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Effect of immunomodulation in turkeys infected with haemorrhagic enteritis virus on the percentage of CD4$^+$ and CD8α$^+$ T lymphocyte subpopulations synthesising IFN-γ

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Abstract

Introduction: Haemorrhagic enteritis virus (HEV) is a common turkey pathogen which suppresses the immune function. The immunosuppressive potential of both field and vaccine strains of HEV makes it necessary to seek substances which can limit or prevent this phenomenon. The aim of the presented work was to investigate the effect of two immunomodulators in the immune response of HEV-infected turkeys. The immunomodulators were synthetic methisoprinol and a natural preparation containing 34.2% β-glucans (β-1,3/1,6) and 12% mannan oligosaccharides (MOS). **Material and Methods:** The synthetic immunomodulator was administered to female Big 6 turkey chicks at a dose of 200 mg/kg b.w. in drinking water i) for 3 days before, ii) for 5 days after, or iii) for 3 days before, on the day of infection, and for 5 days after experimental HEV infection in turkeys. The natural counterpart was also given to female Big 6 turkey chicks at a dose of 500 g/tonne of feed i) for 14 days before, ii) for 5 days after, or iii) for 14 days before, on the day of infection, and for 5 days after infection. Their effect was evaluated on the synthesis of interferon gamma (IFN-γ) by splenic CD4$^+$ and CD8α$^+$ T cells in response to mitogen stimulation *in vitro*. Samples were taken 3, 5 and 7 days after infection and analysed by intracellular cytokine staining assay. **Results:** Methisoprinol was shown to increase the CD4$^+$IFN-γ$^+$ and CD8α$^+$IFN-γ$^+$ T cell count in these birds over the same cell count in control turkeys. A similar effect was obtained in turkeys that received the natural immunomodulator. **Conclusion:** The evaluated immunomodulators may be used to attenuate the effects of immunosuppression in HEV-infected turkeys.

Keywords: haemorrhagic enteritis virus, immune response, immunomodulation, turkeys.

Introduction

The immune system of intensively reared turkeys is constantly exposed to a number of factors impairing its functioning, although it is with viral infections that immunosuppression in these birds is most often associated. A distinctive feature of immunosuppressive pathogens is their special affinity for the organs and cells of the immune system. In Poland, one of the most commonly reported pathogens causing immunosuppression in turkeys is haemorrhagic enteritis virus (HEV), which is a member of the *Siadenovirus* genus in the *Adenoviridae* family (47). Haemorrhagic enteritis in turkeys was first described by Pomeroy and Fenstermacher in the USA in 1937 (36). This disease was first diagnosed and confirmed in Poland in 1987 (22). Infection with the virus causing this disease, which is usually asymptomatic, results in impaired immune function (38, 40, 45, 47). The immunosuppressive effect of HEV on the turkey leads to an aggravation of pre-existing diseases and infections with opportunistic microorganisms, most often *E. coli*, as well as to a reduction of the effectiveness of flock vaccination (12, 27, 33, 35). The most important strategy to combat the infection of turkeys with this virus is to conduct immunoprophylaxis using commercially available vaccines (10, 47). Another key course of action is to search for other agents to mitigate the effects of infection, inhibit viral replication and reduce the risk of immunosuppression (26, 47, 48). These include the various substances called immunomodulators. For several years now, we have seen increasing attention

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paid to methisoprinol as a therapeutic for immunocompromised patients or viral disease sufferers in Poland and around the world. Methisoprinol, also known as inosine pranobex, inosine acedoben dimepranol or inosiplex, is a synthetic compound with antiviral properties. The drug was initially approved in 1971 and is currently marketed in more than 70 countries as a treatment for viral diseases, including subacute sclerosing panencephalitis, acute viral respiratory infections, and measles, and for infection with herpes simplex, varicella zoster, human papillomavirus, cytomegalovirus, and Epstein–Barr virus infections (42). In addition, methisoprinol also has properties modulating the body's natural immunity, which enhances its antiviral effect. Methisoprinol has also been the subject of numerous studies in various bird species (32, 44, 47). However, immunomodulators of natural origin find much greater uptake among poultry producers. They are intended to support the immunity of birds and thus reduce the risk of outbreaks of infectious diseases. They therefore indirectly reduce the amount of antibiotics used in the treatment of infectious poultry diseases. This group of immunomodulators includes β-glucans and mannan oligosaccharides (MOS) extracted from the walls of *Saccharomyces cerevisiae* yeast cells. Beta-glucans are polysaccharides composed of numerous glucose molecules linked by β-1,3 and -1,6 bonds, the former of which are responsible for the immunomodulatory effect. All host immune mechanisms, both specific and non-specific, are affected by β-1,3/1,6 glucans, and their stimulation of cells to intensify cellular production of interleukins indicates their indirect antiviral, antibacterial and even antioncogenic activity (11, 13, 19, 47). Mannan oligosaccharides are prebiotic substances composed of D-mannose molecules and, like β-glucans, they exhibit immunomodulatory properties (55).

The aim of this study was to investigate the influence of two kinds of immunomodulator on the immune response of experimentally HEV-infected turkeys. The immunomodulators were synthetic methisoprinol and natural β-1,3/1,6 glucans with MOS. The preparations were administered before, after, or both before and after HEV infection of turkeys and their effect was observed on IFN-γ synthesis by $CD4^+$ and $CD8\alpha^+$ T cells isolated from turkey spleens in response to mitogenic stimulation *in vitro*.

Material and Methods

Immunomodulators. Two preparations with different mechanisms of action were used to affect the defence mechanisms of turkeys: a synthetic immunomodulator, which was a 20% solution of methisoprinol (inosine-((N, N-dimethylamino-2-propanol)-4-acetamidobenzoate)1:3), in the Isoprivet preparation (VetAgro, Lublin, Poland) administered with drinking water at a dose of 200 mg/kg b.w./day (32, 44); and an immunomodulator of natural origin, which was the Alphamune G commercial preparation providing β-1,3/1,6 glucans with MOS (Alpharma Animal Health, Antwerp, Belgium) at a dose of 500 g/tonne of feed. Alphamune G is a spray-dried and granulated product produced after the autolysis of food-grade *Saccharomyces cerevisiae* yeast. The preparation contained 34.2% β-1,3/1,6 glucans and 12% MOS (according to the manufacturer's certificate for the analysis of batch no. AG91570 of this preparation). The dose used was in accordance with the manufacturer's recommendations.

