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Research paper

## Course of infection and immune responses in the respiratory tract of IBV infected broilers after superinfection with *E. coli*

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## ABSTRACT

Colibacillosis results from infection with avian pathogenic *Escherichia coli* bacteria. Healthy broilers are resistant to inhaled *E. coli*, but previous infection with vaccine or virulent strains of Infectious Bronchitis Virus (IBV) predisposes birds for severe colibacillosis. The aim of this study was to investigate how IBV affects the course of events upon infection with *E. coli*. Broilers were inoculated with IBV H120 vaccine virus or virulent M41 and challenged 5 days later with *E. coli* 506. A PBS and *E. coli* group without previous virus inoculation were included. Sections of trachea, lung and airsacs were stained for CD4, CD8,  $\gamma\delta$ -TCR,  $\alpha\beta$ 1-TCR, and for macrophages (KUL-01) and both pathogens. Changes in the mucociliary barrier of trachea, lung and airsacs did not predispose for bacterial superinfection. The disease in the lungs of the *E. coli* group and both IBV/*E. coli* groups was similar. Lesions in the airsacs were more pronounced and of longer duration in the IBV/*E. coli* groups. The immunocytological changes differed substantially between the *E. coli* group and both IBV/*E. coli* groups. In trachea, lungs and airsacs the CD4+ and CD8+ populations were significantly larger than in the *E. coli* and PBS groups. In the lungs and the airsacs the macrophages were more numerous in the IBV/*E. coli* and the *E. coli* groups than in the PBS group. The presence of high numbers of T cells and macrophages in IBV infected birds most likely induced an altered immune response, which is responsible for the enhanced clinical signs of colibacillosis.

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### 1. Introduction

In humans as well as in animals it is well known that viral infection of the respiratory tract can predispose for bacterial infections (reviewed by Heinzelmann et al., 2002; Matthijs et al., 2003). Two hypotheses for the underlying mechanisms have emerged. One hypothesis is that bacterial superinfection emerges from viral damage to

the respiratory tissue, characterized by loss of cilia and ciliated cells (Bakaletz, 1995), decreased ciliary activity and mucociliary clearance (Wilson et al., 1996), and/or that damage to epithelium may provide more attachment sites to bacteria (El Ahmer et al., 1999). A second hypothesis is that the immune responses after viral infection may increase the susceptibility for bacterial infections. Phagocytosis of bacteria by macrophages (Debets-Ossenkopp et al., 1982) or neutrophils (Engelich et al., 2001; Navarini et al., 2006) has shown to be hampered due to a previous viral infection. Moreover, the innate anti-viral responses, especially type I IFN, may have a severe granulotoxic effect that increases susceptibility to bacterial superinfection (Navarini et al., 2006). The cell populations in tissues of

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superinfected animals differ significantly from cell populations in tissues of animals infected with only one pathogen. In addition, superinfections may result in an overproduction of inflammatory cytokines, which may contribute to immunopathology due to exacerbated immune responses (Beadling and Slifka, 2004; Van der Sluijs et al., 2006; Speshock et al., 2007).

Two hypotheses to explain the enhanced susceptibility have been suggested but the underlying mechanisms are still not fully understood (Navarini et al., 2006). The results are not conclusive and further experimental studies are needed to elucidate this phenomenon. Most experiments concerning superinfections of the respiratory tract have been performed in laboratory animals (Beadling and Slifka, 2004; Van der Sluijs et al., 2006). These experimental models have several advantages, but an important disadvantage is that the animals used are usually not natural host for the infectious agents used.

The use of chickens as experimental animal in combination with avian pathogens could overcome this disadvantage. A superinfection model with Infectious Bronchitis Virus (IBV) and *Escherichia coli* in chickens has been developed (Goren, 1978). Matthijs et al. (2003) used this model and showed increased susceptibility for *E. coli* after previous infection with virulent IBV. Remarkably, this phenomenon was also observed after infection with a mild IBV vaccine virus. This suggested that mechanical tissue damage was not the cause of the enhanced susceptibility to *E. coli*.

This infection model offers the opportunity to investigate how a preceding infection with either a virulent or a mild IBV strain may affect the course of a subsequent *E. coli* infection in the respiratory tract (trachea, lung and airsac) of broilers. The aim of this study was therefore to investigate two possible mechanisms of enhanced susceptibility: tissue damage and alteration of the immune response. The immunocytological changes were observed over a time course from 0.5 h to 7 days after application of *E. coli* in the trachea. During that period dynamics of IBV, *E. coli*, lymphocytes (CD4, CD8,  $\gamma\delta$ -TCR and  $\alpha\beta$ 1-TCR) and macrophages (KUL-01) in the trachea, lung and airsacs of broilers were studied.

