



Immuno-fluorescence staining patterns of leukocyte subsets in the skin of taurine and indicine cattle



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ABSTRACT

The immuno-staining patterns of skin leukocytes were investigated in three breeds of cattle: Holstein–Friesian, Brahman and Santa Gertrudis of similar age before and after tick infestation. The antibodies specific for CD45 and CD45RO reacted with cells in the skin of all Holstein–Friesian cattle but did not react with cells in the skin of any Brahman cattle. The same antibodies reacted with cells from the skin of four (CD45) and seven (CD45RO) of twelve Santa Gertrudis cattle. The antibodies specific for T cells and $\gamma\delta$ subset of T cells recognized cells from all three breeds of cattle. The antibody specific for MHC class II molecules labelled cells of mostly irregular shape, presumably dermal dendritic cells and/or macrophages and Langerhans cells. The antibody specific for granulocytes (mAb CH138) reacted with cells only in sections cut from skin with lesions. The antibody specific for CD25⁺ cells labelled regularly shaped cells that showed a wide range of intensities of staining.

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1. Introduction

The identification of the cells of the immune system *in situ* has become an important tool for both research and diagnosis in recent years (Gutierrez et al., 1999). Although many antibodies specific for various subsets of bovine leukocytes have been produced they are most commonly used in flow cytometry, very few of them have been used to probe tissue sections (Howard and Naessens, 1993; Niku et al., 2006). Consequently, for most of the antibodies that are available, limited or no information is available on the immuno-staining patterns and localization of the cells recognized in tissue sections. Furthermore, most research on immuno-staining so far was carried out on cells from cattle of unspecified breed

(Keresztes et al., 1996; Niku et al., 2006). Things are further complicated by the fact that for many antibodies successful immuno-staining is only accomplished after the optimization of antigen retrieval, fixation, incubation times and dilutions of antibodies etc. (Niku et al., 2006; Polak and Van Noorden, 2003). Furthermore, some cell membrane antigens (such as T-cell sub-set antigens) might not survive routine fixation and wax embedding and can be successfully demonstrated only on frozen sections (Beesley, 1993). Finally, although antibodies that recognize T and B cells in a wide range of mammalian species have been reported, many are species specific (Jones et al., 1993; Niku et al., 2006).

As part of a project investigating the local immune response in cattle infested with ticks (Constantinoiu et al., 2010) we evaluated a panel of antibodies for the identification of immune cells in the skin sections of three breeds of cattle, including representatives of *Bos taurus taurus* (Holstein–Friesian), *Bos taurus indicus* (Brahman) and a stabilised composite breed (Santa Gertrudis, 5/8 *B. t. taurus* and 3/8 *B. t. indicus*). Recent analyses of SNP variation have demonstrated the wide divergence between indicine (*B. t. indicus*) and taurine (*B. t. taurus*) cattle with respect to genetic variability and suggests that antibodies developed for one subspecies may not be suitable for use in another species (Decker et al., 2009; Gibbs et al., 2009). The immuno-staining patterns of cells in the skin of these breeds of cattle produced by a panel of 12 antibodies are reported here.

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2. Materials and methods

2.1. Tissue samples

Tissue samples were collected from the perineum of three Holstein–Friesian cattle (100% *B. t. taurus*), three Brahman cattle (100% *B. t. indicus*) and twelve Santa Gertrudis cattle (5/8 *B. t. taurus* and 3/8 *B. t. indicus*) of similar age (12–24 months) before and after infestation with *Rhipicephalus microplus*. For each mAb and breed of cattle at least 5 sections derived from skin samples collected before and after tick infestation were immuno-stained and analyzed. However, the mAbs specific for CD45 and CD45RO were probed on sections cut from tissue samples collected before tick infestation only. The trial was conducted with the approval of the University of Queensland Animal Ethics Committee for Production and Companion Animals (Approval number: SVS/864/06/CRC and SVS/872/07/CRC).

