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# Serum Levels of Mannan-Binding Lectin in Chickens Prior to and During Experimental Infection with Avian Infectious Bronchitis Virus

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**ABSTRACT** Mannan-binding lectin (MBL) is a glycoprotein and a member of the C-type lectin super family, the collectin family, and the acute phase protein family. The MBL exerts its function by directly binding to microbial surfaces through its carbohydrate recognition domains, followed by direct opsonization or complement activation via MBL-associated serine proteases (MASP) –1 and –2. Thus, MBL plays a major role in the first-line innate defense against pathogens. We investigated the MBL concentrations in serum during experimental infectious bronchitis virus (IBV) infections in chickens. The results showed that the acute phase MBL response to infection with IBV was, to a degree ($P < 0.0068$), dependent on whether the chickens were inoculated after 12 h of rest (dark) or after 12 h of activity (light). The acute phase response in chickens challenged after 12 h of activity peaked after 4.6 d with an increase of 24%, whereas the acute phase response in chickens challenged after 12 h of rest peaked after 3.1 d with an increase of 51%. The specific antibody titer against IBV was also tested, and a difference ($P < 0.0091$) between the two experimental groups was found with peak titer values of 6,816 and 4,349. However, the highest value was found in chickens inoculated after 12 h of activity. Thus, an inverse relation exists between the MBL response and the IBV specific antibody response. The ability of MBL to activate the complement cascade was tested in a heterologous system by deposition of human C4 on the chicken MBL/MASP complex. The complement activation was directly associated with the concentration of MBL in serum, indicating neutralization of the virus before the humoral antibody response took over.

(*Key words*: chicken, viral infection, infectious bronchitis virus, mannan-binding lectin)

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## INTRODUCTION

Mannan-binding lectin (MBL) is a glycoprotein and a member of the collectin family of proteins. Members of the collectin family share identical molecular structures having a collagenous region linked to a C-type carbohydrate recognition domain (CRD). These animal lectins also belong to the C-type lectin super family. Besides MBL, the family includes the lung surfactant proteins A (SP-A) and D (SP-D), conglutinin, and collectin-43 (CL-43). So far, the latter two have only been detected in *Bovidae*. So far MBL has been isolated from mammals (reviewed by Holmskov, 2000), birds (Laursen et al., 1995), and fish (Vitved et al., 2000).

The binding of collectins to carbohydrates on the surface of microorganisms and the binding of the collectins to the collectin receptor on macrophages trigger a number of cellular defense mechanisms including phagocytosis, modulation of cytokines, and immunoglobulin secretion. Collectins have been shown to bind to a variety of bacteria, viruses, fungi, and parasites (Epstein et al., 1996). In addition, only MBL can activate the complement system, independent of antibody and C1q, via the two MBL-associated serine proteases (MASP). This leads to cleavage of the complement components C4 and C2 and thus generates the C3 convertase (Matsushita and Fujita, 1996; Thiel et al., 1997), resulting in opsonization or lysis of the microorganism.

The MBL has been extensively studied in mammals but less so in chickens. Chicken MBL (cMBL) has been isolated from liver and serum by means of cMBL-specific monoclonal antibodies (Oka et al., 1985; Sugii and Hirota, 1994; Laursen et al., 1995). Furthermore, assays with cMBL


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**Abbreviation Key:** cMBL = chicken MBL; IBV = infectious bronchitis virus; IBDV = infectious bursal disease virus; ILTV = infectious laryngotracheitis virus; MASP = MBL-associated serine protease; MBL = mannan-binding lectin; OD = optical density; PI = postinfection.

bound to solid-phase mannan showed that the chicken lectin could mediate an antibody-independent activation of the complement as judged by the activation and deposition of human complement component C4 on the MBL/MASP complex. Deposition of human C4 was dependent on the amount of cMBL added to the assay and the presence of calcium. Nielsen et al. (1998, 1999) investigated the function of cMBL in connection with avian viral infections. They studied the level and distribution of chicken MBL in different tissues during experimental infection with infectious laryngotracheitis virus (ILTV), infectious bursal disease virus (IBDV), and infectious bronchitis virus (IBV). The serum concentration of chicken MBL increased two- to threefold in virus-infected chickens compared with noninfected controls, indicating that chicken MBL is a minor acute phase reactant. In noninfected controls, cMBL was found in the cytoplasm of a few liver cells, whereas more cells were found in the liver from IBDV- or ILTV-infected chickens, indicating an upregulation of the MBL synthesis during acute infection. They also observed cMBL in germinal centers of cecal tonsils of noninfected cells. Furthermore, they found that cMBL had the ability to bind to ILTV-infected tracheal cells and the ability to bind to macrophage-like cells in the spleen from IBDV-infected cells (Nielsen et al., 1998). These results strongly indicate that cMBL plays an active role in the innate immune system. In IBV studies (Nielsen et al., 1999), the MBL concentration peaked 3 to 7 d after infection and returned to normal levels 6 to 10 d after the infection. However, only four of the eight chickens (7 wk old) analyzed had increased serum concentrations of MBL (1.5 to 3-fold) within the first 8 d postinfection (PI). The remaining chickens did not respond at all. Nielsen et al. (1999) proposed that the inconsistent MBL response was possibly a parallel manifestation of the mild distress (no clinical signs observed) caused by the IBV infection.

Infectious bronchitis virus in chickens is a corona virus that induces a highly contagious viral respiratory disease of chickens and is characterized by tracheal rales, coughing, and sneezing. In addition to causing disease in the respiratory tract, the virus also affects the oviduct and other tissues, depending on the virus strain. The infection occurs worldwide and causes great losses to the poultry industry due to the negative impact on production and to vaccine expenses.