## 2. Materials and methods

### 2.1. Experimental chickens

Eighteen-day-incubated eggs originating from a *Mycoplasma gallisepticum*-free broiler parent (Cobb) flock were obtained from a commercial hatchery and hatched at the research facility of the Department of Farm Animal Health (Utrecht University). From day of hatch (day 1), broilers were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, The Netherlands) each with a volume of 0.68 m<sup>3</sup> and fitted with a wire floor of 0.93 m<sup>2</sup>. Isolators were ventilated at a rate of approximately 40 m<sup>3</sup> h<sup>-1</sup>.

Broilers were fed a commercial ration containing 12.4 MJ of metabolically energy per kg and 19.5% crude protein *ad libitum*, but from day 14 onwards feed was restricted to 75% of *ad libitum* intake on 'skip a day base' to

diminish leg disorders and hydrops ascites. Tap water was provided *ad libitum* throughout the experimental period. Up to the start of feed restriction, 22 h light was given per 24 h; thereafter it was reduced to 16 h. From day 4 onwards red light was given to prevent cannibalism. Isolator temperature was gradually decreased from 35 °C at day 1 to 20 °C at day 31. From day 31 to the end of the experiment isolator temperature was kept at approximately 20 °C.

All chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

### 2.2. Inocula

IBV vaccine virus H120 was obtained as commercial freeze-dried 1000 doses vials, which contained at least 10<sup>3.0</sup> EID<sub>50</sub> (egg infective dose 50%) per dose (Nobilis<sup>®</sup> IB H120; batch 041106A). The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, The Netherlands, as freeze-dried vials containing 10<sup>8.3</sup> EID<sub>50</sub>/1.2 ml/vial. The virus had been passaged twice in SPF embryonated eggs. All IBV inocula were prepared in distilled water just prior to use, and contained at least 10<sup>3.0</sup> EID<sub>50</sub>/ml of H120 virus and 10<sup>4.6</sup> EID<sub>50</sub>/ml of IBV M41 virus.

The *E. coli* strain 506 (O78; K80) was isolated from a commercial broiler (Van Eck and Goren, 1991). The *E. coli* culture was prepared as described by Matthijs et al. (2003) and was used at a concentration of 10<sup>7.6</sup> CFU/ml.

### 2.3. Experimental design

At day 1, broilers were randomly divided into four groups of 30 chickens each. Each group was housed in a separate isolator. At 27 days of age all groups were inoculated oculo-nasally (one droplet of 0.05 ml per bird in each eye and nostril) and intra-tracheally (1 ml per bird): groups 1 and 2 received distilled water, group 3 received IBV H120 vaccine and group 4 IBV strain M41. At 32 days of age, groups 2, 3 and 4 were intra-tracheally inoculated with 1 ml *E. coli* culture per bird. Group 1 received 1 ml PBS-diluted glucose broth intra-tracheally per bird.

For convenience, we refer to group 1 (distilled water and PBS broth) as PBS group, group 2 (distilled water and *E. coli* broth) as the *E. coli* group, group 3 (IBV H120 vaccine and *E. coli* broth) as the H120 group, and group 4 (IBV strain M41 and *E. coli* broth) as M41 group.

### 2.4. Clinical and post-mortem examination

Clinical signs of IBV infection were recorded 1, 2, 4 and 5 days after IBV inoculation and after *E. coli* inoculation and just before euthanizing. A bird was recorded as having signs of IBV infection if mucous nasal discharge was observed after mild pressure on the nostrils (Matthijs et al., 2003).

From each group, 5 broilers were electrocuted and bled at 0.5, 3 h, and at days 1, 2, 4 and 7 after *E. coli* inoculation

(hpi/dpi). At each time point, colibacillosis lesions were scored macroscopically. Lesion scoring was performed as described by Van Eck and Goren (1991) in the left and right thoracic airsac, pericardium and liver. Lesion scores ranged from 0 to 3 per organ, leading to a maximum score of 12 per bird (Van Eck and Goren, 1991).

## 2.5. Immunocytochemical staining

After post-mortem examination, trachea, left lung and both thoracic airsacs were collected and snap-frozen in liquid nitrogen for immunocytological examination. Samples for immunocytological staining were taken from the middle of trachea, in the area of the entrance of the mediadorsal secondary bronchi in the lung and of both thoracic airsacs in total. Cryostat sections (6  $\mu\text{m}$ ) were transferred to Superfrost Plus slides (Menzel-Glaser) and stored over silicagel for at least 24 h before use. Slides were fixed in pure acetone, air-dried and incubated with mAbs in appropriate concentrations for 1 h; mouse anti-chicken CD4, CD8 $\beta$ ,  $\gamma\delta$ -TCR,  $\alpha\beta$ 1-TCR and KUL-01 (Southern Biotechnology, Birmingham, USA). All mAbs were diluted in PBS containing 1% BSA (Sigma) and 0.1% sodium azide (PBS/BSA). Slides were washed in PBS and incubated for 1 h with biotinylated horse anti-mouse IgG (Vectastain<sup>®</sup>). Slides were washed with PBS and incubated for 1 h with avidin:biotinylated enzyme complex (Vectastain<sup>®</sup> Elite ABC kit) diluted in PBS. Then the slides were incubated with 1 mg 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma, USA) per ml Tris-HCl buffer (0.05 M, pH 7.6) containing 0.06% H<sub>2</sub>O<sub>2</sub>. To ensure no over- or under staining, slides were monitored during the reaction under the microscope. Slides were washed with PBS, counterstained with haematoxylin and mounted in Glycergel mounting medium (Dako, USA). All incubations were performed at room temperature in a humidified box. Control slides were incubated as described above, except that mAbs were omitted.

