Induction of delayed-type hypersensitivity reactions to *Candida albicans* in pasture-based dairy cows

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Abstract

Delayed-type hypersensitivity (DTH) reactions to *Candida albicans* are regularly used as an *in-vivo* indicator of cell-mediated immune function in cattle. Prior immunisation against *C. albicans* ensures a large clone of antigen-specific memory T cells, resulting in larger DTH responses and positive responses from a greater proportion of cows. Previous experimental results indicate that natural exposure to *C. albicans* is minimal in cows housed indoors and does not interfere with the interpretation of DTH responses to *C. albicans* in pre-immunised cows. However, housed cows may have different natural exposure to *C. albicans* than cows grazing fresh pasture outdoors. To determine if *C. albicans* is an appropriate antigen for inducing measurable DTH responses in pasture-based cows, DTH responses to *C. albicans* were assessed in ten cows pre- immunised with *C. albicans* and ten unimmunised control cows. A greater proportion of immunised versus unimmunised cows mounted a positive DTH response (90% vs. 70%), and responses were larger ($P<0.01$) and more variable ($P<0.05$) in immunised cows. Maximum relative double skinfold thickness increased 4.3 mm (±0.7; SEM) and 1.4mm (±0.2) in immunised and unimmunised cows, respectively. The results of this experiment suggest that pasture-based dairy cows in New Zealand have a low background exposure to *C. albicans* and pre-immunisation with *C. albicans* is required when using DTH responses as an indicator of cell-mediated immune function in pasture-based cows.

Keywords: *Candida albicans*; delayed-type hypersensitivity (DTH); dairy cow; pasture-based; housed; cell-mediated immune response (CMIR)

Introduction

Selection for production traits with little or no emphasis on health traits has led to an increase in the incidence of disease in dairy herds (Oltenacu & Broom 2010). Development of methods to assess immune response phenotypes in pasture-based NZ dairy cattle will allow selection pressure to be placed on health traits, and when used in combination with selection for important production traits, improve productivity, health & welfare in dairy herds. Cell-mediated immunity is crucial to protection against viruses and other intracellular parasites (Baldridge & Ward, 1997). Delayed-type hypersensitivity (DTH) reactions are localised cell-mediated immune responses (CMIR), mediated primarily by antigen-specific CD4+ T-cells which act to recruit macrophages to the reaction site. DTH reactions generally occur in response to intracellular pathogens (Baldridge & Ward 1997, Kobayashi et al. 2001) and are marked by a hard swelling at the site 24-72 hours after antigen exposure (Rosenstreich 1993). A DTH response only occurs if the animal has been exposed to the antigen previously and has memory T-cells specific for the antigen (Rosenstreich 1993).

Delayed-type hypersensitivity is an accepted *in-vivo* measure of CMIR in many species, including humans, rodents and pigs (Hessing et al. 1995; Martin et al. 2008; Neuvonen & Salo 1984). To assess CMIR in cattle, DTH responses to the intracellular yeast *Candida albicans* have been used (Heriazon et al. 2011). Importantly, the use of *C. albicans* does not interfere with the diagnosis of bovine infectious diseases, such as tuberculosis (Heriazon et al. 2009a). *C. albicans* is a common commensal of many mammals and it has been reported that dairy cows produce a mild DTH response to *C. albicans* without priming suggesting previous natural exposure (Heriazon et al. 2009a). To minimise the effects of individual variability in background exposure, most DTH testing protocols involve priming the test animals by immunising with *C. albicans* prior to DTH testing to generate an expanded pool of antigen-specific T cells. Immunised animals respond more consistently and display larger responses than cows that have not been immunised (Heriazon et al. 2009a).

Measurement of DTH responses to *C. albicans* has been used successfully in Canadian and Irish dairy herds to assess CMIR (Begley et al. 2009; Heriazon et al. 2009a; Mallard, 2007) but has not been investigated in NZ dairy herds. Cows assessed in both the Canadian and Irish dairy herds spend a large proportion of their time in housed environments. This is in contrast to NZ dairy herds which are predominantly pasture-based and not housed for extended periods of time. There has been limited research about the prevalence of *C. albicans* in dairy-farm environments because it is not an important cause of disease (Heriazon et al. 2009; Richard et al. 1980); however, it is plausible that housed cows are exposed to different pathogens and

have different commensal micro-flora to their pasture-based counterparts. For example, cows that are permanently housed have a higher prevalence of digital dermatitis than cows with access to pasture (Holzhauer et al. 2012; Laven & Holmes 2008), and the prevalence of mastitis-causing pathogens is different in pasture-based versus housed cows (Laven & Holmes 2008).