The cattle were restrained in a crush and given an epidural injection of 5 mL of Lignocaine 20 mg/mL (Troy Laboratories Pty. Limited, Sydney, Australia) to desensitise the tail head and the escutcheon area. Skin biopsies were collected with 8 mm punches (Paramount Surgimed Ltd., New Delhi, India) and within 10 min of collection were placed in Tissue-Tek O.C.T. compound (Sakura Finetech Co., Tokyo, Japan.) that was frozen in isopentane (Labscan Asia Co., Ltd., Bangkok, Thailand) cooled with liquid nitrogen.

2.2. Monoclonal antibodies evaluated

Twelve different monoclonal antibodies for specific bovine immune cell types were evaluated in this study. Their source, stated specificity and associated references are outlined in Table 1.

2.3. Immuno-fluorescence

Various combinations of antibodies were used by double immuno-fluorescence labelling to investigate the immuno-staining patterns of leukocytes and their location in the skin of the cattle from different breeds. Cryosections, 6 µm thick, were mounted on Polysine™ glass slides (Menzel-GmbH & Co KG, Braunschweig, Germany) and dried overnight at room temperature (RT) with a fan. Next the sections were fixed in cold ethanol (4 °C) for 8 min. Because the method of embedding and fixation can alter the

epitopes of interest or make them inaccessible (Willingham, 1999) and determine whether an antibody labels the target cells or not, the intensity and pattern of staining, and the intensity of the background, four methods of fixation were tried (cold acetone for 10 min, cold methanol for 10 min, cold ethanol for 8 min or dried fixed). Following fixation the background staining was blocked with Image-iT FX signal enhancer (Invitrogen, Carlsbad, California, USA) followed by 10% [v/v] goat serum in 1% [w/v] bovine serum albumin (BSA, Sigma, St Louis, USA), in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ and 1.4 mM KH₂PO₄). The cryosections were further incubated overnight at 4 °C in a humidified chamber with monoclonal antibodies (100 µL per section) for specific leukocyte receptors (Table 1) diluted in 1% [w/v] BSA/PBS. IgG1, IgG2a and IgM negative control mouse monoclonal antibodies (DakoCytomation, Carpinteria, California, USA) in similar concentrations to the receptor specific antibodies were used as negative controls. The cryosections were washed in PBS and incubated with goat anti-mouse isotype-specific antibodies (100 µL per section) conjugated with fluorescein isothiocyanate (FITC) or Texas Red (Invitrogen, Carlsbad, California, USA) (1/400 [v/v] in 1% [w/v] BSA/PBS for 40 min at RT. After washing with PBS the nuclei were stained with DAPI dilactate (100 µL per section) (Invitrogen, Carlsbad, California, USA) and the slides were mounted with mounting medium (KPL, Gaithersburg, Maryland, USA). The slides were examined and photographed using an epifluorescent microscope, Olympus BX 51 (Olympus, Tokyo, Japan), equipped with a digital camera (Model DP 70, Olympus, Tokyo, Japan). The intensity of cell staining was assessed visually and the differences between breeds with regard to the intensity of cell staining were mentioned only when they were obvious. The images to be published were imported into Microsoft Office Picture Manager and the contrast/brightness adjusted similarly for all images.

3. Results

3.1. Sensitivity of the epitopes to the fixatives

Out of the four methods of fixation tried ethanol proved to have a less harsh effect on the epitopes recognized by most of the antibodies (for most antibodies the reaction was more intense and the background reduced after ethanol fixation, the reaction of antibodies CACT80C, IL-A116 and MM61A with their epitopes was abolished

Table 1
Monoclonal antibodies used to characterize the bovine skin immune cells.