## 2.6. Immunocytological analysis of the tissue sections

### 2.6.1. Trachea

Three sections of the trachea per mAbs (CD4, CD8,  $\gamma\delta$ -TCR,  $\alpha\beta$ 1-TCR and KUL-01) were examined by light microscopy magnification. The score given per animal was the cell count of the 3 trachea rings in total divided by three. Cell populations were scored using the following system for CD4, CD8, KUL-01,  $\gamma\delta$ -TCR and  $\alpha\beta$ 1-TCR cells: 0 = 0–3 cells, 1 = 4–10 cells, 2 = 11 cells or 1 cluster of cells, 3 = multiple clusters. A cluster is a group of tens of cells located close together.

### 2.6.2. Left lung

Of each left lung and for each mAbs (CD4, CD8,  $\gamma\delta$ -TCR,  $\alpha\beta$ 1-TCR and KUL-01) three parabronchi in the proximity of the Bronchial Associated Lymphoid Tissue (BALT), but not next to the BALT, were analysed. This area was chosen to examine the same well-defined area in every lung consistently. The score given per animal was the total cell count of three parabronchi divided by three. Cell populations were scored using the following system for CD4:

1 = 0–20 cells, 2 = more than 20 cells and/or 1 cluster, 3 = multiple cell clusters; for CD8: 1 = 0–10 cells, 2 = 11–20 cells, 3 = more than 20 cells; for KUL-01: 1 = 20–50 cells, 2 = 51–200 cells, 3 = more than 200 cells; for  $\gamma\delta$ -TCR and  $\alpha\beta$ 1-TCR: 1 = less than 10 cells, 2 = 11–40 cells, 3 = more than 40 cells.

### 2.6.3. Left and right thoracic airsacs

Because of lack of a well-defined morphological unit in the airsacs the complete section of the airsacs was examined. Four levels of relative cell density were distinguished for all cell subpopulations: 0 = no cell influx, 1 = few scattered cells throughout the stroma, 2 = many cells scattered throughout the stroma, 3 = complete cell infiltration of the stroma of the airsac wall.

## 2.7. Detection of IBV and *E. coli*

### 2.7.1. Production of polyclonal antibodies against *E. coli* 506

The bacterial inocula were prepared by submersing one frozen bead (–70 °C) of a batch containing the *E. coli* 506 strain in trypton soya broth and by subsequent incubation for 20 h at 37 °C. The bacteria were washed with PBS and killed with methanol during 5 min. The solution was centrifuged 10 min at 1500  $\times$  g and the bacteria were washed twice with PBS and dissolved in 10 mg DDA (dimethyl dioctadecylammonium bromide)/ml PBS. Two rabbits were injected 3 times, 21 days between each injection, subcutaneous with 1.5 ml of the PBS/DDA solution containing 10<sup>9</sup> *E. coli* bacteria per ml. Pre-immune and immune sera were tested for specific antibodies against *E. coli* 506 on dot blot, paraffin and cryostat sections. Both live and fixed bacteria were recognized.

### 2.7.2. Tissue staining procedure for IBV and *E. coli*

Trachea and lung were collected in 10% buffered formalin, fixed for 24 h and processed to paraffin. Sections were cut 5- $\mu\text{m}$  thick, deparaffinised and placed in pure methanol with 0.75% H<sub>2</sub>O<sub>2</sub> for 30 min to remove endogenous peroxidase activity. Sections were incubated with rabbit serum specific for *E. coli* (*E. coli* 506) or with mouse sera with mAbs 48.4 against IBV nucleoprotein (Koch et al., 1990) for 1 h. After washing in PBS, the slides were co-incubated for 1 h with rabbit anti-mouse serum (Dako, Denmark) or goat anti-rabbit serum (Dako, Denmark) conjugated to horseradish peroxidase. The slides were washed and stained with 0.5 mg 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma, USA) per ml Tris-HCl buffer (0.05 M, pH 7.6) containing 0.03% H<sub>2</sub>O<sub>2</sub>.

