	10.2	3	12.0	1.11394
36	3	.	.	.	1382	428	-5.0	-12	3.0	0.60206

OBS	SI1	SI2	SI3	SI4	CPMT1	CPMC1	SCC1	SCC2	SIMEAN	LOGSIM
37	21	.	.	.	1722	82	.	.	21.00	1.34242
38	0	0	.	.	153	176	0.9	-1	0.00	0.00000
39	49	.	.	.	3585	73	.	9	49.00	1.69897
40	17	.	.	.	9117	511	1.9	-2	17.00	1.25527
41	5	0	.	.	1192	206	4.5	-4	2.50	0.54407
42	12	.	.	.	647	52	.	4	12.00	1.11394
43	9	10	.	.	3656	378	.	7	9.50	1.02119
44	0	4	.	.	455	317	.	3	2.00	0.47712
45	6	.	.	.	4749	696	0.3	-3	6.00	0.84510
46	25	0	.	.	19144	741	-0.2	1	12.50	1.13033
47	0	6	.	.	278	332	-0.1	-1	3.00	0.60206
48	3	0	0	.	960	263	-0.1	-12	1.00	0.30103
49	2	2	.	.	1185	441	9.7	10	2.00	0.47712
50	0	.	.	.	236	305	.	11	0.00	0.00000
51	3	48	.	.	734	192	.	27	25.50	1.42325
52	27	.	.	.	14164	522	3.7	2	27.00	1.44716
53	15	.	.	.	7566	479	2.7	4	15.00	1.20412
54	131	.	.	.	9894	75	10.4	1	131.00	2.12057
55	2	0	4	.	386	159	6.6	5	2.00	0.47712
56	12	0	.	.	5373	424	.	18	6.00	0.84510
57	0	.	.	.	248	191	1.1	-9	0.00	0.00000
58	3	9	.	.	1897	643	0.3	-3	6.00	0.84510
59	0	0	.	.	92	226	-1.6	0	0.00	0.00000
60	3	29	.	.	1221	349	9.4	16	16.00	1.23045
61	0	.	.	.	273	206	8.9	-4	0.00	0.00000
62	0	14	.	.	537	476	.	1	7.00	0.90309
63	8	3	30	2	2321	285	.	9	10.75	1.07004
64	0	0	.	.	216	155	-0.7	8	0.00	0.00000
65	0	11	.	.	813	430	9.3	8	5.50	0.81291
66	2	.	.	.	490	239	13.8	16	2.00	0.47712
67	4	.	.	.	2791	621	6.0	4	4.00	0.69897
68	12	.	.	.	5587	456	22.6	23	12.00	1.11394
69	5	3	.	.	1040	204	7.9	8	4.00	0.69897
70	9	0	.	.	1564	163	9.5	13	4.50	0.74036
71	20	12	.	.	4523	220	5.0	5	16.00	1.23045
72	21	.	.	.	2261	107	.	12	21.00	1.34242