Monoclonal antibody designation	Source	Antigen specificity	Isotype	Cellular expression	Dilution used	References
CACTB51A	VMRD	CD45	IgG2a	Leukocytes	1/800	(Keresztes et al., 1996; Niku et al., 2006)
IL-A116	VMRD	CD45RO	IgG3	Activated cells	1/400	(Bembridge et al., 1995)
MM1A	VMRD	CD3	IgG1	T cells	1/800	(Davis et al., 1993)
CH138	VMRD	Unknown	IgM	Granulocytes	1/400	(Keresztes et al., 1996; Naessens et al., 1996)
CACT80C	VMRD	CD8	IgG1	T cytotoxic cells	1/50	(Gutierrez et al., 1999)
MCA837G	AbD Serotec	CD8	IgG2a	T cytotoxic cells	1/50	(Gutierrez et al., 1999; Liebana et al., 2007)
HM57	DakoCytomation	CD79α	IgG1	B cells	1/100	(Jones et al., 1993)
IL-A29 ^a	ILRI ^b	γδ form of the T cell receptor	IgG1	γδ T cells	1/25	(Morrison and Davis, 1991)
IL-A21 ^a	ILRI ^b	MHC class II antigen	IgG2a	Macrophages, dendritic cells, B cells, activated T cells	1/200	(Taylor et al., 1993)
IL-A12 ^a	ILRI ^b	CD4	IgG2a	T helper cells	1/25	(Bensaid and Hadam, 1991)
IL-A111 ^a	ILRI ^b	CD25	IgG1	Activated cells (IL2-R bearing cells)	1/25	(Collins et al., 1998)
CC37	ILRI ^b	CD21	IgG1	B cells, follicular dendritic cells	1/25	(Naessens and Howard, 1991)

^a Monoclonal antibodies from tissue culture supernatant.

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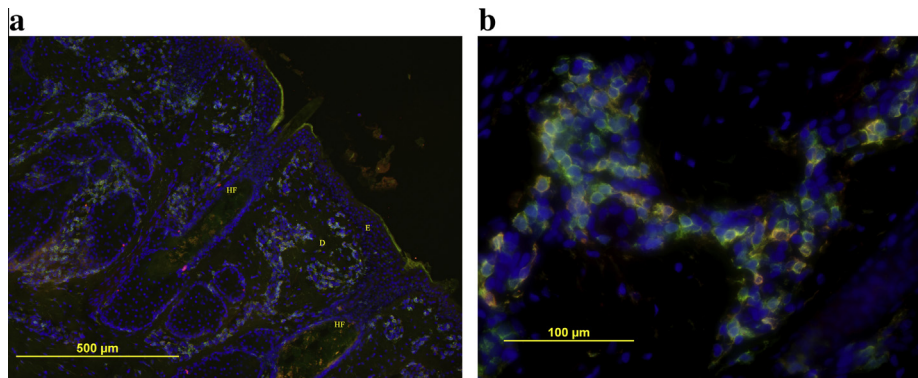


Fig. 1. CD45⁺ and CD45 RO⁺ cells in the skin of a Holstein–Friesian cow. The green cells bear CD45 antigens while the cells with different shades of orange bear both CD45 and CD45RO antigens ((a) overview and (b) detail). Cells nuclei are in blue. E: epidermis, D: dermis, HF: hair follicle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by the methanol fixation etc.) tested and was chosen as the routine fixative for all cryosections. However, there were antibodies (MCA837G, IL-A116, HM57) that stained better on acetone fixed sections. This method also preserved the structure of the tissues better.

3.2. Immuno-staining patterns of the leukocytes

The antibodies specific for CD45 and CD45RO antigens (Fig. 1a and b) reacted with cells in the skin samples of all three Holstein–Friesian cattle but with none in the skin of the three Brahman cattle. The antibody directed at CD45 antigens labelled cells from the skin of four out of the twelve Santa Gertrudis cattle while the antibody directed at CD45RO antigens stained cells of seven out of the twelve Santa Gertrudis cattle. All Santa Gertrudis cattle whose cells were positive for CD45 antigens were also positive for CD45RO antigens. However, three cattle whose cells were positive for CD45RO antigens were negative for CD45 antigens. These results were confirmed by flow cytometry using blood samples from the same animals in this study (Piper et al., 2008, unpublished data). The cells from the skin of the Holstein–Friesian and Santa Gertrudis cattle displayed a strong, similar staining with both antibodies.

