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Hsp70 vaccination-induced primary immune responses in efferent lymph of the draining lymph node

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ABSTRACT

Bovine paratuberculosis is a highly prevalent chronic infection of the small intestine in cattle, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). In earlier studies we showed the protective effect of Hsp70/DDA subunit vaccination against paratuberculosis. In the current study we set out to measure primary immune responses generated at the site of Hsp70 vaccination. Lymph vessel cannulation was performed to obtain efferent lymph from the prescapular lymph node draining the neck area where the vaccine was applied. Hsp70 vaccination induced a significant increase of $CD21^+$ B cells in efferent lymph, accounting for up to 40% of efferent cells post-vaccination. Proliferation ($Ki67^+$) within the $CD21^+$ B cell and $CD4^+$ T cell populations peaked between day 3 and day 5 post-vaccination. From day 7, Hsp70-specific antibody secreting cells (ASCs) could be detected in efferent lymph. Hsp70-specific antibodies, mainly of the IgG1 isotype, were also detected from this time point onwards. However, post-vaccination IFN-γ production in efferent lymph was non-sustained. In conclusion, Hsp70-vaccination induces only limited Th1 type immune responsiveness as reflected in efferent lymph draining the vaccination site. This is in line with our previous observations in peripheral blood. The main primary immunological outcome of the Hsp70/DDA subunit vaccination is B cell activation and abundant Hsp70-specific IgG1 production. This warrants the question whether Hsp70-specific antibodies contribute to the observed protective effect of Hsp70 vaccination in calves.

1. Introduction

Paratuberculosis in ruminants is an infectious disease of the small intestine and a global problem of the livestock industry [1,2], affecting animal welfare and leading to substantial economic losses [3]. The disease is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Currently there is no vaccine registered for use in cattle in the European Union. Experimental studies with whole cell bacterins have shown that infection is not prevented, but occurrence of clinical disease is reduced [4]. The disadvantage of whole cell vaccines is their interference with tuberculosis diagnosis [5].

The development of new vaccines is based on the dogma that induction of an effective cellular response will limit infection. We previously showed that cows naturally infected with MAP mount cellular responses against heat shock protein 70 [6,7]. PBMCs, as well as lymphocytes isolated from the draining mesenteric lymph node, and intra-epithelial lymphocytes of the ileum showed proliferation upon stimulation with Hsp70 in vitro. In contrast, Hsp70-specific antibody responses were marginal in naturally infected animals [8].

In a subsequent experiment calves were vaccinated with Hsp70, using dimethyl dodecyl ammonium bromide (DDA) as an adjuvant, and experimentally challenged with MAP simultaneously [9]. Vaccination resulted in significantly reduced fecal shedding of MAP. In this setting the major immunological outcome was the induction of an Hsp70-specific antibody response. A vaccine-specific interferon-gamma (IFN-γ) response could not be detected in PBMCs. Also Hsp70-specific lymphocyte proliferation did not differ between controls and vaccinated animals.

Abbreviations: MAP, *Mycobacterium avium* subspecies *paratuberculosis*; Hsp70, 70 kDa heat-shock protein; DDA, dimethyl dodecyl ammonium bromide; ASC, antibody secreting cell; ConA, Concanavalin A.

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Follow-up studies analyzed the T and B cell response generated after Hsp70 vaccination with the goal to understand the protective immune response. T cell epitopes of Hsp70 were defined that activate MHC class II restricted $CD4^+$T cells in a large proportion of outbred cattle. Hsp70 vaccination induces short-lived effector T cells, which produce IFN-γ [10]. Recently, it was shown that the Hsp70 molecule is present in the cell wall of MAP and accessible to vaccination-induced antibodies [11].

Combined these studies indicated that vaccination-induced immune responses as measured in peripheral blood did not at all resemble infection-induced immune responses to Hsp70. As peripheral response might not adequately reflect the vaccination-induced immune response, we set out to study early-induced immune responses at the site of Hsp70 vaccination. As the primary response after vaccination is induced in the draining lymph node of the neck region, where the vaccine is applied, we used a prescapular lymph vessel cannulation strategy to directly measure the locally generated primary immune responses [12–15].

2. Materials and methods

2.1. Animals

Three Holstein-Friesian calves (3 months of age), from farms enrolled for over 10 years in the national Veterinary Health Service annual test-and-certification program and with the highest level of paratuberculosis unsuspected status were used in this study. Calves were housed in a conventional animal house, fed according to requirements and checked daily for general health.

The use of animals in the present study was approved by the experimental animal committee of Utrecht University and conducted according to existing regulations.

2.2. Antigens

Recombinant MAP Hsp70 was produced according to methods described in detail earlier [8]. Ovalbumin (control protein) was obtained from Sigma (USA) and purified protein derivative, prepared from *Mycobacterium avium* subspecies *paratuberculosis*, from the Central Veterinary Institute (Lelystad, the Netherlands).