OBS	SI1	SI2	SI3	SI4	CPMT1	CPMC1	SCC1	SCC2	SIMEAN	LOGSIM
73	0	.	.	.	474	322	15.6	18	0.00	0.00000
74	0	.	.	.	331	184	.	13	0.00	0.00000
75	0	.	.	.	159	192	11.9	.	0.00	0.00000
76	0	0	.	.	210	164	3.7	7	0.00	0.00000
77	0	.	.	.	133	82	19.6	23	0.00	0.00000
78	2	.	.	.	295	119	8.0	7	2.00	0.47712
79	0	.	.	.	531	297	-3.4	-9	0.00	0.00000
80	0	0	0	.	341	193	.	10	0.00	0.00000
81	7	0	.	.	22929	3133	.	-4	3.50	0.65321
82	7	13	15	5	2883	388	.	6	10.00	1.04139
83	15	32	2	10	8758	565	.	-6	14.75	1.19728
84	38	.	.	.	20472	538	2.0	11	38.00	1.59106
85	4	.	.	.	977	233	-0.7	5	4.00	0.69897
86	5	.	.	.	357	71	.	.	5.00	0.77815
87	0	0	0	.	311	401	.	-3	0.00	0.00000
88	0	.	.	.	281	185	.	5	0.00	0.00000
89	3	5	0	0	1307	436	.	-6	2.00	0.47712
90	5	.	.	.	374	67	8.4	2	5.00	0.77815
91	8	.	.	.	.	.	.	2	8.00	0.95424
92	7	.	.	.	2432	245	7.0	8	7.00	0.90309
93	2	0	.	.	485	229	.	14	1.00	0.30103
94	8	0	.	.	2557	304	6.4	8	4.00	0.69897
95	11	.	.	.	5215	448	1.0	0	11.00	1.07918
96	2	7	.	.	697	325	.	17	4.50	0.74036
97	2	.	.	.	415	197	11.4	12	2.00	0.47712
98	22	.	.	.	1990	90	3.5	3	22.00	1.36173
99	25	45	.	.	11299	446	.	12	35.00	1.55630
100	2	.	.	.	597	247	8.4	21	2.00	0.47712
101	48	.	.	.	17346	355	.	9	48.00	1.69020
102	0	0	.	.	289	264	3.3	7	0.00	0.00000
103	5	.	.	.	906	169	.	9	5.00	0.77815
104	0	.	.	.	544	386	.	-2	0.00	0.00000

Appendix C

Data from 104 bulls

Correlation analysis

Correlation Analysis

10 'VAR' Variables: SI1 SI2 SI3 SI4 CPMT1 CPMC1 SCC1 SCC2 SIMEAN LOGSIM

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
SI1	104	9.096154	15.985138	946.000000	0	131.000000
SI2	52	7.538462	11.986040	392.000000	0	48.000000
SI3	10	8.800000	12.804513	88.000000	0	33.000000
SI4	6	6.000000	5.176872	36.000000	0	14.000000
CPMT1	103	3133.126214	4752.231160	322712	66.000000	22929
CPMC1	103	330.436893	338.752749	34035	52.000000	3133.000000
SCC1	59	4.818644	6.028938	284.300000	-7.500000	22.600000
SCC2	98	4.979592	8.443847	488.000000	-12.000000	27.000000
SIMEAN	104	9.033654	15.578803	939.500000	0	131.000000
LOGSIM	104	0.702822	0.518455	73.093460	0	2.120574

Correlation Analysis

Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / Number of Observations

	SI1	SI2	SI3	SI4	CPMT1	CPMC1	SCC1	SCC2	SIMEAN	LOGSIM
SI1	1.00000	0.26233	0.70879	0.03416	0.58080	-0.01360	-0.01702	-0.05338	0.96041	0.70265
	0.0	0.0603	0.0217	0.9488	0.0001	0.8915	0.8982	0.6016	0.0001	0.0001
	104	52	10	6	103	103	59	98	104	104
SI2	0.26233	1.00000	0.51430	0.24864	0.16009	0.04035	0.23505	0.12224	0.81158	0.64967
	0.0603	0.0	0.1283	0.6347	0.2569	0.7764	0.2477	0.3928	0.0001	0.0001
	52	52	10	6	52	52	26	51	52	52
SI3	0.70879	0.51430	1.00000	-0.33995	0.49593	-0.08598	1.00000	0.16187	0.77665	0.71793
	0.0217	0.1283	0.0	0.5097	0.1449	0.8133	.	0.6550	0.0082	0.0194
	10	10	10	6	10	10	2	10	10	10
SI4	0.03416	0.24864	-0.33995	1.00000	0.21940	0.28538	.	-0.07629	0.13712	0.33300
	0.9488	0.6347	0.5097	0.0	0.6762	0.5836	.	0.8858	0.7956	0.5190
	6	6	6	6	6	6	0	6	6	6
CPMT1	0.58080	0.16009	0.49593	0.21940	1.00000	0.57401	-0.23913	-0.12798	0.49718	0.59487
	0.0001	0.2569	0.1449	0.6762	0.0	0.0001	0.0681	0.2116	0.0001	0.0001
	103	52	10	6	103	103	59	97	103	103
CPMC1	-0.01360	0.04035	-0.08598	0.28538	0.57401	1.00000	-0.07917	-0.19141	-0.02253	0.13694
	0.8915	0.7764	0.8133	0.5836	0.0001	0.0	0.5512	0.0604	0.8213	0.1678
	103	52	10	6	103	103	59	97	103	103
SCC1	-0.01702	0.23505	1.00000	.	-0.23913	-0.07917	1.00000	0.74675	0.04152	-0.07434
	0.8982	0.2477	.	.	0.0681	0.5512	0.0	0.0001	0.7549	0.5758
	59	26	2	0	59	59	59	58	59	59
SCC2	-0.05338	0.12224	0.16187	-0.07629	-0.12798	-0.19141	0.74675	1.00000	-0.00068	-0.01137
	0.6016	0.3928	0.6550	0.8858	0.2116	0.0604	0.0001	0.0	0.9947	0.9115
	98	51	10	6	97	97	58	98	98	98