The antibodies specific for T cells (CD3 complex) (Fig. 2a and b) and $\gamma\delta$ subset (WC1) of T cells (Fig. 3a–c) reacted with cells from all three breeds of cattle. However, in sections cut from the skin samples collected before tick infestation the cells recognized by the antibody specific to T cells (CD3 complex) and the $\gamma\delta$ subset (WC1) of T cells stained marginally more intensely in Brahman than in Holstein–Friesian cattle.

The antibody IL-A12 (specific for CD4⁺ cells) (Fig. 4a and b) and the antibodies CACT80C and MCA837G (specific for CD8⁺ cells) (Fig. 5a and b) stained cells of regular shape and no obvious differences were observed among the three breeds of cattle. For all breeds of cattle in this trial the antibody specific for MHC class II molecules (mAb IL-A21) (Fig. 2a and b) reacted with cells that had different shapes (most of them irregular shape) located in dermis, presumably dermal dendritic cells (DDC) and/or macrophages and cells from the epidermis, the Langerhans cells (LC) (Larregina and Faló, 2005). Generally, the reaction of this antibody with cells from the dermis was stronger than with cells from the epidermis. No differences between the three breeds were observed for this antibody.

The antibody CH138 (Fig. 3a–c) stained cells only in the sections cut from the samples collected after tick infestation. This antibody reacted with cells from dermis in sections from areas of skin with injuries caused by ticks (tick mouthparts fixed in the skin). The stained cells had a tendency to migrate towards skin injury or accumulate in intra-epidermal vesicles. In contrast this antibody did not recognize any cells in the tissue samples collected from areas of intact skin (before tick infestation) of all cattle from the three breeds but it labelled cells from the lumen of the blood vessels of the skin samples collected from these animals. No differences between the three breeds were observed for this antibody.

Antibody specific for CD25 (mAb IL-A111) cells labelled regular cells that showed a wide range of intensities of staining, from very weak to very strong (probably depending on the number of CD25 molecules on the surface of the cells related to the level of activation of the cells) (Fig. 4a and b). The same range of staining intensities was observed in the three breeds of cattle.

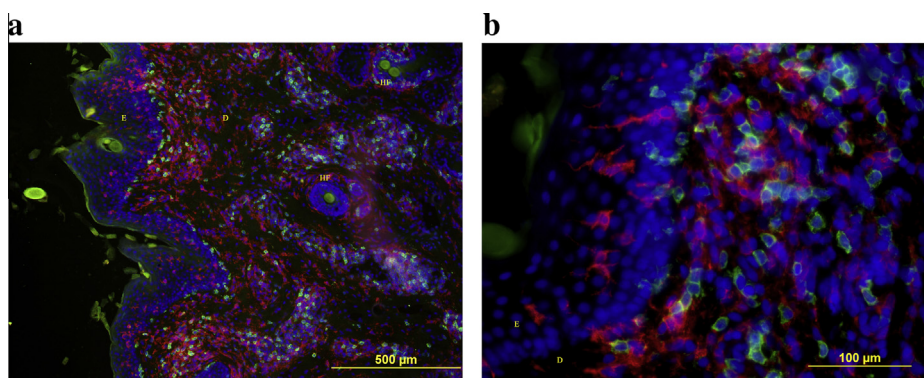


Fig. 2. MHC class II⁺ (red) and CD3⁺ (green) cells in the skin of a Santa Gertrudis cow ((a) overview and (b) detail). Cells nuclei are in blue. E: epidermis, D: dermis, HF: hair follicle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