For IBV and *E. coli* staining in the airsac, cryostat sections (6- $\mu\text{m}$  thick) were made from both thoracic airsacs in total. The procedure was the same as used in the immunohistochemical staining. The complete section of each organ was examined for general pathology and the location of both pathogens. The number of *E. coli* bacteria in different parts of the respiratory tract were recorded and classified. Four classes were distinguished: 0, 1, 2, and 3. For trachea and bronchi, the following classes were distinguished: in class 0 no bacteria were found; in class 1 1–5 bacteria; in class 2 6–10, and in class 3 more than 10 bacteria were counted. For the air sacs the classes were:

class 0 when no bacteria were found; class 1 for 1–20 bacteria; class 2 20–100 bacteria and class 3 when more than 100 were counted.

### 2.8. Statistical analysis

Between-group differences per time point were non-parametrically analysed for mean lesion score (MLS) using the Mann–Whitney test. Nasal discharge, airsacculitis and pericarditis/perihepatitis per time point were non-parametrically analysed between groups with Fischer Exact Test with Bonferroni correction. Lymphocyte subpopulations were analysed with Fischer Exact Test with the Bonferroni correction.

Examination of birds has not taken place for all organs at the same time points. For the statistical analysis of the airsac all time points were used, but for the other organs the time points of 0.5 and 3 hpi are missing. Both airsacs were examined separately. The classification of birds with respect to the number of *E. coli* was 87% identical; in 6% of the birds a difference of one class was found. Therefore only the right airsac was analysed statistically. Because of the low number of birds per group and time point, no distinction was made between the various classes for number of *E. coli* found. So, for the statistical analysis the result per bird was recorded as 'with (at least one) *E. coli*' or 'no *E. coli*' found (Table 3).

A generalized linear model was performed on the number of birds without or with *E. coli* with a Poisson distribution. The explanatory variables were treatment group (*E. coli*, H120 or M41), time and class (with/without) of *E. coli*. The interaction between time and class and group and class represent the relation between the distributions of the number of birds in the classes with *E. coli*. The best model was based on the (lowest) Akaike's Information Criterion (R Development Core Team, 2007; Pawitan, 2001) but the larger model should have at least a difference of two in AIC to be selected as the best model. For the generalized linear model the

**Table 1**

Number of broilers with mucous nasal discharge at different time points after inoculation with Infectious Bronchitis Virus (IBV) vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age.

Group	Days after IBV inoculation							
	1	2	4	5 <sup>a</sup>	6	7	9	12
PBS <sup>b</sup>	0/30 <sup>A</sup>	0/30 <sup>A</sup>	0/30 <sup>A</sup>	0/30 <sup>A</sup>	0/5 <sup>A</sup>	0/5 <sup>A</sup>	0/5 <sup>A</sup>	0/5 <sup>A</sup>
<i>E. coli</i> <sup>c</sup>	0/30 <sup>A</sup>	0/30 <sup>A</sup>	0/30 <sup>A</sup>	0/30 <sup>A</sup>	0/5 <sup>A</sup>	0/5 <sup>A</sup>	0/5 <sup>A</sup>	0/5 <sup>A</sup>
H120	0/30 <sup>A</sup>	1/30 <sup>A</sup>	2/30 <sup>A</sup>	3/30 <sup>A</sup>	1/5 <sup>A</sup>	2/5 <sup>A</sup>	3/5 <sup>A</sup>	0/5 <sup>A</sup>
M41	0/30 <sup>A</sup>	15/30 <sup>B</sup>	28/30 <sup>B</sup>	30/30 <sup>B</sup>	3/5 <sup>A</sup>	3/5 <sup>A</sup>	1/5 <sup>A</sup>	0/5 <sup>A</sup>

Groups with different superscript letters (A and B) within a column are significantly different ( $P < 0.05$ ).

<sup>a</sup> Day of inoculation with *E. coli*.

<sup>b</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.

<sup>c</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.

statistical program R version 2.5.1 (R Development Core Team, 2007) was used.

## 3. Results

After inoculation with IBV, the birds were clinically examined, and mucous nasal discharge was observed from 2 to 9 days p.i. (Table 1). After inoculation with *E. coli*, the birds were examined for clinical signs of colibacillosis, and post mortem examination was performed to quantify colibacillosis lesions. The number of birds with airsacculitis, perihepatitis and/or pericarditis and mean lesion score (macroscopical lesions) are presented in Table 2.

### 3.1. Trachea

#### 3.1.1. Location of pathogens

H120 virus was detected for 4 days after *E. coli* inoculation in 15 broilers, and M41 virus was detected

**Table 2**

Macroscopical colibacillosis lesions: number of broilers with airsacculitis and/or pericarditis/perihepatitis and the mean lesion score (MLS  $\pm$  S.D.). At each time point and within each group five birds were analysed. Birds were inoculated with Infectious Bronchitis Virus (IBV) vaccine H120 (group: H120) or virulent IBV M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age.