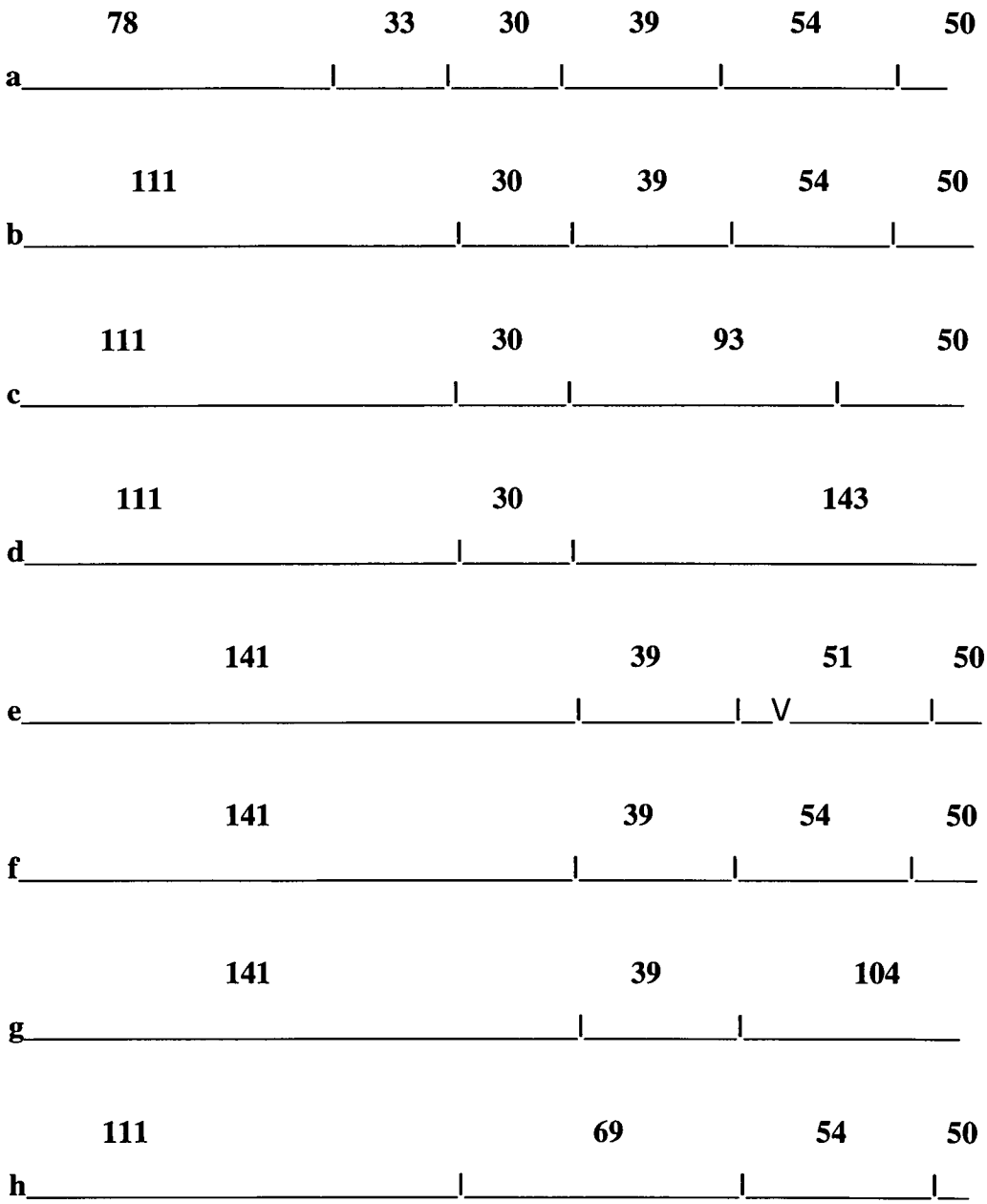
Correlation Analysis

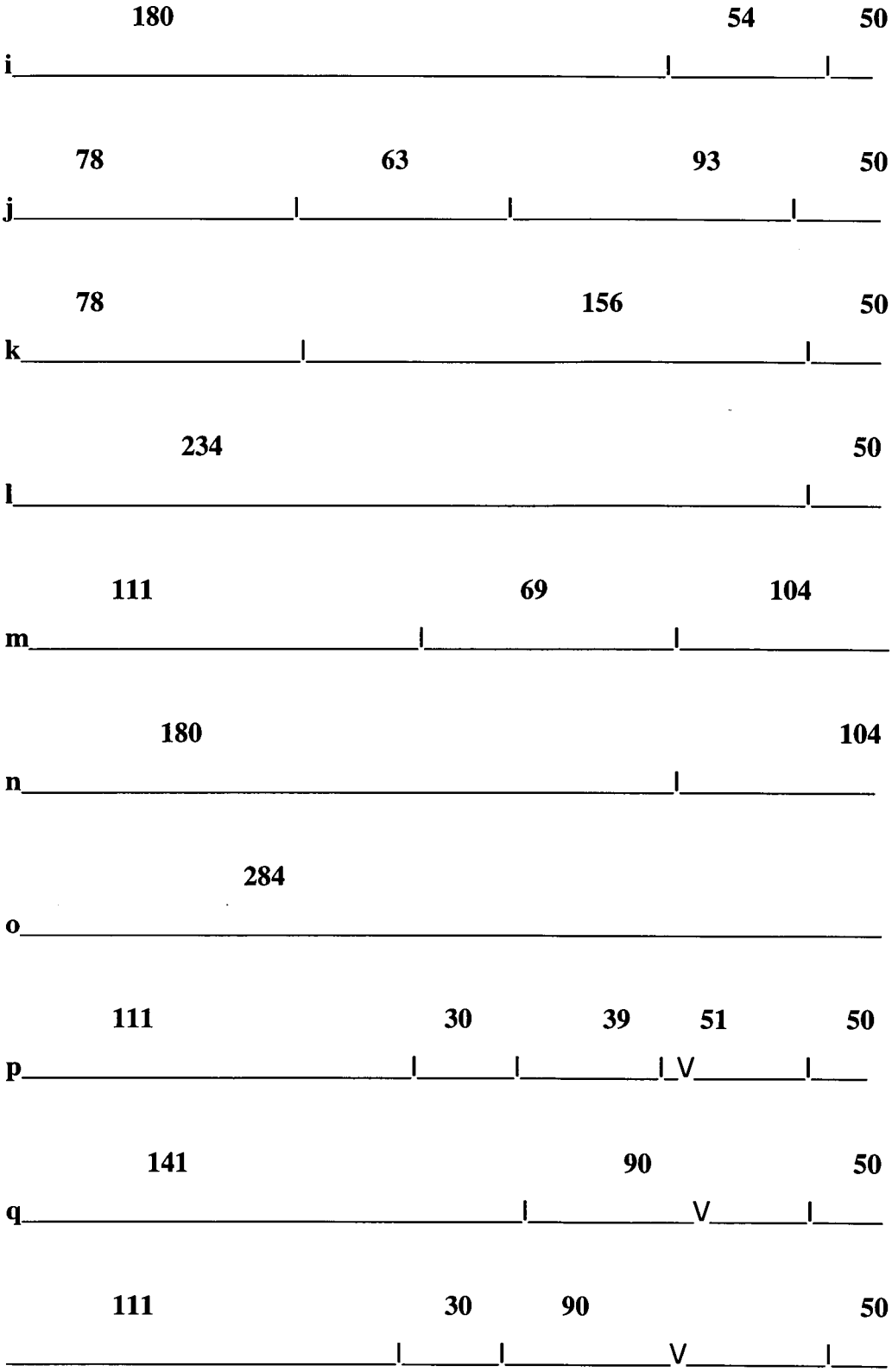
Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / Number of Observations

