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Effects of Mycophenolic Acid (MPA) Treatment on Expression of Fc Receptor (FcRn) and Polymeric Immunoglobulin Receptor (pIgR) mRNA in Adult Sheep Tissues

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Aim. To quantify the expression of Fc receptor (FcRn) and polymeric immunoglobulin receptor (pIgR) mRNA under a long-term influence of mycotoxin mycophenolic acid (MPA), which is used in human transplantation medicine due to its immunosuppressive properties and is a common contaminant in silage.

Method. We applied 300 mg MPA/day in nine sheep for nine weeks and compared them with untreated animals (n = 9). The expression level of Ig receptor mRNA was determined in eight different adult ovine tissues (liver, kidney, jejunum, ileum, spleen, thymus, mesenteric and pharyngeal lymph nodes). For a reliable and sensitive mRNA quantification of Ig receptor subtypes, a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was used with a relative- and tissue-specific efficiency corrected quantification model (REST).

Results. Each tissue exhibited an individual expression pattern of FcRn and pIgR mRNA. Both types of Ig receptors were highly expressed in the liver, kidney, and gastrointestinal tract. Medium-to-low expressions were found in the spleen, thymus, mesenteric and pharyngeal lymph nodes. FcRn mRNA was significantly down-regulated by MPA in the liver ($p = 0.02$). After MPA treatment, a significant up-regulation of pIgR mRNA expression was observed in the ileum and liver ($p = 0.04$ for both). Expression level of FcRn mRNA in the tissues in decreasing order was as follows: liver > kidney > jejunum > ileum > spleen > thymus > mesenterial lymph node > pharyngeal lymph node; whereas expression level for pIgR mRNA was the following: liver > kidney > jejunum > ileum > pharyngeal lymph node > spleen > thymus > mesenterial lymph node.

Conclusion. The MPA exhibited immunomodulatory effects in the liver and ileum of treated sheep. Its possible immunosuppressive effects may be explained by lowering the level of FcRn expression in the liver, which resulted in a lower IgG serum-to-bile transport. However, MPA showed stimulatory effects on pIgR expression in the liver and ileum, suggesting good IgA and IgM transport in these tissues.

Key words: *mycophenolic acid; receptors, Fc; receptors, polymeric immunoglobulin; reverse transcriptase polymerase chain reaction; RNA, messenger; sheep*

Animal health is one of many factors that affect the economic efficiency in livestock production. Grass and maize silages are frequently contaminated with fungi belonging to various genera, which are responsible for serious contamination with mycotoxins (1). *Penicillium roqueforti*, which produces mycophenolic acid (MPA), is considered one of the most important fungal contaminants in the food chain and silages (2,3).

MPA is a weak organic acid with antibacterial, antifungal, antitumor, and antiviral properties (4). It exhibits toxicity in farm animals if applied in relatively high doses. In mouse, the 50% lethal dose (LD_{50}) of orally administrated MPA is 250 μg/g (5). However, 2-morpholinoethyl ester (MMF) of MPA is used in humans for the long-term therapy after organ transplantation (5). Additionally, MPA is known for its immunosuppressive effects on cell proliferation and induction of differentiation in many cell types and cell lines (5). Consequently, feeding livestock with MPA-contaminated silage may modulate the immune system of the animals. Schneweis et al (3) reported that the consumption of immunosuppressive compounds increased the risk of infectious diseases in livestock.

Many mechanisms can protect an individual from the harmful effectors of pathogenic organisms. We investigated the mRNA gene expression of specific immunoglobulin (Ig) receptors responsible for the immune response by regulating the Ig transport and cellular concentrations in various tissues (6-8). In neonates and adults, the Fc receptor (FcRn) is specific for IgG, whereas the polymeric immunoglobulin re-

