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Evaluation of IFN-γ production in bovine hypodermosis using ELISPOT and ELISA

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Abstract

Background: Two enzyme immunoassays, ELISPOT and sandwich ELISA, were compared in order to evaluate the production of interferon gamma (IFN-γ) in peripheral blood mononuclear cell (PBMC) cultures from *Hypoderma* (Diptera: Oestridae) infested cattle.

Methods: Cell cultures from *Hypoderma*-infested (sensitized) and uninfested cattle (non-sensitized) were stimulated with the mitogen phytohemaglutinin A (PHA) and with different *H. lineatum* antigens, crude larval extract (CLE) and its purified fractions (hypodermin A, B and C). IFN-γ secreting cells (SC) were detected using an ELISPOT test, whereas theIFN-γ levels presented in supernatants from parallel cell cultures were measured by a sandwich ELISA; the same bovine specific IFN-γ antibodies were employed in both tests.

Results: The addition of *H. lineatum* antigens had an immunomodulatory effect on PBMC cells from both infested and uninfested cattle, characterized by suppression in the production of IFN-γ. ELISPOT results showed that hypodermin B was the antigen with major immunosuppressive effect on non-sensitized cultures, while CLE had the strongest impact on previously sensitized cultures. Our results revealed that the ELISPOT showed a high sensitivity allowing the determination of IFN-γ-SC frequencies in non-stimulated cultures; in contrast, the sandwich ELISA was not useful for detecting IFN-γ levels in parallel culture supernatants.

Conclusion: The ELISPOT test allows an accurately determination of the frequency of IFN-γ-SC in *ex vivo* PBMCs without the need for extensive re-stimulation *in vitro* with antigen or mitogen over long periods of time.

Keywords: Cattle-arthropoda, *hypoderma*, IFN-γ, ELISPOT, ELISA, cellular responses

Introduction

Warble flies (*Hypodermabovis*, L. and *Hypoderma-lineatum*, De Villers) cause myiasis in cattle from the Northern Hemisphere, leading to a serious negative impact on animal productivity and welfare. Newly-hatched larvae penetrate the skin aided by the secretion of enzymes from the parasites' midgut (hypodermins A, B and C). Then, they migrate through fascial planes of muscles and connective tissue to the oesophageal submucosa, where they over winter before migrating to, and eventually completing their development in, the subdermal tissues of the back [1]. Larval secretions are also implicated in immunomodulatory processes, allowing the survival of the parasite [2].

It is well known that young cattle are the most susceptible to *Hypoderma* infestation, since old cattle develop acquired resistance after repeated exposures to *Hypoderma* larval antigens [3]. This resistance has been recognized as an important factor in controlling larval populations, depending on both the host age and the number of larvae invading the host [4].

Interferon-gamma (IFN-γ), secreted by Th1-type cells, plays an important role in the activation of cell-mediated immunity. It is also considered as the main cytokine implicated in both the activation of phagocytic cells and the production of some IgG sub classes from B cells. This cytokine is produced predominantly by natural killer and natural killer T cells as part of the innate immune response, and by Th1 CD4 and CD8 cytotoxic T lymphocyte effector T cells after the development of antigen-specific immunity [5].

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The profile of production of this cytokine during the course of hypodermosis was investigated in previous studies by sandwich enzyme-linked immunosorbent assay (ELISA) with variable results. In naturally infested cattle, a significant reduction on IFN-γ serum levels during the resting phase of *H. lineatum* larvae in the oesophagus was observed by sandwich ELISA [7]. In contrast, in experimentally infested cattle IFN-γ serum levels remained below the detection limit of the test (1ng/ml) during larvae penetration [6], although immune histochemistry results showed a significant increase of the number of IFN-γ^+ cells detected in the skin.

The enzyme-linked immunospot assay (ELISPOT) has demonstrated to be highly sensitive for the *ex vivo* quantification of cytokine-secreting cells after *in vitro* stimulation with an antigen [8]. In addition, while ELISA shows the final cytokine concentration without revealing the number and rate of cytokine-secreting cells, ELISPOT shows the frequency of antigen specific T-cells [9].

In this study, an ELISPOT assay for the detection of IFN-γ secreting cells (SC) was developed in order to study the immunomodulatory effect of *H. lineatum* larval antigens in both previously sensitized and non-sensitized cattle peripheral blood mononuclear cells (PBMCs). The frequencies of IFN-γ-SC detected by ELISPOT and the levels of IFN-γ in culture supernatants measured by sandwich ELISA were also compared.