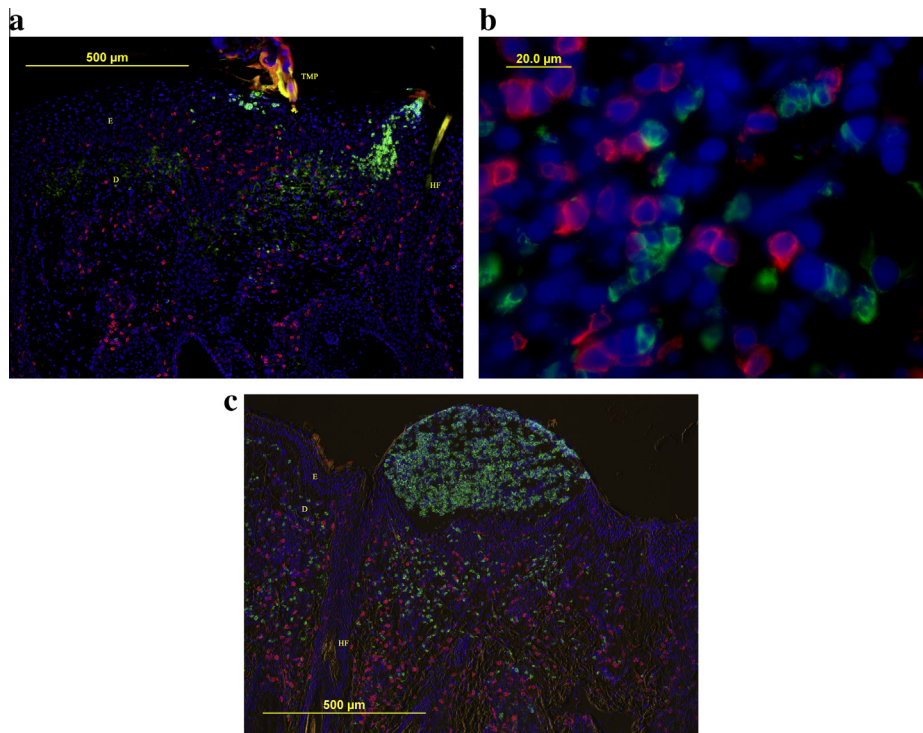


Fig. 3. $\gamma\delta$ T cells (red) and granulocytes (green) in the skin of a Holstein-Friesian cow (a) and a Brahman cow ((b) and (c)) ((a) overview showing two places where ticks were fixed, (b) detail and (c) epidermal vesicle). Cells nuclei are in blue. E: epidermis, D: dermis, HF: hair follicle, TMP: tick mouth parts. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

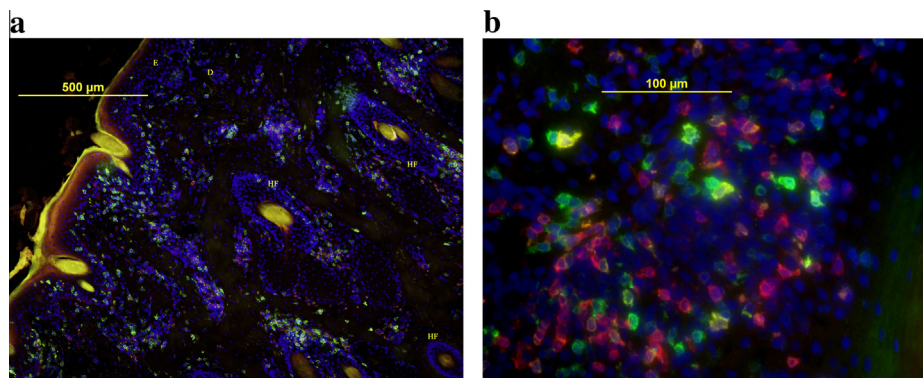


Fig. 4. CD4⁺ cells (red) and CD25⁺ cells (green) in the skin of a Holstein-Friesian cow. The cells with different shades of yellow-orange are likely to be T regulatory cells (CD4⁺/CD25⁺) ((a) overview and (b) detail). Cells nuclei are in blue. E: epidermis, D: dermis, HF: hair follicle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