Birds, general management practices and experiment design. One-day-old female Big 6 turkeys were purchased from a commercial hatchery (Grelavi S.A., Kętrzyn, Poland). The experiment was carried out on 280 turkeys that were randomly divided into 14 groups of 20 birds each. The turkeys were floor raised on straw shavings in isolated pens in the avian experimental infection pavilion of the Department of Poultry Diseases in the Faculty of Veterinary Medicine at the University of Warmia and Mazury in Olsztyn. A three-stage negative pressure cascade was maintained in the experimental boxes and passageways in the pavilion. Air entering and leaving the building was passed through HEPA H13 filters to prevent uncontrolled cross-infections between groups and infections caused by environmental agents. The total number of birds in each group was specified appropriately for the size of the experimental boxes. The temperature and lighting programmes were consistent with the recommendations of the turkey supplier Grelavi, and the birds had free access to feed and water. The investigation was divided into three experiments differing in the timing of the use of immunomodulators relative to the time of the turkeys' infection with HEV. Infected groups were identified as HEV^+ and uninfected as HEV^-.

In the first experiment, turkeys in the I-M/HEV^+ and I-M/HEV^- groups received methisoprinol from 39 to 41 days of life (dol) and turkeys in the I-B/HEV^+ and I-B/HEV^- groups received Alphamune G from 28 to 41 dol. This was before they were experimentally infected with the HE virus, which was carried out at 42 dol.

In the second experiment, turkeys in the II-M/HEV^+ and II-M/HEV^- groups received methisoprinol and turkeys in the II-B/HEV^+ and II-B/HEV^- groups received Alphamune G for 5 days from 43 to 47 dol and therefore after infection with HEV.

In the third experiment, turkeys in the III-M/HEV^+ and III-M/HEV^- groups received methisoprinol from 39 to 47 dol and turkeys in the III-B/HEV^+ and III-B/HEV^- groups received Alphamune G from 28 to 47 dol, which was before and after the HEV infection at 42 dol.

Turkeys in the C/HEV^+ and C/HEV^- groups were controls and did not receive the immunomodulators.

Experimental inoculation with HEV. At 42 dol (in the period of greatest susceptibility to infection), turkeys from all groups for which infection was planned (including controls) were experimentally inoculated with 1 mL of a suspension containing a Polish HEV isolate (23) at a dose of $10^{4.3}$ 50% egg infectious dose

(EID_{50})/bird using an oesophageal cannula. Birds that were not infected with HEV received 1 mL of phosphate-buffered saline (PBS) by the same route.

Sample collection. Sample collection times and experiment design are presented in Table 1. Four turkeys were randomly selected from each group at 45, 47 and 49 dol, or 3, 5, and 7 days post infection (dpi). These birds were euthanised humanely with the use of a professional unit (Uno, Zevenaar, the Netherlands) and their spleens were collected for mononuclear cell isolation and determination of the percentages of $CD4^{+}IFN\text{-}\gamma^{+}$ and $CD8\alpha^{+}IFN\text{-}\gamma^{+}$ T cell subpopulations by flow cytometry. The presence of HEV genetic material was also measured in the spleen samples by PCR.

Isolation of mononuclear cells and determination of the percentage of IFN-γ–synthesising lymphocytes within the $CD4^{+}$ and $CD8\alpha^{+}$ subpopulations. Splenic mononuclear cells were isolated according to a previously described procedure (27). The cells were counted and their viability was evaluated using a Vi-cell XR cell counter (Beckman Coulter, Brea, CA, USA). The percentages of $CD4^{+}$ and $CD8\alpha^{+}$ T lymphocytes synthesising IFN-γ in response to mitogenic stimulation (Leukocyte Activation Cocktail with BD GolgiPlug; BD Pharmingen, Franklin Lakes, NJ, USA) under *in vitro* conditions were determined by intracellular cytokine staining assay and flow cytometry according to a procedure described previously (46). Each sample was analysed in triplicate. Samples were studied with a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA), and data were acquired with FACSDiva version 6.1.3 software (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry datasets were analysed with FlowJo V10 software (FlowJo, Ashford, OR, USA) using the gating hierarchy illustrated in Fig. 1.

DNA isolation and PCR for the hexon gene. Prior to viral DNA isolation, 0.2 g of spleen fragments were placed in sterile 2 mL tubes (Eppendorf, Hamburg, Germany), the tubes were filled with sterile PBS (Sigma-Aldrich, Schnelldorf, Germany) and the contents were homogenised using an automatic TissueLyser II homogeniser (Qiagen, Hilden, Germany).

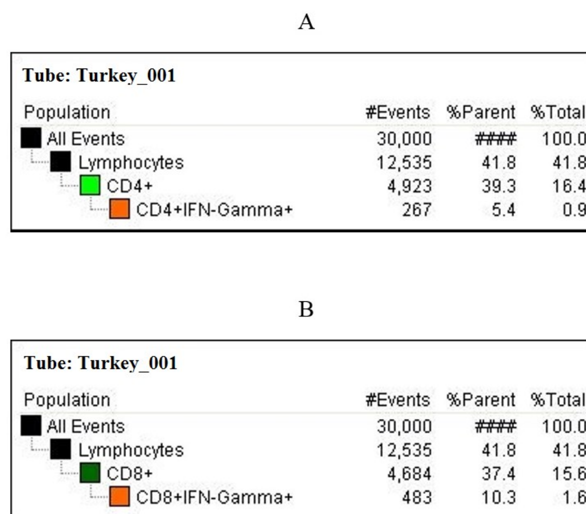

Fig. 1. Gating hierarchy chart in the analysis of the percentage of cells synthesising IFN-γ in response to *in vitro* mitogen stimulation. The cells were in the $CD4^{+}$ (A) and (B) $CD8\alpha^{+}$ T lymphocyte subpopulations isolated from turkey spleen tissue