Differences in pathogen and commensal micro-flora exposure among farming systems may also extend to exposure to *C. albicans.* Yeast are isolated from a lower proportion of milk samples submitted for bacteriological culture in New Zealand (0.3%; Petrovski et al. 2011), than in other countries (1.3% to 25%; Casia dos Santos & Marin 2005; Hayashi et al. 2013; Moretti et al. 1998), indicating that exposure to *C.albicans* might be lower in New Zealand herds. If natural exposure to *C. albicans* is not consistently low in pasture-based cows then background exposure would need to be determined prior to priming with antigen and subsequent DTH testing, or an alternative antigen used to elicit the DTH response. The primary objective of this experiment was to determine the magnitude of background DTH responses to *C. albicans* in New Zealand pasture-based dairy cows and ascertain whether *C. albicans* is a suitable antigen to elicit measurable DTH responses for assessment of immune responsiveness on farm.

Materials and methods

The experiment was conducted at the DairyNZ Lye Farm, Hamilton (37°76'S 175°37'E, 45 m above sea level), during November and December 2010. All procedures were approved by the Ruakura Animal Ethics Committee, Hamilton, New Zealand, in accordance with the New Zealand Animal Welfare Act 1999.

Animals and immunisation

Twenty healthy, mid-lactation dairy cows of mixed age and breed were selected from the Lye Farm research herd (DairyNZ Ltd, Hamilton, New Zealand). The Lye Farm herd is milked twice per day and grazes a ryegrass and white clover pasture, with pasture silage and maize silage offered when pasture is limited. . The cows were run together in their normal herd, and no supplementary feed was offered, for the duration of this experiment. Average milk production at the time of experiment was 22.3kg per cow per day. Cows were grouped on body condition score (BCS) and then cows within each BCS group were randomly assigned to one of two experimental groups; immunised (n=10) or control (n=10). For the immunised group, the mean age was 4.1 ± 1.6 yr (mean ± SD) and mean BCS was 3.8 ± 0.4 (mean ± SD; 1-10 BCS scale, where 1 is emaciated and 10 obese; Roche et al. 2004). For the unimmunised group, the mean age was 4.6 ± 1.9 yr and the mean BCS was 3.8 ± 0.3.

The immune testing protocol involved immunisation on d0 and d14, followed by DTH testing on d21, as described by Heriazon et al. (2009b). Cows in the immunised group received an intramuscular (IM) injection of 0.5 mL of phosphate-buffered saline (PBS, pH7.4) containing 0.5 mg of type 1 antigen *C. albicans* (CaWC, My15 crude whole cell; Greer Laboratories Inc, Lenoir, NC, USA) and 0.5 mg of adjuvant (QuilA, 848051, Brenntag Biosector A/S, Frederikssund, Denmark) on D0 and D14. The injection also contained 0.5 mg of type 2 antigen; either hen egg-white lysozyme (HEWL) or human serum albumin (HSA). Immunisation with HEWL (n=5) or HSA (n=5) did not significantly influence DTH responses (P>0.05), therefore DTH responses from these cows were combined (immunised group, n=10) for analysis. Cows in the control group received an IM injection of 0.5mL PBS on day 0 (d0) and d14 (n=5), or received no injection (n=5). Immunisation with saline or receiving no immunisation did not significantly influence DTH responses (P>0.05), therefore DTH responses from these cows were combined (unimmunised group, n=10) for analysis.

Delayed-type hypersensitivity

The DTH skin test was performed on all cows on d 21, in the caudal skin folds on either side of the tail, as described previously (Heriazon et al. 2009a, Hine et al. 2011). Briefly, 0.1 ml PBS alone (control) or containing 0.1 mg of CaWC (test) were injected intradermally into the skin fold on opposite sides of the tail. Double-skin-fold thickness (DSFT) measures (3 repetitions), were taken at both test and control sites, prior to injection (0 h) and 7, 24 and 47 h after injection, using a spring loaded calliper (Harpenden, Mentone Educational Centre, Victoria, Australia).