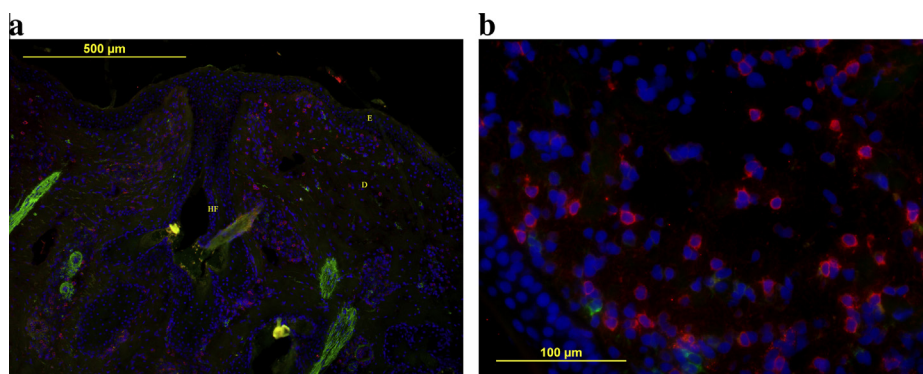


Fig. 5. CD8⁺ cells (red) (MCA837G) and B cells (green) (HM57) in the skin of a Brahman cow ((a) overview and (b) detail). Cells nuclei are in blue. E: epidermis, D: dermis, HF: hair follicle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

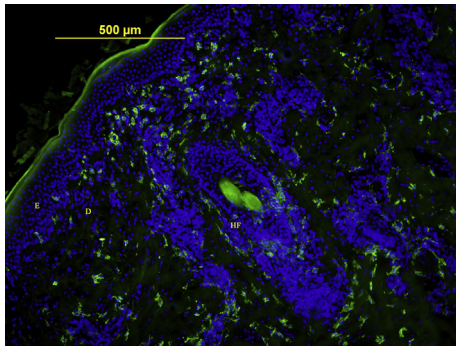


Fig. 6. Immuno-staining pattern of cells recognized by mAb CC37 in skin of a Santa Gertrudis cow. E: epidermis, D: dermis, HF: hair follicle.

For all cattle breeds in the trial a relatively weak reaction was observed with antibody HM57 (CD79 specific) that labelled two types of cells, some that have a circular shape, presumably B cells, and others whose shape resembled that of dendritic cells. Very few B cells could be observed in the skin of all breeds of cattle (Fig. 5a and b).

The antibody designated CC37 stained two types of cells, with regular and irregular shape (Fig. 6). The reactivity of this antibody was checked only with skin samples from Santa Gertrudis cattle so no comparison among the three breeds was carried out.

Generally, in all breeds of cattle the antibodies specific for CD3 cells, $\gamma\delta$ T cells, CD4 cells, CD25 cells (interleukin 2 receptor α -chain (IL-2R α)), CD8 cells and CD21 cells (mAb CC37) labelled cells that were located mainly in the dermis, most of them in the superficial dermis (within 0.5–0.6 mm from the epidermis). Occasionally a few of these cells were observed in the epidermis (mostly $\gamma\delta$ T cells and CD25 cells). Except for mAb CH138 that did not stain any cells in intact skin sections no other differences were observed in the staining patterns of the cells before and after tick infestation.

No reaction was seen on cryosections that were incubated with IgG1, IgG2a and IgM negative control mouse monoclonal antibodies (data not shown).

4. Discussion

Although the largest proportion of material is formalin fixed, paraffin embedded this combination is not the best choice for preserving antigenicity of tissues (Beesley, 1993; Seitzer et al., 2002). Some cell membrane antigens, including those present on some T- and B-cell phenotypes do not survive paraffin embedding and the chemical processing that follows (Beesley, 1993; Polak and Van Noorden, 2003). This is the reason why O.C.T. embedded, frozen tissues are preferred for immune-phenotyping these cells and generally more specific cell types can be detected with frozen sections than with paraffin sections (Beesley, 1993; Ward et al., 2006).