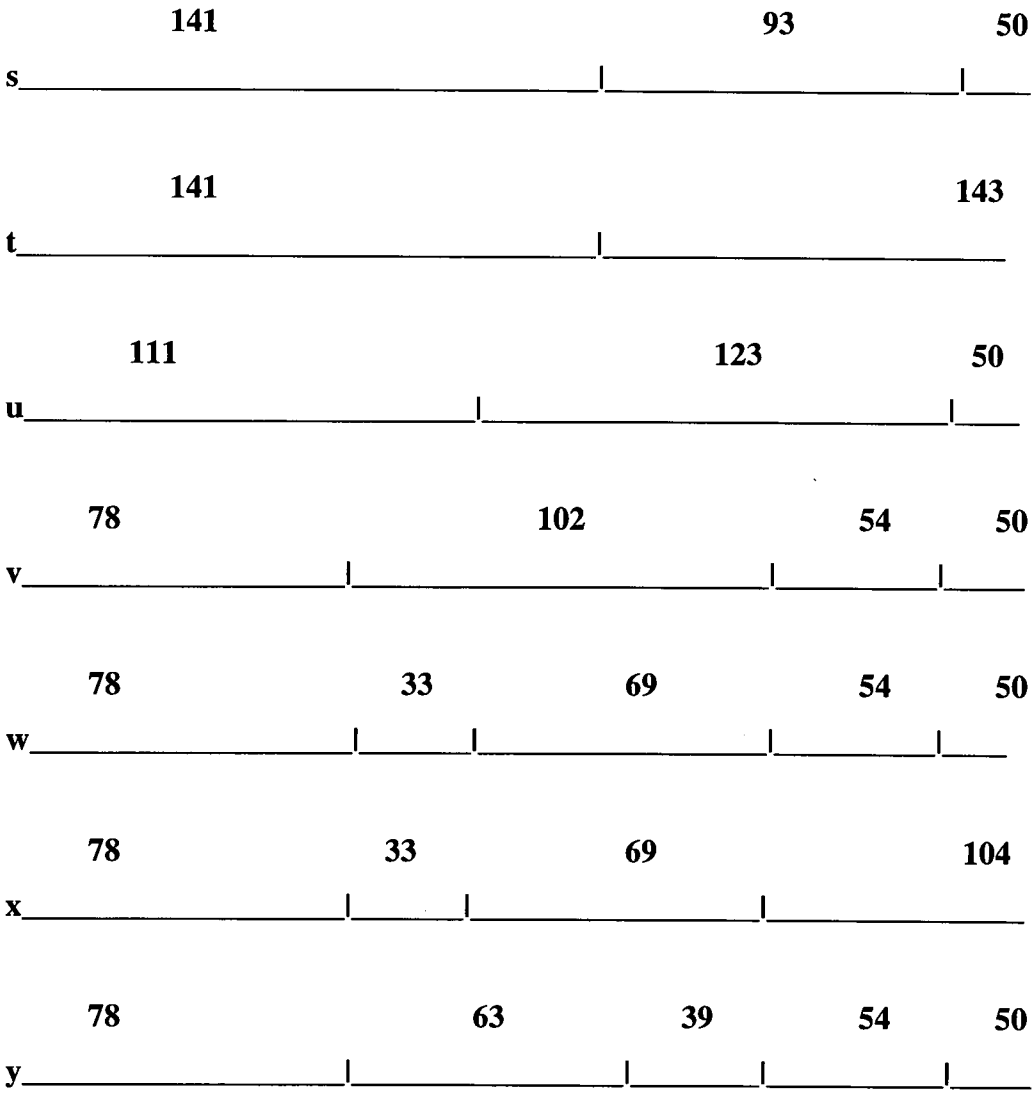
SI1	SI2	SI3	SI4	CPMT1	CPMC1	SCC1	SCC2	SIMEAN	LOGSIM
0.96041	0.81158	0.77665	0.13712	0.49718	-0.02253	0.04152	-0.00068	1.00000	0.72813
0.0001	0.0001	0.0082	0.7956	0.0001	0.8213	0.7549	0.9947	0.0	0.0001
104	52	10	6	103	103	59	98	104	104
0.70265	0.64967	0.71793	0.33300	0.59487	0.13694	-0.07434	-0.01137	0.72813	1.00000
0.0001	0.0001	0.0194	0.5190	0.0001	0.1678	0.5758	0.9115	0.0001	0.0
104	52	10	6	103	103	59	98	104	104