Material and methods

Animals and PBMC collection

Four Frisian cows (4-8 years old) that presented warbles on their back were used in this study as previously sensitized cell donors. Apart from *Hypoderma* infestation, those animals were apparently healthy. Four Frisian calves (6-8 months old), without previous contact with the parasite, were chosen as non-sensitized cell donors.

Blood samples were collected in heparinised tubes by caudal venipuncture and PBMCs were individually isolated by density gradient centrifugation using Ficoll solution (specific gravidity 1.077 g/ml, Biochrome AG) according to a previous study [10]. Viability of cells was determined by Trypan Blue dye exclusion, considering valid a percentage above 90%.

Antigen preparation

The antigens used in this study were prepared from *H. lineatum* first instar larvae (L-1) collected from oesophagi of cattle slaughtered in a local abattoir. Larvae were homogenized with a Polytron tissue homogenizer (Kinematica AG), using 0.1M Tris-HCl pH 7.5 buffer. The homogenate was centrifuged at 10.000 rpm for 5 minutes and the supernatant, that constituted the crude larval extract (CLE), was collected. Hypodermins A, B and C were after wards purified by ion-exchange chromatography, using diethylamino etil (DEAE)-cellulose (DE-52, Whatman) as anion exchanger. After the dialysis of the different fractions, the purity of the hypodermins was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in reduced conditions. The protein concentration for each antigen was determined by the bicinchoninic method (BCA Protein Assay Reagent, Pierce) and adjusted to 100µg/ml. Finally, the different antigens were stored at -20°C until needed.

ELISPOT assay

Membrane microplates (PVDF, Millipore) were prewetted with 50 µl of ethanol 70% for 2 min. After washing with phosphate buffer saline (PBS; pH 7.5), plates were coated with 100 µl/well of the capture antibody diluted in PBS (mAb IgG2 to bovine IFN-γ, Serotec) at 1µg/ml and incubated overnight at 4°C. Plates were then blocked with 300 µl/well of phosphate buffer saline Tween 20-bovine serum albumin (PBST-BSA) 1% and incubated for two hours at room temperature (RT) in agitation.

PBMCs collected from each individual animal were resuspended at a concentration of 2×10^5 cells/well in complete medium (CM) containing Roswell Park Memorial Institute medium (RPMI 1640, Sigma-Aldrich), 10% bovine foetal serum (BFS, Biochrom AG) and 1% antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, USA) andtested in triplicate. PBMCs were stimulated with the mitogen phytohaemagglutinin (PHA) at 6 µg/well and/or different parasitic antigens (CLE, HyA, HyB and HyC) at a final concentration of 100 µg/ml. Optimal concentrations of antigens and mitogen were established in previous studies [10].

Control wells contained complete medium only (CM background control), CM plus cells (cell control) or CM plus cells and mitogen (mitogen control). Plates were incubated at 37°C in 5% CO_2 for 24h. Then, the cells were aspirated and wells were rinsed four times with PBST 0.01%; 100 µl/well of the secondary biotinylated antibody (mAb IgG2 to bovine IFN-γ, Serotec) diluted in PBS at 1µg/mlwere added, and plates were incubated for 2h at RT in agitation. In order to reduce the background, the secondary antibody was filtered through a 0.22µm pore size filter (Millex®-GP, Millipore). Alkaline phosphatase (Sigma-Aldrich) was used as streptavidin conjugate at 1:1000 in Tris buffer saline (TBS) pH8 and colour was developed using 5-bromo-4-chloro-3-indolyl-phosphate, nitro blue tetrazolium (BCIP-NBT, Sigma-Aldrich) as chromogenic substrate. Finally, plates were rinsed with distilled water and let dried completely before analysis. The number of spots/well was counted manually under a stereomicroscope. Scores from 0 to 5 were established using the following criteria: (0) no spots; (1) less than 50 spots/well; (2) 50-100 spots/well; (3) 100-200 spots/well; (4) 200-400 spots/well; (5) more than 400 spots/well.

ELISA assay

Parallel cultures, using the same conditions than in the ELISPOT, were performed in uncoated 96-well flat-bottomed cell culture plates (Deltalab). Cell culture supernatants were removed after plate centrifugation, and the presence of IFN-γ

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was tested by a sandwich ELISA using a previously described method [6]; the bovine specific IFN-γ antibodies employed were the same used in the ELISPOT test.

Statistical analysis

All statistical tests were performed using Statistical package. Student t test for dependent samples was conducted to investigate the presence of significant differences between PHA stimulated and non-stimulated cultures ($P<0.05$). Student t test for independent samples was employed to find significant differences between cultures from both *Hypoderma* sensitized and non-sensitized animals ($P<0.05$).