Broilers with	Group	Time after <i>E. coli</i> 506 strain inoculation					
		0.5 h	3 h	1 day	2 days	4 days	7 days
Airsacculitis	PBS <sup>a</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>
	<i>E. coli</i> <sup>b</sup>	0 <sup>A</sup>	0 <sup>A</sup>	5 <sup>B</sup>	2 <sup>AB</sup>	3 <sup>AB</sup>	0 <sup>A</sup>
	H120	4 <sup>AB</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>
	M41	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	4 <sup>AB</sup>	5 <sup>B</sup>	5 <sup>B</sup>
Perihepatitis and/or pericarditis	PBS <sup>a</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>
	<i>E. coli</i> <sup>b</sup>	0 <sup>A</sup>	0 <sup>A</sup>	1 <sup>A</sup>	0 <sup>A</sup>	2 <sup>A</sup>	0 <sup>A</sup>
	H120	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	2 <sup>A</sup>	1 <sup>A</sup>
	M41	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	3 <sup>A</sup>	2 <sup>A</sup>
MLS	PBS <sup>a</sup>	0.0 $\pm$ 0.0 <sup>A</sup>	0.0 $\pm$ 0.0 <sup>A</sup>	0.0 $\pm$ 0.0 <sup>A</sup>	0.0 $\pm$ 0.0 <sup>A</sup>	0.0 $\pm$ 0.0 <sup>A</sup>	0.0 $\pm$ 0.0 <sup>A</sup>
	<i>E. coli</i> <sup>b</sup>	0.0 $\pm$ 0.0 <sup>A</sup>	0.0 $\pm$ 0.0 <sup>A</sup>	1.6 $\pm$ 1.6 <sup>B</sup>	1.6 $\pm$ 2.2 <sup>AB</sup>	3.4 $\pm$ 3.4 <sup>AB</sup>	0.0 $\pm$ 0.0 <sup>A</sup>
	H120	0.4 $\pm$ 0.2 <sup>A</sup>	0.5 $\pm$ 0.0 <sup>A</sup>	2.4 $\pm$ 1.5 <sup>B</sup>	3.4 $\pm$ 1.9 <sup>B</sup>	6.6 $\pm$ 3.5 <sup>B</sup>	5.0 $\pm$ 4.1 <sup>B</sup>
	M41	0.5 $\pm$ 0.0 <sup>A</sup>	0.5 $\pm$ 0.0	3.4 $\pm$ 0.9 <sup>B</sup>	2.0 $\pm$ 1.9 <sup>B</sup>	6.4 $\pm$ 2.5 <sup>B</sup>	4.5 $\pm$ 3.0 <sup>B</sup>

Groups with different superscript letters (A and B) within a column are significantly different ( $P < 0.05$ ).

<sup>a</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.

<sup>b</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.

**Table 3**

Number of birds with or without *E. coli* bacteria in different parts of the respiratory tract detected by immunostaining. Birds were inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age. At each time point and within each group five birds were analysed.

Tissue	Group	Time after <i>E. coli</i> infection					
		0.5 h	3 h	1 day	2 days	4 days	7 days
Right airsac	PBS	0/5 <sup>a</sup>	0/5	0/5	0/5	0/5	0/5
	<i>E. coli</i>	2/3	2/3	3/2	2/3	2/3	0/5
	H120	2/3	1/4	1/4	1/4	0/5	0/5
	M41	1/4	1/4	1/4	1/4	0/5	0/5
Trachea	PBS	n.d.	n.d.	0/5	0/5	0/5	0/5
	<i>E. coli</i>	n.d.	n.d.	1/4	0/5	0/5	0/5
	H120	n.d.	1/4	1/3	2/2	1/4	0/5
	M41	n.d.	1/4	1/4	0/5	0/4	0/5
Parabronchi	PBS	n.d.	n.d.	0/5	0/5	0/5	0/5
	<i>E. coli</i>	n.d.	n.d.	5/0	5/0	4/1	2/3
	H120	n.d.	3/2	2/3	4/1	5/0	2/3
	M41	n.d.	4/1	3/1	3/1	2/2	2/3
Bronchi	PBS	n.d.	n.d.	0/5	0/5	0/5	0/5
	<i>E. coli</i>	n.d.	n.d.	5/0	4/1	1/3	0/5
	H120	n.d.	4/1	1/3	2/2	2/3	0/5
	M41	n.d.	3/2	2/2	0/5	1/3	0/4

n.d., Not determined.

<sup>a</sup> 0/5: the number of birds with at least one *E. coli* detected/the number of birds without *E. coli* in that part of the respiratory tract.

for 2 days in 6 broilers. Virus was found in the columnar ciliated cells, and in the lamina propria. We did not find any *E. coli* bacteria either attached to the epithelium or in the underlying tissue of the trachea (Table 3). No time effect

was found in number of birds with *E. coli*. In birds in the H120 group, *E. coli* was more often found than in birds of the *E. coli* group and the M41 group. No difference was found in number of birds with *E. coli* between the *E. coli* and M41 groups.