ceptor (pIgR) recognizes dimeric IgA and pentameric IgM. Ig Fc receptors are of special interest because of their ability to transduce signals between bound, cross-linked Igs and the cell interior, leading either to activation or down-regulation of cellular functions (6). There are two well-defined Fc-domain functional classes of mammalian receptors (8,9). One class of receptors transports Igs across epithelial tissues to their sites of action. This class includes FcRn as a transport receptor for maternal IgG to offspring. Upon ingestion of the colostrums, the Igs are transported across the intestinal barrier of the neonate into its blood (10). The other class is the pIgR, which in humans and ruminants transports both IgA and IgM (11), and mediates the transcytosis of polymeric IgA across epithelial cells (12). The pIgR is a type I membrane protein with a large extracellular region arranged in five domains that are homologous to the variable-like domains of the Ig superfamily (12,13), whereas the FcRn is a type I membrane protein related to major histocompatibility complex (MHC) class I molecules (6). Furthermore, pIgR mediates the transcytosis of polymeric IgA and pentameric IgM across epithelial cells, and transports these Igs in the basolateral-to-apical direction (6,12). Expression of the pIgR gene in epithelial cells of mucosal and glandular tissues is an unconditional prerequisite for acquiring mucosal immunity (13). Transportation of IgG is mediated by FcRn and, like pIgR-IgA and pIgR-IgM transport, involves transcellular transport. Recent data indicate that FcRn could be important in immune activation and tolerance (14).

The main objective of this study was to quantify FcRn and pIgR mRNA expression levels in different tissues of adult healthy sheep treated with MPA to determine if these levels could be associated with any immunomodulatory effect. In addition, the comparison of the expression levels between different tissues of untreated animals was also performed.

Material and Methods

Animals

The study was performed on 18 healthy six-month-old male crossbred sheep (Merino Landschaf × Schwarzkopfschaf). Nine sheep were treated with 300 mg MPA/day/sheep orally for nine weeks, and nine animals in the control group received placebo with no MPA. MPA was produced by Hoffman-La Roche Ltd. (Basel, Switzerland). The applied dose was calculated according to the "worst-case scenario" of silage contamination (3). After the treatment period, sheep were slaughtered at the slaughterhouse in Grub (EU official slaughterhouse of the Bayerische Landesanstalt für Tierzucht, Poing, Germany). Tissue samples of immune and gastrointestinal system (spleen, thymus, liver, kidney, ileum, jejunum, and pharyngeal and mesenteric lymph nodes) were collected 10-15 minutes *post mortem* and stored in the liquid nitrogen until the total RNA extraction.

Total mRNA Isolation

The RNA was isolated from 300-400 mg tissue. Tissue samples were homogenized in 2 mL TriPure buffer (Roche Diagnostics, Basel, Switzerland) by using an Ultra-Turax homogenizer (T 25 Janker & Kunkel, Staufen, Germany) according to an established method (15). Manufacturer's instructions were carefully followed to isolate the total RNA from the tissue. The optical density OD_{260} of extracted total RNA was determined in duplicates by using a Biophotometer (Eppendorf, Hamburg, Germany) at three different dilutions of the final RNA preparations. The purity was assessed by nucleic acid/protein ratio at OD_{260}/OD_{280}. The ratio $OD_{260}/OD_{280} > 1.80$ was obtained for all samples.

Reverse Transcription of Ovine mRNA into cDNA

Total RNA was reversely transcribed with 200 U MMLV (Molony Murine Leukemia Virus) reverse transcriptase Rnase H minus (Promega, Madison, WI, USA) from 1 μg RNA by using 100 pmol random hexamer primers (MBI Ferments, St. Leon-Rot, Germany) in a volume of 40 μL on Mastercycler Gradient (Eppendorf, Hamburg, Germany). The resulting complementary DNA (cDNA), which was reversely transcribed from total RNA, served as template for real-time polymerase chain reaction (PCR).

Oligonucleotide Primers for PCR Amplification

Primers for the PCR reactions – pIgR, FcRn, and ß-actin (Table 1) – were designed with the HUSAR software tool by using a multiple sequence alignment called *Clustal* (Analysis Package software version 4.0, DKFZ, Heidelberg, Germany) from ovine or consensus sequences (GenBank, National Center for Biotechnological Information). Each primer pair was selected from different exons to ensure that amplified cDNA could be distinguished from any amplified genomic DNA contamination. The β-actin gene was used as a housekeeping gene to confirm constant expression levels in the targeted cDNA samples. The integrity of isolated RNA was evaluated by the amplification of β-actin cDNA to validate constant housekeeping gene expression levels within the investigated tissues and to assure identical RNA extraction efficiencies.