Table 1. Experimental design

Group	Day of life/day post infection											
	1	2–27	28–38	39–41	42/0	43/1	44/2	45/3 (S)	46/4	47/5 (S)	48/6	49/7 (S)
I-M/HEV^{+}	Random division of chicks into 14 groups of 20 birds each and introduction into experimental boxes	Rearing under standard techniques and procedures	Meth		EI							
I-M/ HEV^{-}					PBS							
I-B/HEV^{+}			β-Glu + MOS		EI							
I-B/HEV^{-}					PBS							
II-M/HEV^{+}					EI	Meth						
II-M/ HEV^{-}					PBS							
II-B/HEV^{+}					EI	β-Glu + MOS						
II-B/ HEV^{-}					PBS							
III-M/HEV^{+}			Meth		EI Meth	Meth						
III-M/HEV^{-}					PBS Meth							
III-B/HEV^{+}			β-Glu + MOS		EI β-Glu+MOS	β-Glu + MOS						
III-B/HEV^{-}					PBS β-Glu+MOS							
C/HEV^{+}					EI							
C/ HEV^{-}					PBS							

I-M – turkeys immunomodulated with methisoprinol prior to infection; I-B – turkeys immunomodulated with β-glucans and mannan oligosaccharides (MOS) prior to infection; II-M – turkeys immunomodulated with methisoprinol after infection; II-B – turkeys immunomodulated with β-glucans and MOS after infection; III-M – turkeys immunomodulated with methisoprinol prior to, at the time of, and after infection; III-B – turkeys immunomodulated with β-glucans and MOS prior to, at the time of, and after infection; C – control turkeys not receiving immunomodulators; HEV^{+} – turkeys infected with haemorrhagic enteritis adenovirus at 42 days of life; HEV^{-} – uninfected turkeys; EI – experimental infection with HEV at a dose of $10^{4.3}$ 50% egg infectious dose (EID_{50}); PBS – uninfected turkeys receiving 1 mL of sterile phosphate buffered saline; Meth – methisoprinol administered in drinking water at 200 mg/kg b.w.; β-Glu+MOS – β-glucans and MOS administered at 500 g of Alphamune G per tonne of feed; S – sampling of the spleen from four turkeys of each group

All homogenised samples were then frozen at −22°C and thawed to +4°C three times to lyse the virus from the cells. Genomic DNA was isolated with the use of a Genomic Mini kit (A&A Biotechnology, Gdańsk, Poland), in accordance with the procedure provided by the manufacturer. Details of the method for detecting the HEV hexon gene fragment in samples were described previously (30).

Statistical analysis. The results were analysed statistically using Statistica v. 10.0 software (StatSoft, Kraków, Poland). The significance of differences between means was determined by Duncan's multiple-range test. Data are presented as means ± SD and the value of $P < 0.05$ was considered statistically significant.

Results

First experiment results. Table 2 shows the percentages of IFN-γ–producing cell subpopulations within the $CD4^+$ T cells isolated from the spleens of 45-, 47- and 49-day-old HEV-infected (HEV^+) and uninfected (HEV^-) turkeys from Groups I-M, I-B and C. Table 3 shows the analogous percentages within the $CD8\alpha^+$ T cells.

At 45 dol, *i.e.* 3 days after HEV infection, a statistically significantly higher proportion of the $CD4^+$ T cell subpopulation was observed to be IFN-γ^+ in cultures of mononuclear cells isolated from the spleens of the group I-B/HEV^- turkeys (7.22 ± 2.50%) in relation to the IFN-γ^+–positivity in these cells cultured from the spleens of birds from the other groups. The lowest percentage of these cells was recorded on that day in birds from the C/HEV^+ control group (2.26 ± 0.41%) and the I-B/HEV^+ experimental group (2.31 ± 0.32%). Five days after infection, a higher proportion of $CD4^+$ T cells was noted to be IFN-γ–synthesising in group I-M/HEV^+ turkeys (10.58 ± 2.44%) than in all other groups, and the margin by which it was larger than this proportion in other groups was statistically significant except compared to that of the I-B/HEV^+ group (9.12 ± 0.87%). At 7 dpi the percentage of $CD4^+$ T cells which were IFN-γ^+ was again highest in turkeys in Group I-M/HEV^+ (11.29 ± 1.15%) and this was a statistically significant difference compared to all other groups. Turkeys from Group I-B/HEV^- receiving β-glucans and MOS had a statistically significantly higher percentage of these cells at the first (7.22 ± 2.50%) and third (8.74 ± 0.94%) sample collections (at 45 and 49 dol) than birds from the C/HEV^- control group, which at these sample collection times had 5.47 ± 1.45% and 4.52 ± 0.17% of cells IFN-γ^+, respectively. In non-HEV infected turkeys which received methisoprinol (Group I-M/HEV^-), a statistically significantly lower proportion of $CD4^+$ T cells was confirmed to be IFN-γ^+ at the first (3.04 ± 1.36%) and second (3.69 ± 0.38%) collections (45 and 47 dol) compared to the $CD4^+$ cells of C/HEV^- control turkeys, of which 5.47 ± 1.45% and 7.03 ± 0.98% were IFN-γ^+, respectively, at these intervals.

