IFN-γ production from NK cells: implications for diagnostic testing

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INTRODUCTION

The IFN-γ test is used to detect bovine tuberculosis and paratuberculosis at an early stage of the infection before the development of clinical signs. The rationale for measuring IFN-γ production as a diagnostic parameter is that T-cells, previously sensitised through the T-cell receptor/MHC complex, will recognise antigens specific for a particular infection. It has been demonstrated however that young calves especially often respond to both avian and bovine PPD in the IFN-γ test without having any evidence of infection (McDonald et al. 1999, Jungersen et al. 2002). An explanation for these observations can be that the exposure of the newborn animal to a variety of new antigens stimulate the immune system and may result in a higher degree of activated cells in peripheral blood. These activated cells could subsequently be more easily triggered to produce IFN-γ by innate mechanisms. The fact that young animals are more prone than adult cattle to elicit this non-specific IFN-γ production in response to PPD makes it unlikely that it is caused by environmental mycobacteria sensitisation. We have previously shown that neither CD4+, nor CD8+ nor γδT-cells were responsible for IFN-γ production in non-infected cattle (Olsen and Storset 2001). A possible source is thus the NK cells that are involved in the early immune responses against intracellular pathogens and are known to secrete large amounts of IFN-γ. Bovine NK cells have recently been characterised and a monoclonal antibody against the NK cell specific receptor, NKP46, is available (Storset et al. 2003, Storset et al. 2004). The aim of the present work was to study the influence of NK cells on the IFN-γ production in young calves.

MATERIALS AND METHODS

Antigens.

A 14 kDa secreted antigen (MPP14) was purified from M. avium subsp. paratuberculosis as described previously (Olsen et al. 2000) Purified Protein Derivative (PPD) from M. tuberculosis was obtained from the National Veterinary Institute, Oslo, Norway. Recombinant ESAT-6 was kindly provided by Dr. John Pollock Veterinary Science Division, Stormont, Belfast and Dr. Peter Andersen, Statens Serum Institute, Copenhagen. Native MPB70 was provided from Prof. Morten Harboe, The National Hospital, Oslo. MPP14, ESAT-6 and MPB70 were used at a concentration of 2 μg/ml and PPD at 10 μg/ml.

Animals.

Clinically healthy Norwegian Red dairy cattle in areas free from paratuberculosis and bovine tuberculosis were used in the present study.

IFN-γ production in whole blood.

Heparinised whole blood was dispensed into 24-well tissue culture trays (1 ml/well) and stimulated with purified MPP14, ESAT-6, MPB70 and PPD and incubated at 37°C in 5% CO₂ in air for 24 hours. The plasma was removed and assayed for IFN-γ using the bovine IFN-γ EASIA (Bovine IFN-γ EASIA, Biosource, Nivelles, Belgium) according to the manufacturer’s instructions.
Depletion of NK cells.
Peripheral blood mononuclear cells (PBMC) were isolated from EDTA blood by density gradient centrifugation (1150 x g, 20 min) on Lymphoprep (Nycomed Pharma, Oslo, Norway). NK cells were removed using MACS system with an LD column (Miltenyi Biotec Ltd., Bisley, UK). The cells were stimulated with MPP14, ESAT-6, PPD or medium alone and incubated at 37°C in 5% CO₂ in air for 24 hours. The supernatant was assayed for IFN-γ.

Intracellular staining for IFN-γ
Performed as described previously (Olsen and Storset 2001)

RESULTS
Altogether 31% of the animals had an IFN-γ response to ESAT-6, 50% to MPP14 while 37% responded to PPD. In contrast, no IFN-γ response was detected in response to MPB70. In general, the animals responding to MPP14 also responded to ESAT-6 and PPD. ESAT-6 is present in the M. tuberculosis complex and has also been detected in M. kansasii and M. flavens (Harboe et al. 1996) while MPP14 was detected by Western blotting in the M. avium complex and M. scrofulaceum (Olsen et al. 2000).

To identify the cells producing IFN-γ, intracellular staining for IFN-γ together with labelling for surface markers and analysis by flow cytometry was used (Olsen et al. 2005). The method identified NK cells as the main IFN-γ producing cell-type while little IFN-γ was produced by CD4+ cells (Fig. 1). Furthermore PBMC from responding animals were depleted of NK cells using a monoclonal antibody directed against NKp46, followed by secondary antibodies coupled to magnetic beads. The removal of NK cells completely abolished the IFN-γ production in response to MPP14, ESAT-6 and PPD confirming that NK cells are a source for IFN-γ production.

DISCUSSION
The IFN-γ test has been used in several countries to diagnose early stages of bovine tuberculosis. The use of specific proteins like ESAT-6 has clearly increased the specificity of this test (Pollock and Andersen 1997, Buddle et al. 2003). However, it is widely recognised that the test cannot be used in young animals due to non-specific IFN-γ production (McDonald et al. 1999, Jungersen et al. 2002). This was confirmed in the present study where one third of young cattle from areas free from both bovine tuberculosis and paratuberculosis responded with IFN-γ production to various antigens was tested. It is unlikely that such a large percentage of study animals were sensitised to mycobacteria harbouring both MPP14 and ESAT-6, and this suggested that other mechanisms for IFN-γ production were present.
Fig. 1. Flow cytometry plots showing IFN-γ production by NK cells. Whole blood was stimulated with MPP14, ESAT-6 or left unstimulated for 6 hours. The cells were labelled with mAbs against; A) CD4 and B) the NK-cell marker NKp46 followed by permabilisation and labelling for intracellular IFN-γ. Numbers in upper right quadrant indicate the percentages of total lymphocytes that are IFN-γ producing CD4+ (A), IFN-γ producing NK cells (B).

In conclusion, our results demonstrated that NK cells are a source of innate IFN-γ production, and this provides an explanation for the problems encountered in the young calves. This is also in agreement with the finding that young calves had the highest proportion of NK cells in peripheral blood (Kulberg et al. 2004). Consequently, it is likely that NK cells are responsible for the majority of the non-specific IFN-γ production observed in young cattle. Methods that inhibit IFN-γ production from NK cells would therefore lead to an improvement of the IFN-γ test for detection of bovine mycobacterial diseases. This should be a focus of further research in addition to the identification of specific antigens from M. a. paratuberculosis.

REFERENCES


A full length paper of these results has been published in Infection and Immunity. 2005. 73: 5628-5635 Olsen I, Boysen P, Kulberg S, Hope JC, Jungersen G and Storset AK. Bovine NK cells can produce IFN-γ in response to the secreted mycobacterial proteins ESAT-6 and MPP14, but not in response to MPB70.