PCR Amplification of Ovine Immunoglobulin Receptor cDNA

Real-time PCR was performed on the LightCycler platform, as described previously, with slight modification (16). Briefly, the PCR mixture for amplification of cDNA was performed in a total volume of 10 μL containing 25 ng cDNA, 4 mmol/L $MgCl_2$, 0.4 μmol/L of forward (sense) primer, 0.4 μmol/L of reverse (anti-sense) primer, and 1xLightCycler DNA FastStart SYBER Green I mix (Roche Diagnostics, Mannheim, Germany). After DNA denaturation and polymerase activation at 95°C for 10 minutes, the temperature cycling consisted of 40 repeated cycles composed of 4 segments as follows: denaturation (95°C for 15 s); PCR-product-specific annealing temperature (64°C for FcRn and pIgR; and 62°C for β-actin) for 10 s; polymerase extension (72°C for 25 s); and fluorescence acquisition for 5 s (88°C for FcRn; 84°C for pIgR; and 87°C for ß-actin). The fluorescence acquisition in the 4th segment at the increased temperature was done to eliminate non-specific fluorescence signal and to ensure the accuracy of the desired product quantification (16). We performed "Second Derivate Maximum Method" to determine the crossing point using LightCycler Software 3.5 (Roche Molecular Biochemicals). Crossing point is defined as the point at which the fluorescence rises appreciably above the background fluorescence (17). In the "Second Derivate Maximum Method", a second derivate maximum within the exponential phase of the amplification curve is linearly related to a starting concentration of

Table 1. Oligonucleotide primers designed to specifically detect ovine immunoglobulin receptors FcRn and pIgR and housekeeping gene (β–actin)

Receptors	Primer sequence (5'-3')*	Type	PCR product (bp)	Acc. No.
FcRn	f-TGAACGGCGAGGAGTTCATG	Ovine	288	AJ313190[†]
	r-GCTCAGGTGGGTAGAAGGAGAA			OAR313190[†]
pIgR	f-ATGTGAGCCTGGAGGTCAGCCA	Ovine	353	AJ313189[†]
	r-CTCCAGCACCTGGAGGTCAA			OAR313189[†]
β -actin	f-AACTCCATCATGAAGTGTGAC	Consensus	234	AF935774
	r-GATCCACATCTGCTGGAAGG			U02295

*f – forward; r – reverse.
[†]Accession number in GenBank and EMBL databases.

template cDNA molecules (17). The PCR-product length was verified by gel electrophoresis as a single band at the expected length (data not shown). The specificity of the band was confirmed by the melting curve analysis of LightCycler Software 3.5. The detailed sequence analyses of the PCR products for the ovine FcRn and pIgR were done by Medigenomics GmbH (Martinsried, Germany). Both newly elucidated ovine FcRn and pIgR sequence were submitted directly to the sequence database GenBank and European Molecular Biology Laboratory (EMBL) with the accession numbers AJ313190/OAR313190 and AJ313 89/OAR31389, respectively (Table 1).

Mathematical Analysis and Statistical Evaluation

The relative expression software tool *REST-XL* (18) was used for the calculation of relative expression levels in real-time PCR. The mathematical model was based on the PCR efficiency and the group mean crossing point difference between the MPA-treated versus the control group. The corresponding real-time PCR efficiency (E) in the exponential phase was calculated using the equation $E = 10^{[-1/slope]}$, applied to a dilution series ranging from 0.20 pg to 50 ng cDNA in triplicate (17). The target gene expression (FcRn and pIgR) was normalized via the β-actin as a reference, according to the following equation:

$$\text{ratio} = \frac{(E_{\text{target gene}})^{\Delta CP_{\text{target gene }(mean\ control - mean\ MPA)}}}{(E_{\text{beta-actin}})^{\Delta CP_{\text{beta actin }(mean\ control - mean\ MPA)}}}$$

The effects of MPA treatment on FcRn and pIgR expression level were calculated according to the crossing point (CP) group means and the corresponding tissue-specific real-time PCR efficiencies. Data for group differences were presented as mean ± standard deviation (SD) values.