**3.1.2. Immunocytological changes**

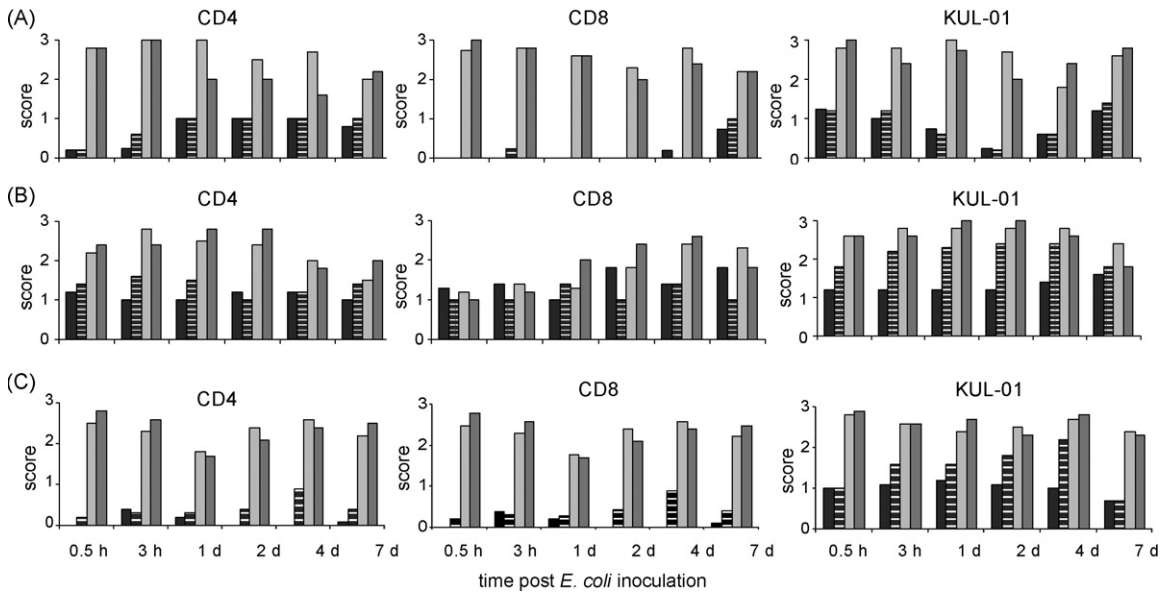
In the PBS and the *E. coli* group no influx of lymphocytes was found. The few lymphoid cells found in the lamina propria were CD4+ cells and macrophages. In contrast, in the H120 and the M41 group massive lymphocyte infiltrations were found at all time points after *E. coli* challenge. These infiltrates consisted of CD4+ and CD8+ lymphocytes, and macrophages (Fig. 1A). The CD4+ cells expressed the  $\alpha\beta$ 1-TCR+, whereas the CD8+ cells expressed either the  $\gamma\delta$ -TCR+ or  $\alpha\beta$ 1-TCR+. No significant differences in number of CD4+ and CD8+ cells were found between the H120 and the M41 groups. From 2 dpi onwards the expression of the T cell receptors, both  $\gamma\delta$ -TCR and  $\alpha\beta$ 1-TCR, reduced drastically. The expression of CD4 and CD8 in tissues of birds in these groups remained high (data not shown).

All cell subpopulations were found scattered throughout the tissue, but CD4+ and CD8+ cells were also found in clusters. The CD8+ cells were found around B cell follicles, and CD4+ cells were located within B cell follicles.

**3.2. Lung**

**3.2.1. Location of pathogens**

IBV (H120 and M41) was found in the columnar ciliated cells of the epithelium in the bronchi, but not in the



**Fig. 1.** Number of CD4+, CD8+ and KUL-01+ cells in different parts of the respiratory tract of broilers inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41). All birds except those in the PBS group were inoculated with *E. coli* at day 5 after IBV inoculation. At each time point and within each group five birds were analysed. Black bars: PBS group; hatched bars: *E. coli* group; grey bars: H120 group; dark bars: M41 group. (A, trachea) Mean cell count per three trachea rings. CD4+, CD8+, KUL-01+ cells: 0: 0–3 cells, 1: 4–10 cells, 2: 11 cells or 1 cluster of cells, 3: more than 1 cell cluster. A cluster is a group of tens of cells located close together. (B, lungs) Mean cell count per three parabronchi. CD4+ cells: 1: 0–20 cells, 2: more than 20 cells and 1 cluster, 3: multiple cell clusters, for CD8+ cells: 1: 0–10 cells, 2: 11–20 cells, 3: more than 20 cells, for KUL-01: 1: 20–50, 2: 51–200, 3: more than 200 cells. (C, airsacs) The complete section of the airsac was examined. CD4, CD8, KUL-01: 0 = no cell influx, 1 = few scattered cells throughout stroma, 2 = many cells scattered throughout stroma, 3 = massive cell infiltration of stroma of airsac wall.

parabronchus. *E. coli* was found as free bacteria and agglomerates in the capillary area, atria, and pus within parabronchial and bronchial lumina (Table 3). At 2 and 4 dpi *E. coli* was mainly found as small agglomerates in the capillary part and lumen of the parabronchus. The presence of bacteria was accompanied by inflammatory changes of the parabronchi (data not shown). In both the parabronchi and secondary bronchi no time or group effect was found in the number of broilers with *E. coli*.