Hine et al, (2011) defined a positive DTH response to CaWC as an increase in DSFT of >1mm at the test site relative to the control site. A similar criterion was used to define positive DTH responses in the current study. The number of cows that exhibited a positive response was calculated for each experimental group. The responses of all cows were included in statistical analyses, regardless of whether they met the threshold for a positive response.

Statistical analyses

For analysis, responses to DTH testing were expressed as the change in log of (DSFT at test site/DSFT at control site) from 0 h, as described by Hine et al. (2011):

$$\text{Response} = \log(T_h/C_h) - \log(T_0/C_0)$$

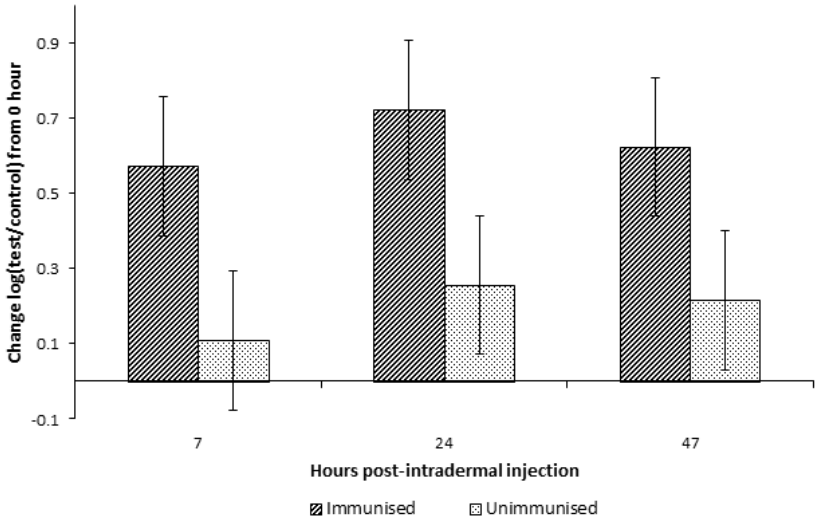
Where T_h = mean test DSFT at time h; T_0 = mean test DSFT at 0 h; C_h = mean control DSFT at time h; C_0 = mean control DSFT at 0 h; and time h was 7, 24 or 48 h. This response variable was used for all statistical tests. Responses were analysed in SAS® software (version 9.3, SAS Institute, Cary, NC, USA), using mixed models approach to repeated measures with time, treatment, and their interaction included as fixed effects. Tukey's test was used for pairwise

comparisons. F-tests were used to determine whether the variability of DTH responses differed between immunised cows and unimmunised cows, and between 24 and 47 h for immunised cows. Pearson product-moment correlation coefficients were computed between 0 h DSFT of the test fold and maximum relative DSFT, and between 24 and 47 h for immunised cows. Significance is declared if $P<0.05$.

Results

All cows had increased DSFT at the test site relative to the control site following the CaWC injection at 0 h. Seven of the ten unimmunised cows and nine of the ten immunised cows produced a positive DTH reaction. No positive reactions at control injection sites were observed. The responses observed at the test sites were limited to swelling of the caudal fold, with no apparent changes to the skin surface of any injection sites.

Figure 1 Delayed-type hypersensitivity (DTH) response to Candida albicans whole-cell, in dairy cows previously sensitised via immunisation (, n=10), and in unimmunised cows (, n=10). Responses are expressed as change in the log of (double skin fold thickness at the test site/doubles skin fold thickness at the control site) from 0 h, measured at 7, 24 and 47 h post-injection. Error bars indicate 95% confidence intervals. Responses in immunised cows were significantly larger than responses in unimmunised cows at every time point ($P<0.05$).



Mean maximum relative DSFT increases were 4.3 mm (±0.7; SEM) and 1.4 mm (±0.2) for immunised and unimmunised cows, respectively. Responses were larger in immunized cows at all time-points relative to unimmunised cows (Fig. 1). The increase in relative DSFT from 0 h to 7 h was five-fold greater in immunised cows than in unimmunised cows. The maximum response was more variable in immunised relative to unimmunised cows ($P<0.01$).