The $CD8\alpha^+$IFN-γ^+ T lymphocyte subpopulation percentage at 45 dol was the highest in Group I-B/HEV^- and was 10.54 ± 3.55%. The percentages of these cells making up these subpopulations in the other groups of turkeys were statistically significantly lower. In contrast, compared to birds from other groups, the highest percentages of IFN-γ–synthesising cells within the $CD8\alpha^+$ T lymphocyte subpopulation were detected at 5 dpi in both control groups of turkeys, where in C/HEV^+ it was 15.17 ± 0.88% and in C/HEV^- it was 14.47 ± 2.11%. These percentages exceeded those of other groups by a statistically significant margin. At the last sampling (7 dpi), statistically significantly higher percentages of these cells were found in HEV-infected turkeys from Groups I-M (15.99 ± 0.86%) and I-B (10.42 ± 0.89%) and in non-infected turkeys from the control groups (11.20 ± 0.93%) compared to birds from the other groups. At the second (47 dol) and third (49 dol) samplings, the $CD8\alpha^+$ T lymphocyte subpopulation which was IFN-γ^+ accounted for a significantly lower proportion in turkeys uninfected with HEV which received immunomodulators (Groups I-M/HEV^- and I-B/HEV^-) than in control turkeys of the C/HEV^- group. These values were statistically significantly lower in turkeys treated with methisoprinol than in control turkeys and birds treated with Alphamune G.

Second experiment results. Table 4 shows the percentages of IFN-γ–producing cell subpopulations within the $CD4^+$ T cells isolated from the spleens of 45-, 47- and 49-day-old HEV-infected (HEV^+) and uninfected (HEV^-) turkeys from Groups II-M, II-B, and C. Table 5 shows the analogous percentages within the $CD8\alpha^+$ T cells.

On day 45, there was no statistical difference between the proportions of $CD4^+$IFN-γ^+ T cell subpopulations in the infected and immunomodulated (II-M/HEV^+ and II-B/HEV^+) and infected and non-immunomodulated (C/HEV^+) groups or between uninfected and immunomodulated (II-M/HEV^- and II-B/HEV^-) and uninfected and non-immunomodulated (C/HEV^-) groups. However, the percentage of subpopulations of these cells in turkeys from the infected groups (II-M/HEV^+, II-B/HEV^+ and C/HEV^+) was statistically significantly lower than in the non-infected groups (II-M/HEV^-, II-B/HEV^- and C/HEV^-). Two days later, the percentage of cells synthesising IFN-γ in the $CD4^+$ T cell subpopulation was statistically significantly higher in the II-M/HEV^+ (10.67 ± 1.12%) and II-B/HEV^- (10.41 ± 2.08%) groups relative to the C/HEV^+ (7.20 ± 1.32%) and C/HEV^- (7.03 ± 0.98%) control groups. At the last collection (49 dol), the $CD4^+$IFN-γ^+ cell subpopulation sizes in the non-immunomodulated control groups and in the II-B/HEV^+ group of turkeys which received Alphamune G after HEV infection were significantly smaller than those in the II-M/HEV^+ and II-M/HEV^- groups of turkeys receiving methisoprinol and those in the II-B/HEV^- group.

Table 2. Mean percentage of IFN-γ$^+$ cells (±SD) in splenic CD4$^+$ T lymphocytes collected from the examined turkeys receiving immunomodulators before the day of infection with HEV

Group	Mean percentage of CD4$^+$IFN-γ$^+$ T cells (±SD)		
	3 dpi	5 dpi	7 dpi
I-M/HEV$^+$	3.64^c (±0.45)	10.58^a (±2.44)	11.29^a (±1.15)
I-M/HEV$^-$	3.04^c (±1.36)	3.69^c (±0.38)	4.75^{cd} (±0.90)
I-B/HEV$^+$	2.31^c (±0.32)	9.12^{ab} (±0.87)	6.38^{bc} (±1.85)
I-B/HEV$^-$	7.22^a (±2.50)	5.77^{cd} (±0.92)	8.74^b (±0.94)
C/HEV$^+$	2.26^c (±0.41)	7.20^{bd} (±1.32)	3.54^{cd} (±0.45)
C/HEV$^-$	5.47^b (±1.45)	7.03^{bd} (±0.98)	4.52^{cd} (±0.17)

IFN-γ$^+$ – interferon gamma–positive; SD – standard deviation; dpi – days post infection; I-M – turkeys immunomodulated with methisoprinol prior to infection; I-B – turkeys immunomodulated with β-glucans and MOS prior to infection; C – control turkeys not receiving immunomodulators; HEV$^+$ – turkeys infected with haemorrhagic enteritis adenovirus at 42 days of life; HEV$^-$ – uninfected turkeys. Values in the same column with different superscripts (a–d) differ significantly at $P < 0.05$ in Duncan's multiple-range test

Table 3. Mean percentage of IFN-γ$^+$ cells (±SD) in splenic CD8α$^+$ T lymphocytes collected from the examined turkeys receiving immunomodulators before the day of infection with HEV

Group	Mean percentage of CD8α$^+$IFN-γ$^+$ T cells (±SD)		
	3 dpi	5 dpi	7 dpi
I-M/HEV$^+$	2.78^{cd} (±0.88)	12.00^b (±0.94)	15.99^a (±0.86)
I-M/HEV$^-$	5.33^{bd} (±0.91)	6.01^d (±0.50)	4.37^c (±0.95)
I-B/HEV$^+$	2.73^c (±0.61)	9.34^{cd} (±1.24)	10.42^a (±0.89)
I-B/HEV$^-$	10.54^a (±3.55)	9.94^{bc} (±1.09)	7.69^b (±1.11)
C/HEV$^+$	2.66^c (±0.33)	15.17^a (±0.88)	5.78^c (±0.87)
C/HEV$^-$	6.43^b (±1.33)	14.47^a (±2.11)	11.20^a (±0.93)

IFN-γ$^+$ – interferon gamma–positive; SD – standard deviation; dpi – days post infection; I-M – turkeys immunomodulated with methisoprinol prior to infection; I-B – turkeys immunomodulated with β-glucans and MOS prior to infection; C – control turkeys not receiving immunomodulators; HEV$^+$ – turkeys infected with haemorrhagic enteritis adenovirus at 42 days of life; HEV$^-$ – uninfected turkeys. Values in the same column with different superscripts (a–d) differ significantly at $P < 0.05$ in Duncan's multiple-range test