### 3.2.2. Immunocytological changes

At 0.5 hpi many CD4+ cells and macrophages were present in lungs of IBV infected birds. At that time no difference in number of CD8+ cells was found between the different groups (Fig. 1B). From 1 to 7 days after *E. coli* infection, an increase in CD8+ cells was detected in the M41 group and at 4 and 7 dpi in the H120 group. The numbers of CD4+, CD8+ and KUL-01+ cells in H120 and M41 groups significantly exceeded the numbers of these cells in the *E. coli* group.

In H120 and M41 groups many macrophages were present until 4 dpi. In the *E. coli* group, the number of macrophages increased after inoculation, but did not equal the levels in the H120 and M41 groups. At 7 dpi the number of macrophages was decreased in the *E. coli* and M41 groups to the level found in the PBS group, but it remained high in the H120 group. Up to 2 dpi most macrophages were elongated, whereas from 4 dpi most macrophages were enlarged and rounded up suggesting activation of these cells.

No significant differences in the number of  $\gamma\delta$ -TCR+ cells in the lung were found between the four groups. In the H120 and M41 groups, the number of  $\alpha\beta$ 1-TCR+ cells increased 4 dpi coinciding with the increase of CD8+ cells (TCR data not shown; Fig. 1B). No significant difference between H120 and M41 groups were found in numbers of CD4+, CD8+, KUL-01+,  $\gamma\delta$ -TCR+ and  $\alpha\beta$ 1-TCR+ cells.

## 3.3. Airsac

### 3.3.1. Location of pathogens

IBV was found in the columnar ciliated cells of respiratory epithelium in the airsacs of birds in the H120 and M41 groups; H120 virus was detected for 2 days after *E. coli* inoculation in 14 birds, and M41 virus was detected for 3 h after *E. coli* inoculation in 9. *E. coli* was found predominantly in pus located at the respiratory surface in the airsacs. For the right airsac no group effect was found on number of birds with *E. coli* (Table 3).

### 3.3.2. Immunocytological changes

In the PBS and *E. coli* groups only few CD4+ cells were observed. In contrast, massive infiltrations of CD4+ and CD8+ lymphocytes were found in birds in the H120 and M41 groups from 0.5 hpi till 7 dpi (Fig. 1C). Macrophages were present in H120 and M41 groups at 0.5 hpi, and their number remained high up to 7 dpi (Fig. 1C). In the PBS and *E. coli* groups, few macrophages were present at 0.5 hpi. Only in the *E. coli* group a substantial increase was found which was highest at 4 dpi. Nevertheless, the number of macrophages in this group remained lower than the

number of macrophages in the H120 and M41 groups. Seven dpi the number of macrophages in the *E. coli* group decreased to a level comparable to the level of the birds in the PBS group (Fig. 1C). The numbers of CD4+, CD8+ and KUL-01+ cells in H120 and M41 groups exceeded significantly the numbers of these cells in the *E. coli* group.

In the H120 and M41 groups the number of  $\gamma\delta$ -TCR+ cells and  $\alpha\beta$ 1-TCR+ cells was high from 0.5 hpi till the end of the experimental period. In the *E. coli* group an increase of  $\gamma\delta$ -TCR+ cells occurred from 0.5 hpi to 4 dpi occurred, which was decreased at 7 dpi. Few  $\gamma\delta$ -TCR+ cells were found in the PBS group and hardly any  $\alpha\beta$ 1-TCR+ cells were found in the PBS and *E. coli* groups. No significant differences in number of CD4+, CD8+, KUL-01+,  $\gamma\delta$ -TCR+ and  $\alpha\beta$ 1-TCR+ cells were detected between the H120 and M41 groups.

## 4. Discussion

In this study the course of an *E. coli* infection after a previous infection with a virulent (M41) or vaccine (H120) strain of IBV in trachea, lungs and airsacs of broilers was examined. The aim of this study was to investigate two possible mechanisms of enhanced susceptibility: tissue damage and alteration of the immune response.

Despite significant differences in number of birds with nasal discharge 2–5 days after inoculation with either IBV strain, comparable clinical, macroscopical and microscopical changes were observed after infection with *E. coli* in both groups. Remarkably, 4 dpi mean lesion scores were comparable in both the *E. coli* and IBV groups, whereas at 7 dpi birds in the *E. coli* group were fully recovered but birds in both IBV groups still showed signs of colibacillosis. These findings suggest that due to a previous infection with either IBV strain birds were less capable of conquer the damage of the *E. coli* infection.