2.3. Experimental design and surgery

Surgical procedures were described previously [15], with the modification that the efferent lymph vessel of the prescapular lymph node was cannulated without surgical removal of the prescapular lymph node. Efferent lymph was continuously collected in tissue culture grade plastic flasks (Corning) with a maximum collection volume of 300 ml containing 5 ml PBS, supplemented with 50 IU/ml penicillin and 50 mg/ml streptomycin (Gibco) and 1000 IU heparin (Leo Pharmaceuticals).

Animals were vaccinated once, subcutaneously in the neck region when the lymph flow stabilized, with 200 μg Hsp70 in 10 mg/ml DDA, formulated as published previously [9]. Lymph flow was collected up to 17 days post-vaccination.

Venous blood was drawn from the jugular vein at day 0 and 18. Serum was collected and stored at −20 °C until analyzed.

2.4. Hsp70 protein ELISA

Hsp70-specific antibodies were measured in lymph and serum by ELISA, described in detail earlier [16]. Lymph was used in a 1:2 and 1:10 dilution in ELISA blocking buffer (Roche).

2.5. Antibody secreting cell (ASC) ELISPOT

For the analysis of Hsp70-specific plasma cells we used the protocol of Lefevre et al. [17], with minor modifications. Wells were coated with 100 μl of 20 μg/ml Hsp70. We used alkaline phosphatase-labeled sheep anti-bovine IgG (Serotec) as secondary antibody (1:1000) and developed with NBT-BCIP.Lymph cells were added in two-fold serial dilution, starting from 5×10^5 cells/well. Spots were counted using an automated EliSpot reader (Aelvis). Results were expressed as spots per 1×10^6 efferent lymph cells.

2.6. Multiplex MSD cytokine assay

Simultaneous detection of IFN-γ, IL-1β, IL-4, IL-10, IL-12, and MIP-1β was performed in direct ex vivo efferent lymph supernatants using a custom bovine 7-plex cytokine/chemokine assay (MSD), according to methods published previously [18]. TNF-α was not analyzed, because of lack of assay reproducibility.

2.7. IFN-γ ELISPOT

Detection of Interferon-gamma producing cells was analyzed by ELISPOT. Interferon-gamma specific antibodies were purchased from Mabtech (Sweden), and the assay was performed according to their instructions. A total of 4×10^5 efferent lymph cells were added per well. Stimulants were added to 96-well filter plates (Milipore) in 100 μl volumes: mitogen Concanavalin A (ConA) at 2.5 μg/ml; PPDP and ovalbumin at 10 μg/ml; Hsp70 at 10, 2 and 0.4 μg/ml. Medium was used to evaluate background spots. All tests were done in triplicate. Results were expressed as delta spot forming units (SFU), the mean spot value of medium control wells was subtracted from the antigen stimulated wells, and expressed per 1×10^6 efferent lymph cells.

2.8. Lymphocyte stimulation assay

Antigens were added to 96-well flat bottom culture plates (Corning): ConA at 2.5 μg/ml; Hsp70 and ovalbumin at 10 μg/ml. Medium was used to evaluate background proliferation. Lymph cells were added at 4×10^5 cells per well and incubated in a 5% CO_2 incubator at 37 °C for 3 days. Proliferation was measured by a 7 h pulse of 1 μCi of [^{3}H]thymidine before cells were harvested and counted for β-emissions. Stimulation indices (SI) represent the mean counts per minute of triplicate wells stimulated with antigen divided by the mean counts per minute of triplicate wells stimulated with medium. Outlier values of triplicates were omitted when the coefficient of variance exceeded 1.0.

2.9. Flow cytometry

The following primary antibodies were used for flow cytometry analysis: anti-CD4-PE (CC8, Serotec), anti-CD8-PE (CC63, Serotec), anti-N24 (γδ T cell receptor, gb21-A, IgG2b, VMRD), anti-CD3 (MM1A, IgG1, VMRD), anti-CD21-like (B cells, GB25A, IgG1, VMRD), anti-Ki67-Alexa647 (b56, BD), anti-CD45RO (IL-A116, IgG3, VMRD). Secondary antibodies used were: goat-anti-mouse IgG1-biotin, goat-anti-mouse IgG2-PE, goat-anti-mouse IgG3-FITC (Southern Biotech). The secondary reagent used to visualize biotin-conjugated antibodies was streptavidin-eFluor® 450 (eBioscience). Matched isotype-controls were used if appropriate.

In short, 1×10^6 lymph cells were stained with antibodies diluted in 100 μl FACS buffer (PBS with 2% FCS and 0.01% azide). Cells were labeled with antibodies for 30 min on ice and washed twice in between with 200 μl FACS buffer. Intracellular staining of Ki67 was performed with the Cytofix/Cytoperm intracellular staining kit (BD Biosciences). FACS analysis was conducted using a