Monoclonal antibodies CACTB51A (specific for CD45) and mAb IL-A116 specific for CD45RO have been used in the past for labelling cattle leukocytes by both flow cytometry (Bembridge et al., 1995; Pelan-Mattocks et al., 2001) and immuno-histochemistry (Niku et al., 2006) but no differences in the reactivity of these antibodies in relation to the genetic composition of cattle have been reported. These differences might affect the interpretation of research involving *B. t. indicus* cattle and their hybrids as well as the results of research comparing the immune response mounted against various pathogens by *B. t. taurus* and *B. t. indicus* cattle. Furthermore, CD45 expression on bovine leukocytes using the same mAb as in the present study (CACTB51A) has been proposed as a tool for differentiation of lymphocytes and monocytes in leukograms

(Pelan-Mattocks et al., 2001). Our data suggest that the antibody is not suitable for that application in *B. t. indicus* breeds and would at best give inaccurate results.

These antibodies have been reported by the producer (VMRD, Inc., Pullman, USA) to react with leukocytes from water buffalo (*Bubalus bubalis*) and Cape buffalo (*Syncerus caffer*) (information sheet for mAb CACTB51A) or sheep (information sheet for mAb IL-A116), which are more distantly related to *B. t. taurus* than *B. t. indicus*. However, it has been shown that there is allelic polymorphism in the gene encoding CD45 among cattle (Ballingal et al., 2001). Ballingal et al. (2001) also demonstrated considerable polymorphism among European *B. t. taurus*, African *B. t. taurus* and Asian *B. t. indicus* with distinct genotype families common to each group. These genotype families were associated with specific cellular staining patterns in flow cytometric analyses of PBL. African and European taurine cattle stained uniformly with mAb IL-A116 (as used in the current study) and also with mAb IL-A150 (another antibody that recognizes bovine CD45RO). In contrast the indicine Boran and Sahiwal cattle stained in a highly variable manner. The results of the present study are highly consistent with the findings of Ballingal et al. (2001), suggesting that further investigation of the immune phenotype of animals with divergent CD45 genotypes would be warranted. Furthermore, the small number of Brahman and Holstein–Friesian cattle in this experiment warrants trials including larger number of animals from all breeds as well as a larger panel of antibodies specific for CD45/CD45RO antigens.

The higher intensity of staining of T cells and $\gamma\delta$ subset of T cells in skin of Brahman cattle suggests higher abundance of these antigens on the surface of cells from Brahman cattle than on the surface of cells from Holstein–Friesian cattle. However, this observation requires further studies to be confirmed as the fluorescence of individual cells from the two breeds was not quantified. CD3 proteins are associated with T cell receptor (TCR) and T cells that express fewer TCRs might be less responsive to antigen activation (Viola and Lanzavecchia, 1996). Activated T cells, including $\gamma\delta$ T cells, might express MHC class II molecules (Bujdoso et al., 1993) but in our experiments little overlap if any between T cells (CD3 receptor) and cells bearing MHC class II molecules (IL-A21) was observed in the skin cells of all breeds (Fig. 2a and b). This is in contrast to previous research carried out by flow cytometry with antibody IL-A21 (specific for MHC class II molecules), which identified class II proteins on the majority of activated CD4⁺ and CD8⁺ T cells cultured for 4–5 days in the presence of IL-2 and either phytohemagglutinin or pokeweed mitogen (Taylor et al., 1993). Currently we have no explanation for this but in the present trial activated cells (CD45RO or CD25 positive) were identified in large numbers in the skin of cattle from all breeds. Furthermore, the lower analytical sensitivity of immuno-fluorescence in comparison to flow cytometry might not be the cause of the differences between these two trials as in the present trial the mAb IL-A21 at the dilution used reacted very intensely with many cells in the skin of all breeds of cattle (Fig. 2a and b).

Dual fluorescence experiments showed that not all cells bearing CD4 antigens expressed CD3 antigens while all CD8⁺ cells expressed CD3 antigens. The CD4⁺ cells that did not express CD3 complex might have been monocytes or macrophages that can also bear CD4 antigens (Janeway, 2005). Furthermore, some $\gamma\delta$ T cells expressed CD8 antigens but none of them expressed CD4 antigens.