Statistical Analysis

Statistical analysis of group differences was done by Pair Wise Fixed Reallocation Randomization Test©, which is implemented in the REST-XL software (18). Differences in expression between control and treated samples were assessed in group means for statistical significance by randomization tests.

Calculation of the Mean Expression Level of FcRn and pIgR

To get an overview of the tissue-specific FcRn and pIgR mRNA, tissue-specific expression levels were compared in untreated animals (n = 9). The tissue-specific β–actin expression value was used for normalization in each tissue (ΔCP), according to the following equation:

$$\Delta CP = \text{mean } CP_{\text{target gene}} - \text{mean } CP_{\beta\text{-actin}}$$

The ΔCP value represented the difference between the ΔCP in the specific tissue and the tissue with the lowest expression, ie, pharyngeal and mesenteric lymph node for FcRn and pIgR, respectively:

$$\Delta\Delta CP = \Delta CP_{\text{tissue with the lowest expression}} - \Delta CP_{\text{specific tissue expression}}$$

Relative expression level (fold-differences), compared with the lowest expression (equal to 1.0), were given by the arithmetic formula $E^{\Delta\Delta CP}$, calculated with the determined tissue-specific amplification efficiency given in Table 2:

Table 2. Tissue specific real-time polymerase chain reaction (PCR) efficiencies of reference gene (β-actin) and target genes (FcRn and pIgR)*

	PCR efficiency		
Tissue	β-actin	FcRn	pIgR
Ileum	1.70	1.87	1.74
Jejunum	1.63	1.85	1.75
Kidney	1.63	1.67	1.84
Liver	1.80	2.01	1.65
Spleen	1.74	1.66	1.90
Thymus	1.56	1.70	1.86
Pharyngeal lymph node	1.56	1.67	1.91
Mesenterial lymph node	1.55	1.71	1.95

*The corresponding real-time PCR efficiency (E) in the exponential phase was calculated by using the equation $E = 10^{[-1/slope]}$, applied to a dilution series ranging from 0.20 pg to 50 ng cDNA in triplicate (17).

Tissue mean expression level (x-fold) = $E^{\Delta\Delta CP}$

Results

Extracted total RNA contents showed no significant variations during MPA treatment compared with the control group (data not shown). FcRn, pIgR, and β-actin mRNA were abundant in all investigated tissues including spleen, thymus, liver, kidney, jejunum, ileum, pharyngeal and mesenteric lymph nodes. β-actin mRNA expression was not significantly different between MPA-treated and control group and served as an optimal housekeeping gene (data not shown). Homology of the newly elucidated ovine FcRn and pIgR sequences (AJ313190 and AJ31389) at the mRNA level was 99% and 100%, respectively, which is comparable to the published bovine sequences for FcRn (AF162866) and pIgR (X81371).

To determine the overall expression level of FcRn and pIgR mRNA in the adult tissues, we calculated normalized mean expression levels (Table 3). The lowest expression level of FcRn mRNA was obtained in the pharyngeal lymph node ($1.67^{\Delta\Delta CP} = 1.0$-fold) and the highest in the liver ($2.01^{6.83} = 117.7$-fold). In MPA-treated animals, a significant down-regulation of FcRn mRNA was observed in the liver (0.14-fold; $p = 0.02$), whereas in the remaining tissues no significant changes were found in comparison with the control group (Fig. 1). Constant expression levels were observed in jejunum (0.79), thymus (1.07), ileum (1.37), mesenteric lymph node (1.45), pharyngeal lymph node (1.56), spleen (1.74) and kidney (1.74).