The average DTH response for both groups peaked at 24 h but was not significantly greater than responses observed at 47 h (immunised*:* $P=0.649$; unimmunised*:* $P=0.989$). There was no difference in variability of 24 and 47 h responses in immunised cows ($P=0.788$). Responses at 24 and 47 h were highly correlated in immunised cows ($r^2=0.79$, $P<0.01$). There was no correlation between maximum relative DSFT increase and 0 h test DSFT in the immunised group ($r^2=0.08$, $P>0.05$).

The time-dependent DTH responses for individual animals in each group depicted in Fig. 2 illustrate the increased variability of response magnitude in immunised cows. In the immunised group, the maximum relative DSFT increase was at 24 h for six cows, 47 h for three cows, and 7 h for one cow. In the unimmunised group, the maximum relative DSFT increase was at 24 h for four cows, 47 h for five cows, and 7 h for one cow (Fig.2).

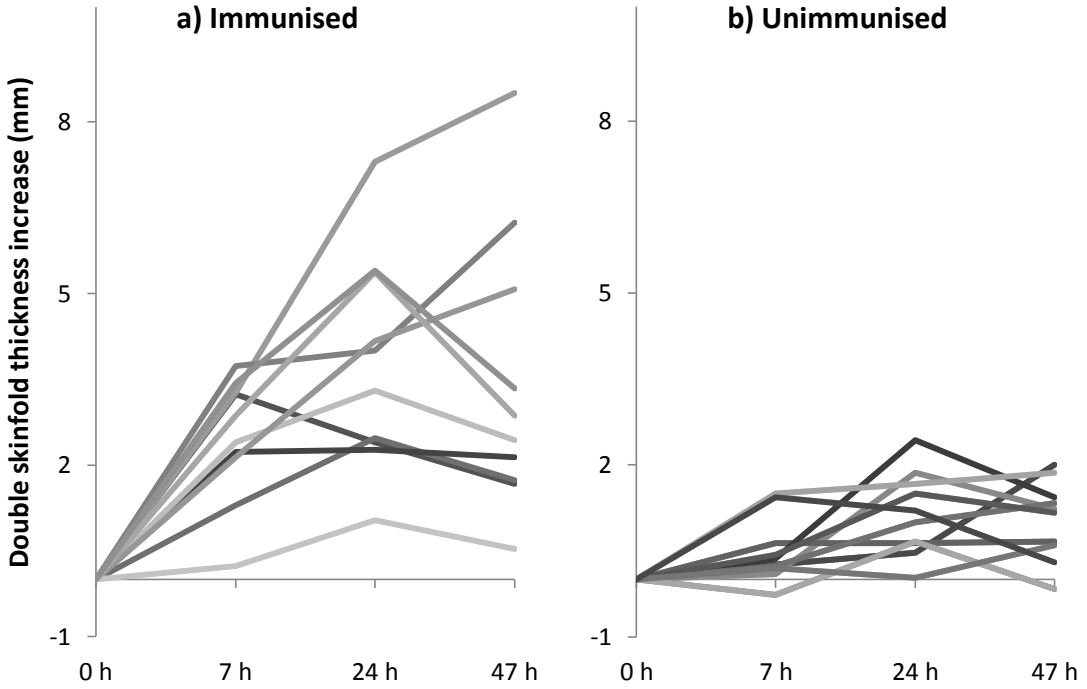
Discussion

Delayed-type hypersensitivity reactions to *C. albicans* have been used extensively in North-American dairy cattle to assess cell-mediated immune responsiveness (Heriazon et al. 2011, Heriazon et al. 2009a, Heriazon et al. 2009b, Hine et al. 2011). Studies have shown that these largely housed dairy cows produce mild DTH responses to *C. albicans* as a consequence of natural exposure. Nevertheless, previous experiments have included priming the immune system by immunising with *C. albicans* prior to DTH testing to minimise the effects of individual variation in natural exposure and to stimulate greater DTH responses. Natural *C.albicans* exposure of cows in New Zealand's pasture-based dairy herds may be different to their largely housed counterparts. Therefore, the objectives of this experiment were to investigate both background and pre-primed DTH responses of pasture-based dairy cows and to determine if DTH testing using *C. albicans* can be used successfully as an indicator of CMIR in pasture-based cows.