The antibody CH138 (Fig. 3a–c) is believed to react with granulocytes but the exact identity of the cells recognized by this antibody is unknown (information sheet for mAb CH138, VMRD, Inc., Pullman, USA). If it had not been for the lesions induced by the ticks no reaction with this antibody would have been observed in the layers of the skin of cattle from this trial. Under certain circumstances granulocytes might be induced to express MHC class II antigens on their surface (Culshaw et al., 2008; Gosselin et al.,

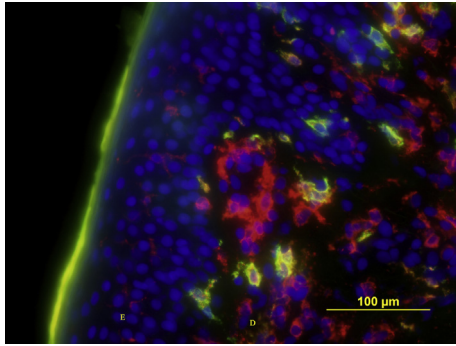


Fig. 7. Cells bearing MHCII molecules (red) and CD21 molecules (green, mAb CC37) in the skin of a Santa Gertrudis cow. E: epidermis, D: dermis, HF: hair follicle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1993). However, in the present trial the cells that reacted with mAb CH138 did not bear MHC class II antigens.

CD25 (interleukin 2 receptor α -chain) is expressed on activated cells, including T cells, B cells and monocytes as well as on T regulatory (T reg) cells (Barclay et al., 1997; Belkaid, 2007). The different intensities of staining of cells by mAb mAb IL-A111 might reflect different densities of interleukin 2 receptor α -chain on the surface of the cells that relates to the level of cell activation.

In addition to the CD79 α specific antibody (mAb HM57, DakoCytomation) two other antibodies specific for B cells (BAQ155A, VMRD and IL-A30, ILRI) were used to probe the skin sections but neither of them labelled any cells at all. However, under the same conditions all three antibodies showed a strong reactivity with cells of germinal centers on cryosections cut from the spleen of a *B. t. taurus* calf (data not shown), indicating that the lack of detectable B cells in skin was not an artefact.

The antibody designated CC37 has previously been shown to react with B cells and follicular dendritic cells (Naessens and Howard, 1991). Considering that few B cells seem to reside in the skin of these cattle it is likely that this antibody reacted mainly with dendritic cells. Some of the cells recognized by this antibody expressed MHC class II molecules recognized by the antibody IL-A21 while others did not (Fig. 7).

Apart from the differences in the staining patterns among breeds described in the present paper, differences in the numbers of cells in the skin of the three breeds were reported. The numbers of CD4⁺, CD8⁺, CD25⁺ and $\gamma\delta$ T cells were significantly higher in the skin of *B. t. indicus* cattle than in the skin of *B. t. taurus* while the numbers of CD3⁺ cells tended to be higher in the skin of *B. t. indicus* cattle but not significantly higher (Constantinoiu et al., 2010). Furthermore, the numbers of CD4⁺, CD8⁺, CD25⁺, CD3⁺ and $\gamma\delta$ T cells were significantly higher in the skin of *B. t. indicus* than in the skin of Santa-Gertrudis cattle (Constantinoiu et al., unpublished data).

5. Conclusions

This paper describes immuno-staining patterns of cells labelled by twelve monoclonal antibodies (Table 1) in the skin of cattle and the associations of the epitopes recognized by these antibodies on different cell subpopulations. The findings of this paper add to current knowledge of leukocyte markers for immuno-staining of cattle cells, especially of those from *B. t. indicus* breeds and they will be very helpful for future research investigating the immune response in the skin of cattle. The differences observed in the reactivity of the antibodies tested with cell populations from three breeds of cattle are also presented. The results of this paper show that the epitopes recognized by some antibodies (CACTB51A and IL-A116)

might not be present on the cell populations from all breeds of cattle or might be expressed in different levels (MM1A and IL-A29). This suggests that caution should be exercised when using some antibodies to compare the immune response in different breeds of cattle or when extrapolating the results obtained within one breed to other breeds. Furthermore the cells recognized by some antibodies might be present in the skin only under specific circumstances as it happened with mAb CH138 that labelled cells only in the skin with lesions.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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