The lowest expression level of pIgR mRNA was observed in mesenteric lymph node (Table 3). The highest mean expression level was observed in liver ($1.65^{8.92} = 87.1$-fold). A significant up-regulation of pIgR mRNA was shown in liver (2.41; $p = 0.04$) and in ileum (4.23, $p = 0.04$) of MPA-treated sheep (Fig. 2). Constant expression levels of pIgR mRNA were observed in kidney (0.86), pharyngeal lymph node

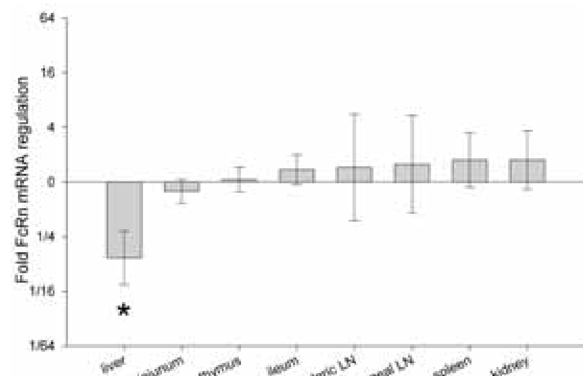

Figure 1. Effect of mycophenolic acid (MPA) treatment with 300 mg per day per sheep on Ig receptors FcRn mRNA expression level in ovine tissues (liver, thymus, jejunum, ileum, pharyngeal and mesenteric lymph node, kidney and spleen; each n = 9) in comparison with untreated control (each n = 9). Expression changes were shown as n-fold up- or down-regulation (mean±SD). Significant changes are indicated by an asterisk (p = 0.02). LN – lymph nodes.

Table 3. Mean tissue specific expression level of FcRn and pIgR mRNA in various tissues of untreated animals

	Expression (CP±SD) of			Comparison (fold-difference)	
Tissue	FcRn	pIgR	β-actin	FcRn vs pharyngeal lymph node	pIgR vs mesenteric lymph node
Ileum	28.88±2.17	30.90±3.11	20.34±2.16	5.05	30.8
Jejunum	28.71±1.51	30.63±2.28	20.73±2.47	6.94	46.0
Kidney	22.09±5.66	27.74±1.65	17.60±1.71	17.8	56.3
Liver	28.09±3.67	31.61±2.27	23.79±2.30	117.7	87.1
Spleen	28.56±3.10	33.78±4.57	19.58±5.11	3.0	5.1
Thymus	30.94±3.21	36.93±4.67	21.45±2.64	2.4	1.5
Pharyngeal lymph node*	30.06±2.55	31.88±3.30	18.93±5.13	1.0	11.7
Mesenteric lymph node*	27.89±3.19	34.93±3.55	18.17±7.16	2.1	1.0

*Calculation is based on crossing point data of animals in the experiment, normalized via the internal housekeeping gene expression of β-actin and converted to fold-difference of expression compared to tissue with the lowest expression level (1.0-fold) on the basis of tissue specific real-time PCR efficiencies given in Table 2 (see equations in the text).

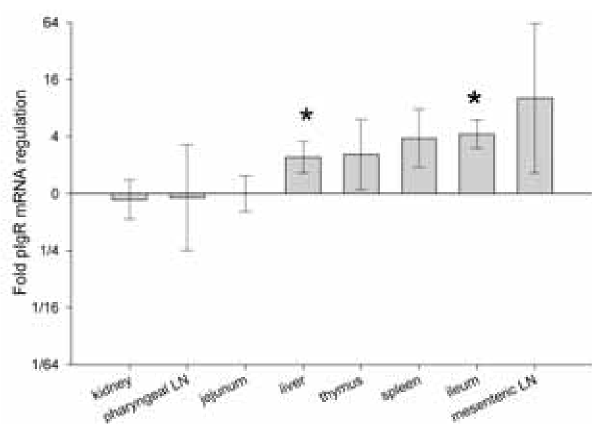

Figure 2. Effect of mycophenolic acid (MPA) treatment (300 mg MPS/day per sheep) on polymeric immunoglobulin receptor (pIgR) mRNA expression level in ovine tissues (liver, thymus, jejunum, ileum, pharyngeal and mesenteric lymph node, kidney and spleen; each n = 9) in comparison with untreated control (each n = 9). Expression changes were shown as n-fold up- or down-regulation (mean±SD). Significant changes in liver and ileum are indicated by an asterisk (p = 0.04). LN – lymph nodes.

(0.91), jejunum (1.0), thymus (2.58), spleen (3.84) and mesenteric lymph node (10.2).