Appendix D

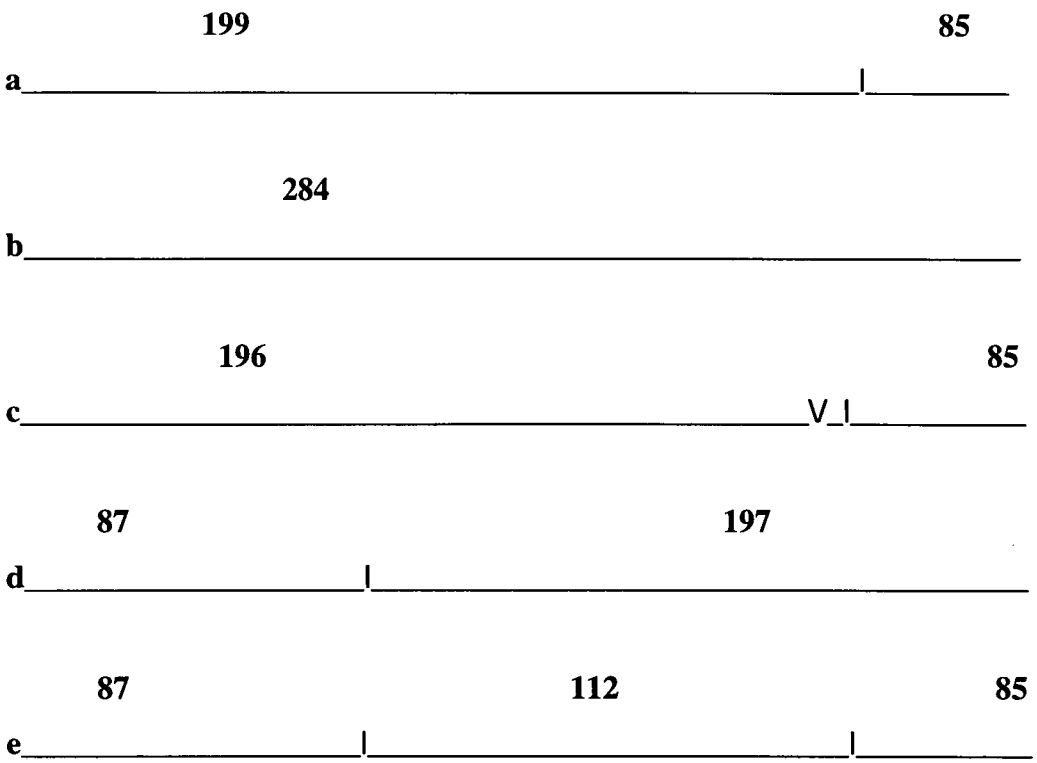
Restriction Fragment Length Polymorphism Patterns of BoLA DRB3 created by enzymatic digestion with *Rsa* I. Patterns A to S, van Eijk *et al.*, 1992; patterns T and U, Gelhaus *et al.*, 1995; patterns V to Y, based on new allele sequences.