Table 4. Mean percentage of IFN-γ$^+$ cells (±SD) in splenic CD4$^+$ T lymphocytes collected from the examined turkeys receiving immunomodulators after the day of infection with HEV

Group	Mean percentage of CD4$^+$IFN-γ$^+$ T cells (±SD)		
	3 dpi	5 dpi	7 dpi
II-M/HEV$^+$	2.33^b (±0.48)	10.67^a (±1.12)	5.73^b (±0.93)
II-M/HEV$^-$	6.13^a (±0.43)	8.06^{ab} (±2.46)	7.94^{ab} (±0.91)
II-B/HEV$^+$	1.83^b (±0.23)	8.81^{ab} (±0.44)	3.52^c (±0.45)
II-B/HEV$^-$	4.69^a (±0.49)	10.41^a (±2.08)	7.86^{ab} (±3.29)
C/HEV$^+$	2.26^b (±0.41)	7.20^b (±1.32)	3.54^c (±0.45)
C/HEV$^-$	5.47^a (±1.45)	7.03^b (±0.98)	4.52^c (±0.17)

IFN-γ$^+$ – interferon gamma–positive; SD – standard deviation; dpi – days post infection; II-M – turkeys immunomodulated with methisoprinol after infection; II-B – turkeys immunomodulated with β-glucans and MOS after infection; C – control turkeys not receiving immunomodulators; HEV$^+$ – turkeys infected with haemorrhagic enteritis adenovirus at 42 days of life; HEV$^-$ – uninfected turkeys. Values in the same column with different superscripts (a–d) differ significantly at $P < 0.05$ in Duncan's multiple-range test

Table 5. Mean percentage of IFN-γ$^+$ cells (±SD) within splenic CD8α$^+$ T lymphocytes collected from the examined turkeys receiving immunomodulators after the day of infection with HEV

Group	Mean percentage of CD8$^+$IFN-γ$^+$ T cells (±SD)		
	3 dpi	5 dpi	7 dpi
II-M/HEV$^+$	2.74^b (±0.35)	17.12^a (±0.96)	10.60^a (±1.16)
II-M/HEV$^-$	7.16^a (±0.49)	13.02^{bc} (±2.49)	7.61^{bc} (±0.87)
II-B/HEV$^+$	3.57^b (±0.24)	15.38^{ac} (±0.90)	8.32^b (±1.44)
II-B/HEV$^-$	5.73^a (±0.82)	7.21^b (±2.40)	5.70^c (±1.32)
C/HEV$^+$	2.66^b (±0.33)	15.17^{ac} (±0.88)	5.78^c (±0.87)
C/HEV$^-$	6.43^a (±1.33)	14.47^{ac} (±2.11)	11.20^a (±0.93)

IFN-γ$^+$ – interferon gamma–positive; SD – standard deviation; dpi – days post infection; II-M – turkeys immunomodulated with methisoprinol after infection; II-B – turkeys immunomodulated with β-glucans and MOS after infection; C – control turkeys not receiving immunomodulators; HEV$^+$ – turkeys infected with haemorrhagic enteritis adenovirus at 42 days of life; HEV$^-$ – uninfected turkeys. Values in the same column with different superscripts (a–d) differ significantly at $P < 0.05$ in Duncan's multiple-range test

Table 6. Mean percentage of IFN-γ^+ cells (±SD) in splenic $CD4^+$ T lymphocytes collected from the examined turkeys receiving immunomodulators prior to, at the time of, and after infection with HEV

Group	Mean percentage of $CD4^+IFN\text{-}\gamma^+$ T cells (±SD)		
	3 dpi	5 dpi	7 dpi
III-M/HEV$^+$	3.56^{cd} (±0.72)	8.19^{cd} (±0.95)	10.17^{a} (±0.80)
III-M/HEV$^-$	8.36^{a} (±0.74)	7.67^{cd} (±0.99)	6.69^{bc} (±0.19)
III-B/HEV$^+$	2.43^{c} (±0.36)	9.91^{abd} (±0.76)	8.37^{abc} (±0.87)
III-B/HEV$^-$	4.71^{bd} (±0.88)	10.98^{a} (±0.51)	8.02^{ac} (±1.73)
C/HEV$^+$	2.26^{c} (±0.41)	7.20^{c} (±1.32)	3.54^{d} (±0.45)
C/HEV$^-$	5.47^{b} (±1.45)	7.03^{c} (±0.98)	4.52^{d} (±0.17)

IFN-γ^+ – interferon gamma–positive; SD – standard deviation; dpi – days post infection; III-M – turkeys immunomodulated with methisoprinol prior to, at the time of, and after infection; III-B – turkeys immunomodulated with β-glucans and MOS prior to, at the time of, and after infection; C – control turkeys not receiving immunomodulators; HEV$^+$ – turkeys infected with haemorrhagic enteritis adenovirus at 42 days of life; HEV$^-$ – uninfected turkeys. Values in the same column with different superscripts (a–d) differ significantly at $P < 0.05$ in Duncan's multiple-range test

Table 7. Mean percentage of IFN-γ^+ cells (±SD) within splenic $CD8\alpha^+$ T lymphocytes collected from the examined turkeys receiving immunomodulators prior to, at the time of, and after infection with HEV

Group	Mean percentage of IFN-γ^+ $CD8^+$ T cells (±SD)		
	3 dpi	5 dpi	7 dpi
III-M/HEV$^+$	5.60^{bd} (±1.94)	12.55^{bc} (±1.08)	11.42^{a} (±0.89)
III-M/HEV$^-$	9.99^{a} (±1.93)	11.84^{c} (±1.07)	6.95^{b} (±1.30)
III-B/HEV$^+$	3.22^{cd} (±0.87)	16.42^{a} (±0.88)	11.46^{a} (±0.84)
III-B/HEV$^-$	4.35^{bcd} (±0.84)	7.51^{d} (±1.91)	6.87^{b} (±0.84)
C/HEV$^+$	2.66^{c} (±0.33)	15.17^{ab} (±0.88)	5.78^{b} (±0.87)
C/HEV$^-$	6.43^{b} (±1.33)	14.47^{ab} (±2.11)	11.20^{a} (±0.93)