Discussion

We tested a number of lymphoid organs and gastrointestinal tissues in adult sheep each receiving nutritionally-relevant MPA treatment, ie, 300 mg MPA per day, to determine whether there was an immunosuppressive effect on the expression level of two Ig receptors, FcRn and pIgR. During the MPA treatment, we observed no dose effect on the amount of total RNA extracted from tested tissues. We found good responses to Ig receptor expression, despite the low abundance of these mRNA concentrations in immunological tissues. We performed a relative quantification using real-time RT-PCR to see whether the regulation through MPA occurred at the FcRn and pIgR mRNA level. Although FcRn was highly abundant in the liver, kidney, and the gastro-intestinal tract, a significant down-regulation of FcRn expression was observed in the liver only. It has been speculated that the function of the FcRn in the liver is associated with serum-to-bile IgG transport, or that the receptor might serve as a protective mechanism against catabolism in hepatocytes (7). Current evidence suggests that FcRn is the catabolic receptor that controls the lifetime of IgG in the serum (19). Mayer et al (10) described the expression and localization of the FcRn in the small intestine of the newborn lamb. Our data showed high expression levels in adult gastrointestinal tissues with only slight FcRn mRNA changes in the jejunum and ileum. Thus, it seems that FcRn is used for IgG transport at the sites other than the liver in adult as well as neonate animals (10).

Recently published animal studies of pIgR and FcRn expression mainly used a single tissue (19) of rodents (20-22) or pig (23), and no direct comparison between the expression levels in these tissues could be made. The studies performed in ruminates were mainly focused on the mammary gland cells and small intestine of neonatal lambs (10,12,14). Ig transmission through mammary epithelial cells has been studied in detail, but there are no data available for other tissues or organs (10). A clear understanding of the regulation of receptor level in the adult tissue could be useful for understanding serum Ig levels and for development of new strategies for passive immunization.

High MPA concentrations reported in the liver and kidney tissues are probably the consequence of the detoxification and excretion function of these organs (24). The sites of interconversion and excretion of MPA are the liver, bile, and intestine (25). Because the liver, kidney, and intestine are highly exposed to MPA, we expected there would be an immunomodulatory or immunosuppressive effect on the regulation of mRNA expression of FcRn and pIgR.

Significant regulation of pIgR can be explained by the role of the pIgR as protector of the mucosal barrier from colonization and invasion of epithelium or as an active transporter (6,7). In our study, MPA resulted in significant up-regulation of pIgR in the liver and ileum. Therefore, high transport rates of IgA and IgM in bile and gastrointestinal tract might be expected.

Although FcRn and pIgR are expressed in the bovine and ovine mammary gland (12,26), their precise localization has not been investigated. Our result, demonstrating significant up-regulation of pIgR in the ileum, is in line with recently published data (27), which bring the ovine system closer to fetal diversification in swine, rabbit, primates, and mice (28). For pIgR mRNA, we found high increase in expression levels between treated and control group, but the dif-

ferences were not significant due to high interanimal variability. Wide variations in pIgR mRNA expression were especially prevalent in the immunologically relevant tissues, such as thymus, spleen, and lymph nodes, probably due to intra-animal variations activation of the immune functions by antigens, or even variations at the level of a single lymph node. A multiple sampling of various mesenteric lymph nodes might improve the homogeneity of expression results.

To avoid further errors in mRNA quantification, we chose a relative quantification system (16,17), normalized by a constantly expressed, or housekeeping, gene. In addition, we applied an efficiency correction to different amplification efficiencies appearing in different tissues, due to tissue-specific PCR inhibitors (29).

We demonstrated the presence of the FcRn and pIgR mRNA expression in all investigated tissues of adult sheep. Both Ig receptor subtypes were highly expressed in the liver. MPA treatment showed divergent expression changes for FcRn mRNA in liver, as for pIgR in liver and ileum. The down-regulation of FcRn mRNA expression in liver may result in immuno-suppressive effects, because IgG serum-to-bile transport rate may decrease. MPA has stimulatory effects on the pIgR expression in liver and ileum, leading to a higher protection rate of IgA and IgM and/or higher transport rate into bile. To conclude, a long term MPA treatment was demonstrated to have potential immunomodulatory effects at nutritionally relevant concentrations in ruminants.

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