Background DTH responses to C.albicans

Results of the current study demonstrate that the pasture-based NZ dairy cows in the herd tested had low-level background DTH responses to *C. albicans* indicating low-level natural exposure to *C. albicans*. Although several unimmunised cows produced mild DTH responses, responses measured in immunised

Figure 2. Delayed-type hypersensitivity responses to *Candida albicans* of individual dairy cows that were previously immunised with *C.albicans* (a) or unimmunised (b). Responses are expressed as relative increase in double skin fold thickness from 0 h relative to the control site, at 0, 7, 24 and 47 h post-injection.



cows were much larger. Furthermore, responses were five-fold larger in immunised cows relative to unimmunised cows at 7 h post-injection. This contrasts with the results of Heriazon et al. (2009a), who reported that the magnitude of responses at 6 h were similar in immunised and unimmunized cows. Heriazon et al. (2009b) reported that cows immunised twice exhibited skin lesions such as pustules, crusting and dermatitis at the *C. albicans* injection site. In the current experiment, all DTH responses were limited to swelling of the caudal fold, with no apparent changes to the skin surface in immunised cows. These results indicate that the natural exposure to *C. albicans* was lower in the pasture-based cows tested here relative to housed cows and, therefore, it is likely not necessary to determine background DTH prior to priming with *C.albicans*. The number of cows used to determine background DTH in this experiment was small (n=10), and the results pertain to a single farm. Testing of background DTH to *C.albicans* in other pasture-based herds, in varied geographical locations and operating under various management systems will be required to confirm that natural exposure to *C.albicans* is typically low in pasture-based herds in New Zealand.

Pre-primed DTH responses in immunised cows

Results demonstrated that immunisation prior to conducting DTH testing successfully enhanced DTH responses. A greater proportion of immunised cows produced positive DTH responses, and their responses were three-fold larger at 24 and 47 h than in unimmunised cows. Testing of unimmunised cows showed that background sensitivity to *C. albicans* was consistently very low, therefore, the high variability observed in DTH responses from immunised cows is likely due to individual differences in immune responsiveness, rather than from differences in previous natural exposure to *C. albicans.* These results confirm that *C. albicans* can be successfully used to elicit measurable DTH responses in pasture-based cows to rank animals based on their cell-mediated immune responsiveness.

Kinetics of pre-primed DTHs

In the current study, peak DTH response times varied among cows indicating that measurement at both 24 and 48 h may be better than either time-point alone. Delayed-type hypersensitivity reactions are characterised by a hard swelling at the site 24-72 hours post antigen exposure (Rosenstreich 1993). While DTH responses at 7 h were significantly higher in immunised versus control cows, responses observed at 7 h are not expected to be indicative of true DTH responses but rather indicative of earlier-phase immune reactions (Black 1999; Hernández et al. 2005). Begley et al. (2009) identified 48 h after antigen exposure as the optimal time to assess DTH response. However, other researchers have measured DTH at both 24 and 48 h and found no significant difference between the magnitude of DTH responses at each time-point (Heriazon et al. 2011; Heriazon et al. 2009a; Heriazon et al. 2009b; Hernandez et al. 2003). Heriazon et al. (2009b) reported that cell infiltration to the DTH site following injection with *C.albicans* was

similar at 24 and 48 h and concluded that the response observed at both time points represented a classic DTH reaction. Hernandez et al. (2003) suggested that using the average of 24 and 48 h or selecting the time-point with greatest variability between cows is appropriate when using DTH responses to classify cows. In this experiment, 24-h responses were highly correlated with 47-h responses, however, for individual cows the time of peak response occurred equally at 24 and 47 h and ranking of cows based on the magnitude of the DTH response was variable between 24 and 47 h. Additionally, the variability was the same at 24 and 47 h. While measuring at 24 h would capture the peak response for most of the cows in the current experiment, our results suggest that DSFT should be measured at both time points when using DTH response to classify cows, as suggested by Hernandez et al. (2003).

Conclusion

Background exposure to *C. albicans* was minimal in the pasture-based New Zealand herd tested and DTH reactions to *C. albicans* can be successfully used as a measure of cell-mediated immune responsiveness. Cows produced only mild DTH responses to CaWC, when not pre-immunised with *C. albicans.* Furthermore immunised cows had a greater proportion of positive DTH responses, and produced significantly larger and more variable DTH responses than non-immunised cows.

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