IFN-γ^+ – interferon gamma–positive; SD – standard deviation; dpi – days post infection; III-M – turkeys immunomodulated with methisoprinol prior to, at the time of, and after infection; III-B – turkeys immunomodulated with β-glucans and MOS prior to, at the time of, and after infection; C – control turkeys not receiving immunomodulators; HEV$^+$ – turkeys infected with haemorrhagic enteritis adenovirus at 42 days of life; HEV$^-$ – uninfected turkeys. Values in the same column with different superscripts (a–d) differ significantly at $P < 0.05$ in Duncan's multiple-range test

Table 8. Results of PCR amplification of the HE virus hexon gene fragment in spleen samples taken from four turkeys from each group

Group	% of samples positive for HEV genetic material by dol/dpi		
	45/3	47/5	49/7
I-M/HEV$^+$	0	50	25
I-M/HEV$^-$	0	0	0
I-B/HEV$^+$	25	75	25
I-B/HEV$^-$	0	0	0
II-M/HEV$^+$	25	75	50
II-M/HEV$^-$	0	0	0
II-B/HEV$^+$	50	100	75
II-B/HEV$^-$	0	0	0
III-M/HEV$^+$	0	75	25
III-M/HEV$^-$	0	0	0
III-B/HEV$^+$	0	75	50
III-B/HEV$^-$	0	0	0
C/HEV$^+$	50	100	100
C/HEV$^-$	0	0	0

dol – day of life; dpi – days post infection; I-M – turkeys immunomodulated with methisoprinol prior to infection; I-B – turkeys immunomodulated with β-glucans and mannan oligosaccharides (MOS) prior to infection; II-M – turkeys immunomodulated with methisoprinol after infection; II-B – turkeys immunomodulated with β-glucans and MOS after infection; III-M – turkeys immunomodulated with methisoprinol prior to, at the time of, and after infection; III-B – III-B – turkeys immunomodulated with β-glucans and MOS prior to, at the time of, and after infection; C – control turkeys not receiving immunomodulators; HEV$^+$ – turkeys infected with haemorrhagic enteritis adenovirus at 42 days of life; HEV$^-$ – uninfected turkeys; 25%, 50%, 75% and 100% mean that HEV genetic material was respectively detected in the 1st, in the 1st and 2nd, in the 1st, 2nd, and 3rd, and in all 4 spleens collected from four turkeys in a group on a given day

On day 45, there were no statistically significant differences between the proportions of CD8α^{+}IFN-γ^{+} T cell subpopulations in the three HEV-infected groups. The same was noted for the three uninfected groups. However, the percentage of subpopulations of these cells in turkeys from the infected groups was statistically significantly lower than in uninfected turkeys. At 47 dol, the percentage of cells synthesising IFN-γ in the CD8α^{+} T lymphocyte subpopulation was significantly higher in HEV-infected turkeys than in uninfected turkeys. The highest value of this percentage (17.12 ± 0.96%) was recorded on this day in the II-M/HEV^{+} group of turkeys receiving methisoprinol after HEV infection, but it was not statistically significantly higher than that in the C/HEV^{+} (15.17 ± 0.88%) or C/HEV^{-} (14.47 ± 2.11%) control groups. At the last collection (49 dol), the CD8α^{+}IFN-γ^{+} T cell subpopulation sizes in Group II-M/HEV^{+} (10.60 ± 1.16%) and in uninfected C/HEV^{-} control group turkeys (11.20 ± 0.93%) were significantly larger than in the other groups.

Third experiment results. Table 6 shows the percentages of IFN-γ–producing cell subpopulations in the CD4^{+} T cells isolated from the spleens of 45-, 47- and 49-day-old HEV-infected (HEV^{+}) and uninfected (HEV^{-}) turkeys from Groups III-M, III-B and C. Table 7 shows the analogous percentages within the CD8α^{+} T cells.

At the first collection (45 dol), the percentage of cells synthesising IFN-γ in the CD4^{+} T cell subpopulation was lower in all infected turkey groups than in the uninfected HEV groups. There were no statistically significant differences between immunomodulated HEV-infected turkeys and control turkeys. The highest percentage (disregarding uninfected turkeys) (3.56 ± 0.72%) was found in birds in Group III-M/HEV^{+}) receiving methisoprinol before and after HEV infection. At 47 days of life, a statistically significantly higher proportion of these cells was found in Groups III-B/HEV^{-} (10.98 ± 0.51%) and III-B/HEV^{+} (9.91 ± 0.76%), which received Alphamune G, than in the C/HEV^{-} (7.03 ± 0.98%) and C/HEV^{+} (7.20 ± 1.32%) control groups. At the last sampling, the CD4^{+}IFN-γ^{+} T cell subpopulation percentage was statistically significantly higher in all groups of turkeys receiving the studied immunomodulators before and after HEV infection than in control birds. The highest proportion (10.17 ± 0.80%) of T cells being CD4^{+}IFN-γ^{+} at 49 dol was reported in the group of turkeys receiving methisoprinol pre- and post HEV infection (III-M/HEV^{+}). Additionally, the III-B/HEV^{+} group of turkeys which received Alphamune G before and after HEV infection had a statistically significantly higher proportion of these cells than control birds.

At 45 dol, the percentage of cells synthesising IFN-γ within the CD8α^{+} T cell subpopulation was statistically significantly higher in Group III-M/HEV^{-} turkeys (9.99 ± 1.93%) than in birds from other groups. In the infected group of turkeys that received methisoprinol before and after infection, the percentage of these cells (5.60 ± 1.94%) was significantly higher compared to birds in the control group (2.66 ± 0.33%). After two days (at 47 dol), there were no statistically significant differences between any HEV-infected turkeys treated with the test immunomodulators and control birds. At the last sampling, statistically significantly higher proportions of CD8α^{+}IFN-γ^{+} T cells, 11.42 ± 0.89% for Group III-M/HEV^{+} and 11.46 ± 0.88% for Group III-B/HEV^{+}, were observed in HEV^{-} infected turkeys treated with the studied immunomodulators compared to the proportion in C/HEV^{+} control birds, 5.78 ± 0.87%.