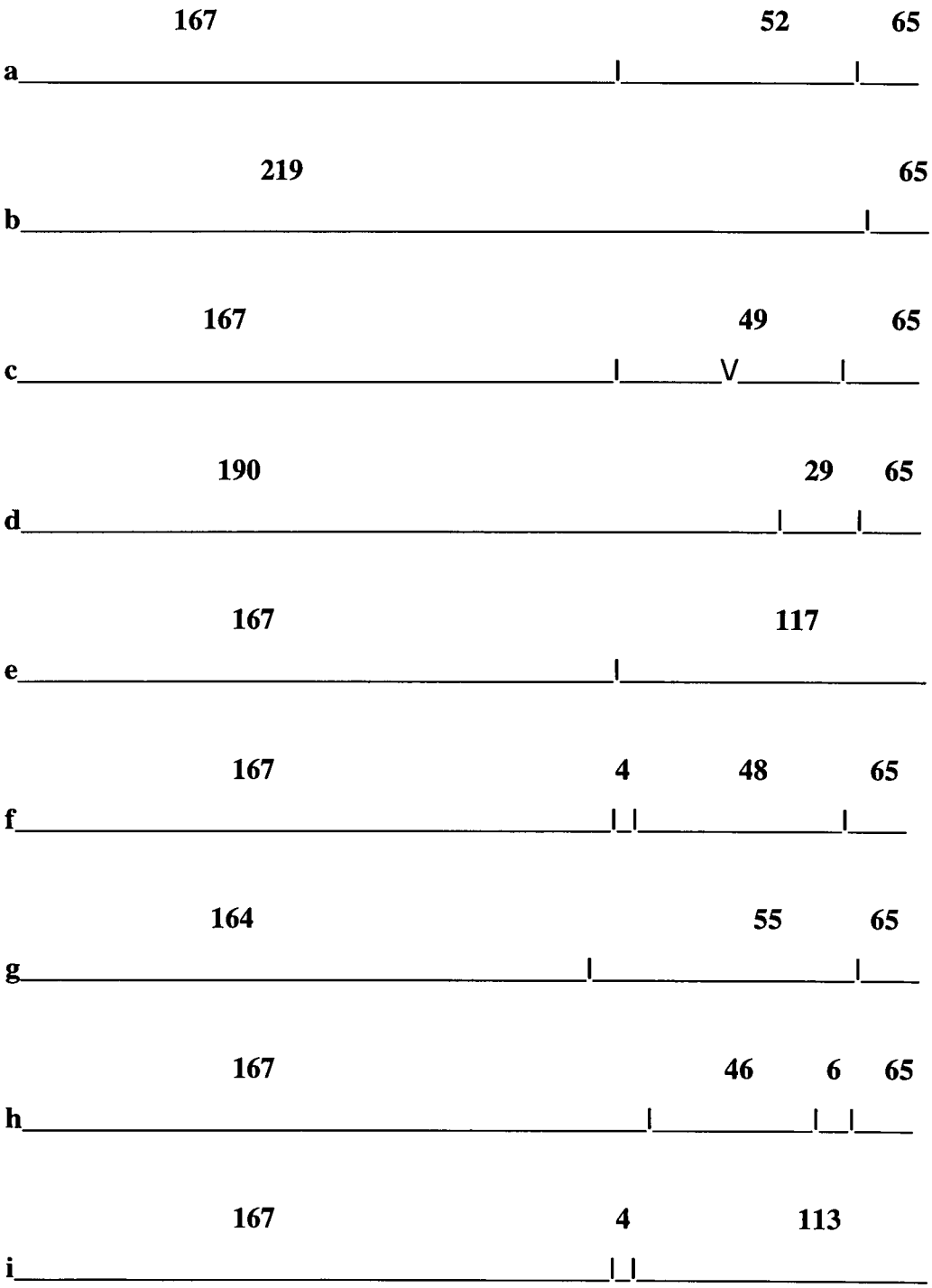

180
54
50
i
78
63
93
50
j
78
156
50
k
234
50
l
111
69
104
m
180
104
n
284
o
111
30
39
51
50
p
141
90
50
q
111
30
90
50