Results

Determination of IFN-γ secreting PBMCs by ELISPOT

Figure 1 shows the effect of the mitogen PHA and different *H. lineatum* antigens on the number of IFN-γ-SC. Spontaneous secretion was observed in mitogen unstimulated cultures, although the number of IFN-γ-SC was higher in non-sensitized PBMC. The stimulation with the mitogen PHA increased the number of IFN-γ-SC in both sensitized and non-sensitized PBMC cultures, with a more pronounced effect in the former. The incubation of PBMC with *Hypoderma* antigens reduced the number of IFN-γ-SC in both groups. In previously sensitized cultures, the highest reduction in the number of IFN-γ-SC was caused by CLE, followed by HyB and HyA. In contrast, HyB was the fraction with major immunosuppressive effect in non-sensitized cultures, followed by CLE and HyA; HyC barely affected the number of IFN-γ-SC in both groups. Nevertheless, no significant differences ($P>0.05$) were detected in response to stimulation with the mitogen and/or the antigens, even in cultures from previously sensitized animals.

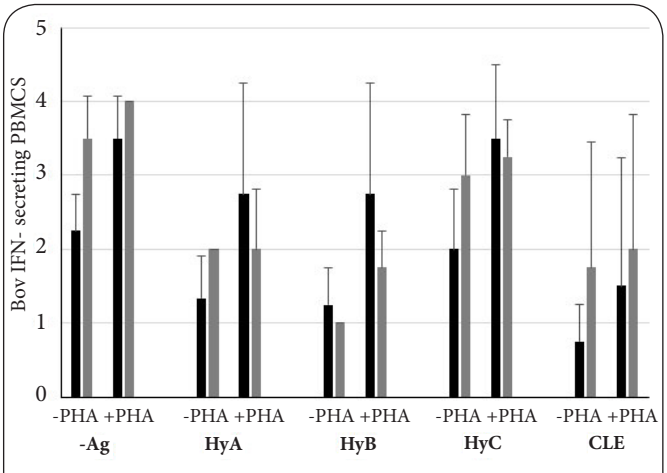

Figure 1. IFN-γ secreting cells detected by ELISPOT in bovine PBMCs stimulated with *H. lineatum* antigens. Cells from non-sensitized (□) and sensitized (▪) cattle were incubated in absence (−PHA) and presence (+PHA) of the mitogen PHA. Results are presented as the average and standard deviation. Axis y: Score 0: no spots. Score 1: <50 spots/well. Score 2: 50-100 spots/well. Score 3: 100-200 spots/well. Score 4: 200-400 spots/well. Score 5: >400 spots/well.

Determination of IFN-γ secretion in cell culture supernatants by sandwich ELISA

Figure 2 shows the effect of the mitogen PHA and different *H. lineatum* antigens on IFN-γ levels from culture supernatants using a sandwich ELISA. As occurred with the ELISPOT, the spontaneous secretion of IFN-γ was higher in PBMC from naïve animals; in contrast, the stimulatory effect of the PHA was higher in previously sensitized cultures. However, these differences were not significant ($P>0.05$).

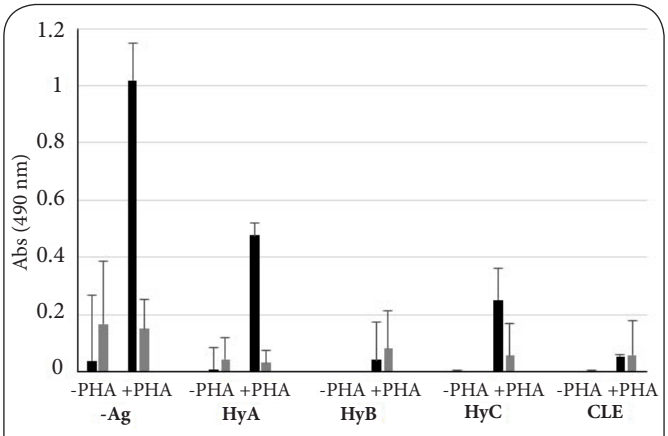

Figure 2. IFN-γ levels detected by sandwich ELISA in bovine PBMC culture supernatants stimulated with *H. lineatum* antigens. Cells from non-sensitized (□) and sensitized (▪) cattle were incubated in absence (−PHA) and presence (+PHA) of the mitogen PHA. Results are presented as the average and standard deviation.

The addition of larval antigens reduced IFN-γ levels in both groups of cultures, but the differences were only significant in previously sensitized PBMC cultures (HyA, $P=0.030$; HyB, $P=0.044$; HyC, $P=0.045$; CLE, $P=0.030$). IFN-γ levels were very low in no mitogen-stimulated cultures, where as IFN-γ levels were below the detection limit of the test (1 ng/ml) in non-sensitized cultures stimulated with HyB and CLE. In general, HyA was the antigen showing the least effect on IFN-γ secretion, where as CLE was the most immunosuppressive fraction in previously sensitized cultures and HyB in non-sensitized ones.