Detection of the HE virus hexon gene fragment in the spleen. The results of the PCR testing of the spleen fragments to detect the presence of the HE hexon gene fragment are presented in Table 8. No HE virus genetic material was found in any spleen samples collected at 45, 47 or 49 dol from turkeys in uninfected groups.

Discussion

During the experiment, no fatalities occurred in infected or uninfected HEV turkeys. The level of biosecurity and the physical means of segregation in the pavilion where the experiment was conducted effectively prevented the transmission of HEV from infected to uninfected groups, as evidenced by the results of the PCR examination of the spleen samples presented in Table 8. The Polish HEV isolate used in the experiment is among the strains with low pathogenicity, which explains why there were no cases in turkeys infected with it (47). Despite the absence of cases with typical symptoms of this disease in this experiment, even non-virulent HEV strains can cause transient immunosuppression, usually resulting in secondary infections within 2–4 weeks of infection (27, 35).

The possibility of induction of immunosuppression in turkeys by both field and HEV vaccine strains imposes the need to search for any and all measures to reduce or prevent this phenomenon. Attempts to develop safe next-generation HEV vaccines are ongoing (4). Unfortunately, these vaccines were never commercially available at any time in the past and neither are they currently. Manufacturers of the classical live-HEV vaccines available in Europe expressly recommend prophylactic administration of broad-spectrum antibiotics to turkeys inoculated with their preparations. This is contrary to the intention of global programmes for the protection of the efficacy of antibiotics, which address the antibiotic use leading to an increase in antibiotic resistance. Various synthetic and natural substances are known to have a beneficial effect on the immune system of turkeys. To abate the risk of antibiotic resistance, research is underway on the possibility of prophylactic use of such substances to mitigate the effects of immunosuppression resulting from infection or HEV vaccination and to impart an adjuvant effect at the same time (25, 48, 49). The effect immunomodulators can have is the net effect of the turkeys' immune status at the time of therapy initiation, the dose, the route of administration, the number of doses in the series, and the timing of their administration relative to the instant when the infection occurred or the action of the immunosuppressive

agent began. Excessively long use or administration of unnecessarily high doses of immunomodulators may result in immune system weakness or even immunosuppression. Therefore, this research was divided into three parallel experiments in which the tested turkeys received immunomodulators before (in the first experiment), after (in the second experiment) or before and after (in the third experiment) intentional HEV infection. The effect of immunomodulation on IFN-γ synthesis by $CD4^+$ and $CD8\alpha^+$ T cells isolated from the spleens of HEV-infected turkeys was investigated. Interferon secretion by different cells is a major mechanism of innate resistance to virus infections. Three types of type I interferons (α, β and λ) and type II IFN-γ have been identified in chickens (21). Turkey and chicken IFN-γ have amino acid sequences which are 97% identical, and for this reason chicken IFN shows high biological reactivity in turkeys (20, 28). It was decided to use commercially available anti-chicken IFN-γ antibodies with high cross-reactivity with turkey IFN-γ in the studies of the turkey immune system conducted in our department (22). Similar experiments are very often conducted in humans and in laboratory animals to evaluate various substances, and they seek to increase the effectiveness of vaccines or to determine the body's response to infection (15, 34, 50). Most often, analyses of the synthesis of IFN-γ in birds within different subpopulations of cells or organs are performed by molecular methods and indicate an increase in the expression of the gene encoding IFN-γ while not confirming an increase in the level of IFN production or secretion (8, 43, 52, 53). Studies on the synthesis of IFN or certain cytokines by specific subpopulations of lymphocytes are performed extremely rarely in birds due to the high cost and degree of complexity of labelling and the need for very expensive equipment (46, 48). It is therefore difficult to relate the results of the present studies to the results of similar studies in birds available in the literature. Studies by the authors have shown that both in the $CD4^+$ and $CD8\alpha^+$ T lymphocyte subpopulations, IFN-γ–synthesising cells are found in response to mitogen stimulation under *in vitro* conditions. The studies have also shown that the immunomodulators used have an influence on the size of these cell subpopulations which is clearly discernible but which also depends on the experiment design.

One of the immunomodulators used in the present studies was the commercial preparation Alphamune G, containing as its two active components β-glucans (β-1,3/1,6) and MOS extracted from the wall of *Saccharomyces cerevisiae* yeast cells. The β-glucans and MOS contained in Alphamune G have a high take-up by poultry producers and veterinarians. They are used in feed to increase the effectiveness of vaccinations and prophylactically during periods of increased susceptibility of birds to certain diseases, as well as during treatment and recovery after diseases associated with immunosuppression (14, 18, 24, 41, 52, 55). Their use reduces the amount of antibiotics administered to birds throughout the production cycle. Beta-1,3/1,6 glucans stimulate macrophages to produce increased amounts of cytokines and eicosanoids, which trigger the proliferation and activity of $CD4^+$ and $CD8^+$ T lymphocytes and antibody synthesis (1, 13, 54, 55). It has been demonstrated that MOS clearly modulate immune processes at the level of the gut-associated lymphoid tissue, which may confer significant health benefits (24, 51). Innate effector cells are capable of recognising yeast cell wall molecules directly through a variety of pattern-recognition receptors, including mannose, β-glucan, and complement receptors (3, 6, 18, 55). It has been shown that the addition of β-glucans to feed for Leghorn chickens caused an increase in the phagocytic activity of heterophils in relation to *Salmonella* Enteritidis bacteria (29). In turn, the addition of purified β-glucans at a dose of 20 g per ton of feed for chickens which were experimentally infected with E. coli reduced the number of fatalities and prevented weight loss (17). It was also shown that β-glucans had the most powerful effect on the immune system of these chickens when they were used for not less than seven days, which correlates with the results of the present studies. In the second experiment, in which turkeys received β-glucans in feed for five days after HEV infection, there was a markedly reduced stimulatory effect on $CD4^+$ T cells to synthesise IFN-γ compared to the effect in the first and third experiments, in which turkeys received β-glucans and MOS for 14 and 20 days, respectively. In addition, MOS have been evaluated for their effectiveness in controlling necrotic enteritis in chickens. Supplementation of feed with MOS combined with a freeze-dried lactic acid bacterial preparation reduced mortality and subclinical effects of *Clostridium perfringens* on poultry feed efficiency, in a similar way to bacitracin methylene disalicylate's effect on birds (16). By administering 0.25 and 0.5% MOS in feed to turkeys experimentally infected with HEV and *E. coli* bacilli, Koncicki *et al.* (24) observed colibacteriosis to have a milder course and noted a lower mortality rate in these birds compared to control turkeys.