In the trachea, epithelial damage and mononuclear cell infiltration due to infection with IBV was found, however, no additional effect of the subsequent infection with *E. coli* was noticed indicating that the apparent changes did not result in predisposition of the trachea for bacterial superinfection. In the lungs of both the *E. coli* group and the IBV groups acute purulent pneumonia of similar severity was observed, suggesting no additional effect of IBV on acute pneumonia. Moreover, the course of the disease in the lungs in both the *E. coli* group and the IBV groups was similar and birds of these groups were nearly completely recovered from pneumonia at 7 dpi. Although apoptosis of granulocytes and subsequent leukopenia is described after viral infection (McCullers, 2006; Navarini et al., 2006) the massive granulocyte infiltration in the lungs and the presence of pus in the airsacs in both *E. coli* and the IBV-*E. coli* infected birds did not clearly suggest a reduction in the participation of granulocytes in the process. These findings suggest that neither trachea nor lungs (as suggested by DeRosa et al., 1992; Smith et al., 1985) were predilection sites for bacterial superinfection.

Lesions in the airsacs of both IBV groups were more pronounced and of longer duration than in the *E. coli* group. In contrast to the lungs, the airsacs from IBV infected birds were not recovered at 7 dpi whereas the airsacs of only *E.*

*coli* infected birds were. The airsacs were the only location where both pathogens were found at the same time. Our results indicate that the difference in reaction upon superinfection with *E. coli* in IBV infected broilers compared to an *E. coli* infection without preceding virus infection is not caused by damage to the mucociliary barrier facilitating bacterial adhesion or penetration or by reduced clearance of bacteria.

The second hypothesis was that alteration of the immune response by a virus infection could make birds more susceptible to subsequent bacterial infections. At time of infection with *E. coli*, great numbers of CD4 cells, CD8 cells and macrophages were found in trachea and airsacs of birds of the IBV groups, whereas great numbers of CD4 cells and macrophages were found in the lung. The pronounced presence of macrophages at time of challenge with *E. coli* most likely increased the clearance of the bacteria, since fewer bacteria were found in lungs and airsacs of IBV infected groups. This finding suggests a reduced susceptibility for a superinfection with *E. coli*. However, macrophages produce type I IFNs (IFN- $\alpha/\beta$ ) after viral infection which have potent effects on viral replication, including that of IBV, but these cytokines can inhibit host defence against both Gram-positive and Gram-negative bacteria such as *Listeria monocytogenes* (O'Connell et al., 2004), *Mycobacterium tuberculosis* in the lungs (Manca et al., 2001), and *Salmonella typhimurium* (Navarini et al., 2006), and possibly also against *E. coli* in broilers. The CD4 cells and CD8 cells also present in large numbers in IBV groups can produce type II IFN (IFN- $\gamma$ ) which is known to have a protective role in both viral and bacterial infections through the induction of a Th1 cell immune response (Decker et al., 2002). However, its role in the activation of macrophages and airway epithelial cells can lead to an exaggerated inflammatory response (Konno et al., 2002). Both T cells and macrophages present at time of *E. coli* inoculation might therefore be responsible for the enhanced colibacillosis, due to overproduction of inflammatory cytokines.

In the airsacs, the numbers of macrophages remained high in the IBV groups, whereas the number of macrophages in the *E. coli* group decreased at 7 dpi to the level of the PBS group. Although both IBV and *E. coli* were cleared in all groups, the macrophages were still present in the airsacs at 7 dpi in the IBV groups. It is unclear whether the macrophages and T cells in the trachea, lungs and airsacs are functionally altered by the IBV infection, but the clear difference in the presence of these cells in IBV infected birds both at time of challenge and in the airsacs at 7 dpi might suggest that an altered microenvironment resulted in altered immune responses, as for example demonstrated in mice where lymphocytes were detrimental during the early innate immune responses against *L. monocytogenes* due to increased apoptosis inhibiting effector reactions (Carrero et al., 2006). Both T cells and macrophages present at the time of *E. coli* inoculation might therefore be responsible for the increase of both the severity and the duration of the inflammatory reactions found in the airsacs in both IBV-*E. coli* infected birds, due to overproduction of inflammatory cytokines, as demonstrated in a study with turkeys (Rautenschlein et al., 1998).

Ariaans et al. (2008) found that the phagocytic capacity and NO production of peripheral blood mononuclear cells and splenocytes was not affected by prior exposure to IBV, but that the proinflammatory response in the spleen of IBV infected birds seemed severely impaired compared to the only *E. coli* infected birds, and suggested that the virus modulated the innate immunity of the birds. Our study only showed immunocytological changes, as no functional study was performed.

In this study, only a limited number of samples were examined. However, the samples were considered representative, because they were taken in a uniform and reproducible manner and the lesions within each compartment were distributed evenly.

It is concluded that a preceding infection of the respiratory tract with IBV does not predispose for bacterial superinfection with *E. coli* by altering the mucociliary barrier but likely moderates the immune response.

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