The frequencies of IFN-γ-SC could not be correlated with the levels of secreted IFN-γ because the latter were often below the detection limit of the ELISA.

Discussion

Spontaneous secretion of IFN-γ was detected in all unstimulated cultures by both techniques, being higher in cultures from non-infested calves, in which IFN-γ is secreted by cells of the innate immune system [11,12]. Innate IFN-γ production is mainly found in young animals, although older animals may also respond in a similar way [13].

According to previous *in vitro* studies [10], the addition of *Hypoderma* antigens has a suppressive effect on IFN-γ secretion. These results suggest that cattle infested by *Hypoderma* under natural conditions display a significant reduction of IFN-γ-SC, which may contribute to larval survival in the host.

The number of antigen-specific T cells is frequently very low *ex vivo*; in such situations, *in vitro* T cell expansion strategies, as the addition of a mitogen, are needed. The stimulation of PBMC cultures with the mitogen PHA enhanced the frequency of IFN-γ-SC, especially in previously sensitized cultures. In this sense, cattle repeatedly exposed to *Hypoderma* presented a more intense cellular response to the antigens and mitogens [4] and acquire a certain degree of resistance to this parasite that results in fewer warbles on their back. The subpopulation of PBMC associated with the *in vitro* proliferative response to PHA has been shown to have characteristics of T cells which are known to be responsible for cell-mediated immune responses [14].

Hypodermin B was the fraction that most reduced the secretion of IFN-γ in non-sensitized cultures and, in general, the HyA was the antigen with minor suppressive effect on IFN-γ secretion. These findings contrast with previous studies that reported that HyA induced a significant elevation of IFN-γ production by bovine PBMC [10], being the antigen that most inhibited proliferative responses to themitogens [2,15,16]. By contrast, CLE, composed by a mixture of the three fractions, showed the strongest suppressor effect on IFN-γ secretion in antigen-sensitized cultures. This immune suppressive effect of CLE in mitogen-stimulated PBMC cultures from naïve and previously sensitized cattle has been previously reported [10].

Hypoderm in C barely affected to the synthesis of IFN-γ in all PBMC cultures. These results coincide with other authors [10,17], who proved that HyC did not show proliferative, cytostatic or toxic activity on bovine lymphocyte cultures. Furthermore, it was demonstrated that the administration of HyC to previously *Hypoderma*-infested and uninfested cattle presented no detectable effects on cellular and humoral responses [17].

The high sensitivity of the ELISPOT allowed the determination of IFN-γ-SC frequencies in non-stimulated cultures, where as the sandwich ELISA has proven to be ineffective in detecting IFN-γ levels in parallel culture supernatants. Those results confirm the ability of the ELISPOT to accurately determine the frequency of IFN-γ-SC in *ex vivo* PBMCs without the need for extensive restimulation *in vitro* with antigen or mitogen over long periods of time.

However, the ELISPOT also presents some inconvenient, since itis more time consuming and expensive than the ELISA. Moreover, ELISPOT showed a noticeable variability in the overall reactivity of PBMCs obtained from different donor animals. That deviation may be due to differences in PBMC activation status or simply to the variation expected between animals. It should be considered that even a slight increase in the number of secreting cells becomes detectable with this technique. Previous investigations reported that immune responses to antigen and mitogenic stimulation were extremely variable between animals, especially in the reinfested ones [8].

Conclusion

The ELISPOT assay has an unsurpassed sensitivity to detect low frequency antigen-specific T cells secreting effector molecules such as cytokines. The secreted cytokine is captured by the antibodies coated on the ELISPOT plates, avoiding their diffusion and dilution on the supernatant, as occurs in the ELISA [8].

Further studies should be performed in order to detect cells secreting other cytokines such as IL-10 or IL-4, leading to a better understanding of the mechanisms of susceptibility or resistance to *Hypoderma*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	EC	CL	PD	APC	MPM	PDB	RP
Research concept and design	✓	--	--	--	--	✓	✓
Collection and/or assembly of data	✓	✓	✓	✓	✓	--	✓
Data analysis and interpretation	✓	✓	--	--	--	✓	✓
Writing the article	✓	--	--	-	--	--	✓
Critical revision of the article	--	✓	✓	✓	✓	✓	--
Final approval of article	✓	✓	✓	✓	✓	✓	✓
Statistical analysis	✓	✓	--	--	--	--	--

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