In human medicine, for several years great attention has been paid to methisoprinol in Poland and around the world for the treatment of immunocompromised patients or sufferers of diseases caused by certain viruses. Recently, it has also been used in adjuvant therapy in people infected with the SARS-CoV-2 virus (2). Studies on methisoprinol have demonstrated that its immunomodulatory activity is characterised by enhanced lymphocyte proliferation, cytokine and IFN-γ production, and natural killer cell cytotoxicity (7, 31, 39, 42).

In the present research, both methisoprinol in all three experiments and β-glucans and MOS in first and third experiments were shown to increase IFN-γ synthesis by $CD4^+$ T cells in such cells isolated from the spleens of HEV^- infected turkeys compared to this synthesis in control birds which were infected but received no immunomodulators. These results are in line with those obtained by other authors, who showed that regardless of whether it was used *in vivo* or *in vitro*, methisoprinol stimulated leukocyte synthesis of IFN-γ in

humans (9), mice (7) and chickens (32). Moya *et al.* (32) administered methisoprinol at doses of 150 and 300 mg/kg b.w. to chickens before and/or after infecting them with Newcastle disease (ND), avian influenza (AI) and infectious bronchitis (IB) viruses and demonstrated an increase compared to control birds in serum IFN levels in individuals which received a higher dose of this immunomodulator. Cilliari *et al.* (7) measured a statistically significantly higher level of IFN-γ secreted by cells isolated from experimental mouse spleens in response to stimulation with concanavalin A in *in vitro* culture. Experimental animals were administered 1 mg/mL methisoprinol in drinking water. Testifying to the effect of the natural rather than the synthetic immunomodulator, in an experiment by Zhang *et al.* (54), the addition of β-glucans (derived from *S. cerevisiae*) at doses of 50 and 75 mg/kg of chicken feed caused a significant increase in the level of IFN-γ in their serum. Cox *et al.* (8) found an opposite effect: that feed supplementation with 0.1% β-glucans also obtained from the wall of *S. cerevisiae* yeast cells significantly reduced the expression of the IFN gene in the duodenal, cecal and ilial walls of 7-day-old chickens. These latter results, in turn, correspond to the results observed in the present study with respect to the $CD8\alpha^+IFN\text{-}\gamma^+$ T lymphocyte subpopulation, the size of which was lower in the uninfected groups receiving β-glucans than in the C/HEV^- control group. In contrast, Yitbarek *et al.* (52) showed that the addition of MOS at a dose of 2 g/kg feed for chickens experimentally infected with *Clostridium perfringens* induced statistically significantly greater IFN-γ gene expression in the amygdala of the cecum and in the wall of the ilium compared to birds infected with this bacterium which did not receive MOS. Cetin *et al.* (5) demonstrated that MOS supplementation at 1g/kg of fodder resulted in significant increases in the serum IgG and IgM levels and significant decreases in the peripheral blood T lymphocyte percentage in 15-week-old experimental turkeys, compared with those of the control group. Confirmation that many fungal and yeast extracts are potent inducers of IFN-γ expression came from research carried out by Ranta *et al.* (37) *in vitro* on mononuclear cells isolated from human peripheral blood, and the authors posited that these extracts could therefore be used as T-helper 1 cell (Th1)–inducing adjuvants.

In all experimental configurations in the present investigation, samples from HEV-infected turkeys which received methisoprinol or β-glucans and MOS contained larger $CD8\alpha^+IFN\text{-}\gamma^+$ subpopulations than the C/HEV^+ control group did at 3 and 7 dpi. The size difference was statistically significant at 7 dpi, whereas it was not at 3 dpi. The $CD8\alpha^+IFN\text{-}\gamma^+$ T cell count was higher at 5 and 7 dpi in the HEV-infected immunomodulated groups than in the uninfected groups treated with the studied immunomodulators. This indicates that the antiviral response mechanisms were triggered by the secretion of interferons by immune cells of the HEV-infected turkeys. This is in line with the results of Rautenschlein and Sharma (38), who found a statistically significantly higher level of IFN-γ in the culture medium of *in vitro* splenocytes isolated from samples from HEV-infected turkeys taken at 3 dpi compared to the same medium from uninfected bird sample cultures. Besides IFN-γ, those authors also found the presence of type I IFN in the test supernatant. In the investigation undertaken by the present authors, it was shown that samples from turkeys receiving the studied immunomodulators generally yielded higher percentages of $CD4^+IFN\text{-}\gamma^+$ and $CD8\alpha^+IFN\text{-}\gamma^+$ T cell subpopulations in the first and third experiments compared to infected and non-immunomodulated infected turkeys. In many samples, these values were statistically significantly higher. This indicates stimulation of the studied T lymphocyte subpopulations to secrete IFN-γ by the immunomodulators used in the experiment.

Immunomodulation may be an effective tool in alleviating the effects of immunosuppression in HEV-infected turkeys. The use of natural and synthetic immunomodulators opens up new opportunities in veterinary practice, especially in poultry flocks, and gives hope for improving the health of turkeys and reducing the administration of antibiotics to flocks, thereby improving consumer safety.

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