141
93
50
s
141
143
t
111
123
50
u
78
102
54
50
v
78
33
69
54
50
w
78
33
69
104
x
78
63
39
54
50
y

Restriction Fragment Length Polymorphism Patterns of BoLA DRB3 created by enzymatic digestion with *Bst* YI. Patterns A to E, van Eijk *et al.*, 1992



Restriction Fragment Length Polymorphism Patterns of BoLA DRB3 created by enzymatic digestion with *Hae* III. Patterns A to F, van Eijk *et al.*, 1992; patterns G and H, Gelhaus *et al.*, 1995; pattern I, based on allele DRB3*25011.



Allele	DRB3 PCR-RFLP	*Rsa* I, *Bst* Y1, *Hae* III patterns
DRB3*0501	01	aaa
DRB3*0503	01	aaa
DRB3*1301	02	bba
DRB3*1001	03	bbb
DRB3*1002	03	bbb
No sequence	04	caa
DRB3*3301	05	rcc
DRB3*2201	06	daa
DRB3*2202	06	daa
DRB3*0201	07	ecc
DRB3*1201	08	faa
DRB3*0301	09	fda
DRB3*0302	09	fda
DRB3*1601	10	fba
DRB3*1602	10	fba
DRB3*0901	11	gea
DRB3*0902	11	gea
DRB3*1202	11	gea
DRB3*1701	12	haa
DRB3*1702	12	haa
DRB3*3201	12	haa
DRB3*3202	12	haa
DRB3*3203	12	haa
DRB3*0401	13	hba
No sequence	14	hbb
DRB3*20011	15	iba
DRB3*20012	15	iba
DRB3*2002	15	iba
DRB3*2003	15	iba
DRB3*1501	16	jbd
DRB3*1502	16	jbd
DRB3*4301	16	kbb
No sequence	17	lbf
DRB3*1801	18	lbf
DRB3*1802	18	sbb
DRB3*2601	19	lbb
DRB3*2301	20	lbb
DRB3*2901	20	lbb
DRB3*3601	20	lbe
DRB3*0801	21	mba
DRB3*1101	22	nba
DRB3*2701	23	nba
DRB3*2702	23	nba

Allele	DRB3 PCR-RFLP	*Rsa* I, *Bst* Y1, *Hae* III patterns
DRB3*2703	23	nba
DRB3*2705	23	nba
DRB3*2706	23	nba
DRB3*2707	23	nba
DRB3*0101	24	nbb
DRB3*0102	24	nbb
No sequence	25	oaa
DRB3*0601	26	oab
DRB3*14011	27	obf
DRB3*14012	27	obf
DRB3*3101	27	obf
DRB3*0701	28	obb
DRB3*4101	29	pcc
Genotyping error	30	qcc
DRB3*2801	31	ibf
DRB3*2401	32	maa
DRB3*2704	33	nbf
DRB3*3001	34	lab
DRB3*3002	34	lab
DRB3*2101	35	cbb
DRB05	36	lba
DRB07	37	oba
DRB18	38	bda
No sequence	39	tba
No sequence	40	uba
DRB3*0502	41	aba
DRB3*1901	41	aba
DRB3*3801	41	aba
DRB3*2802	42	hbf
DRB3*25012	43	kbf
DRB3*25011	44	kbi
DRB3*3401	45	sdb
DRB3*3402	45	sdb
DRB3*3501	46	vba
DRB3*1703	47	waa
DRB3*3901	48	wba
DRB3*3701	49	wbe
DRB3*4001	50	xba
DRB3*4201	51	gaa
DRB3*0303	52	sda
DRB3*1902	53	yba

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