To gain further insight into the MBL acute phase response, we have investigated the serum MBL concentrations, the specific IBV antibody titer, and the capacity of complement activation during experimental IBV infections in chickens.

## MATERIALS AND METHODS

### Experimental Chickens

The experimental chickens were hatched from specific-pathogen-free eggs.[2] After hatch, the chickens were wing-banded and transferred to positive-pressure isolators where they were kept until the end of the experiments. Thus, the chickens were individually identified during the experiments. Water and commercial chicken feed were supplied ad libitum. During the initial 2 wk of the experiments, the lighting period was reduced from constant light to 12 h beginning at 0700 h. Thus, a 12-h period of total darkness started at 1900 h.

### Virus

Experimental infections were performed with the M41 strain of IBV.[3] The virus was propagated and titrated by inoculation in the allantoic cavities of 9-d-old specific-pathogen-free chicken embryos.

### Experimental Infections and Design

The experimental infections were performed in two independent identical trials (Experiments 1 and 2), each including 30 chickens. The time between trials was 3 mo. In each trial, the chickens were allocated to three groups of 10 birds each and placed in three isolators. At 42 d of age, all chickens were inoculated nasally and orally with 0.2 mL of inoculum. One group was mock inoculated with sterile allantoic fluid at 0900 h. The other two groups were inoculated at 0900 h (light) and at 2100 h (dark), respectively, and each chicken was given $10^{6.25}$ egg lethal $dose_{50}$ of IBV.

### Serum Samples

Serum samples were collected from all chickens when they were 14, 21, 28, 35, and 38 d old, which corresponded to 28, 21, 14, 7, and 4 d before inoculation with the virus. Five chickens in each group were bled on Days 1, 3, 5, 9, 14, and 18 PI, and five chickens were bled on Days 2, 4, 7, 11, 16, and 21 PI.

### ELISA for Measurement of Serum MBL

Microtiter plates[4] were coated with 5 $\mu$g/mL of the monoclonal anti-chicken MBL antibody HYB182-1 diluted in PBS according to the method described in Laursen et al. (1998) and then placed overnight in a humid chamber at 4 C. Upon emptying the wells, nonspecific binding was blocked by incubation with 10 mg/mL of BSA in TBS (10 m*M* Tris base, 140 m*M* NaCl, pH 7.6) for 2 h at room temperature. The plates were washed with TBS-Tween, after which duplicates of 100 $\mu$L of serum dilutions were added to the wells. The serum samples were diluted from 200 to 800 times in TBS-Tween-EDTA. To determine MBL content in the samples, standard chicken serum with a known MBL concentration was diluted 1.5-fold in eight steps and transferred in duplicate to wells in the middle of the plate. Plates were incubated overnight at 4 C in a humid chamber. After the plates were washed, wells were incubated for 2 h at room temperature with 1 $\mu$g/mL of biotinylated HYB182-1. Another wash

[2]Lohmann Tierzucht, Cuxhaven, Germany.
[3]Veterinary Laboratories Agency, Weybridge, UK.
[4]Polysorp, Nunc A/S, Roskilde, Denmark.

was followed by a 2-h incubation at room temperature with 0.125 $\mu$g/mL alkaline phosphatase-conjugated avidin. After 1 h of incubation at 37 C with the substrate paranitrophenylphosphate (1 mg/mL, dissolved in diethanalamine buffer, pH 9.8), the optical density (OD) at 405 nm was measured. Using OD values corresponding to each of the eight dilutions of the 'standard' serum, a calibration curve was constructed. OD values of samples were transformed to MBL concentrations by readings from this standard curve. The interassay CV was 10.6% (n = 42 d), the inter plate CV was 11.9% (n = 2 plates), and the intra plate CV was 9.0% [n = two controls; 9.91 and 11.24 $\mu$g/mL)].

For figure presentation, the basic cMBL level of each bird was determined as a mean value of the five samples taken before the infection, i.e., on Days 28, 21, 14, 7, and 4 before infection. The percentage increase in MBL concentration was calculated as X $\mu$g/mL $\times$ 100%/Y $\mu$g/mL, where X is the actual measurement, and Y is the basic level of cMBL in the particular bird.

### ELISA for Measurement of Serum Antibody Titers to IBV

The ProFLOK IBV ELISA Test Kit[5] was used to measure serum antibody titers to IBV. The ELISA assay was performed according to the kit manual.

### Complement Deposition

The complement deposition was performed as an ELISA and was essentially measured as described by Petersen et al. (2001) with a few modifications. Two randomly chosen birds were analyzed from each of the three experimental groups bled on Days –7, –4, 1, 3, 5, 9, 14, and 18 PI. Microtiter wells were coated with 0.2 $\mu$g mannan[6] in 100 $\mu$L carbonate buffer, pH 9.6, and blocked by 200 $\mu$L 0.1% BSA for at least 2 h. After an overnight incubation with serum samples, the wells received 0.25 $\mu$g human complement factor C4[7] per well. Another incubation was performed for 1.5 h to activate C4 and for C4b deposition onto the MBL/MASP complex. Deposited C4b was detected by means of two biotinylated monoclonal antibodies[8] using 0.03 $\mu$g of each antibody per well. Streptavidin conjugated with horseradish peroxidase[9] was added at a 1:10,000 dilution, and the presence of horseradish peroxidase was detected by adding 3.3',5.5"tetramethylbenzidin (<0.05% wt/wt TMB). After 15 min, the color development was stopped by adding 1 *M* $H_2SO_4$, and the OD was read at 450 nm with 650 nm as a reference. Normal chicken serum (stored at –80 C in aliquots) was used as a standard, and wells receiving only buffer were used as blanks.

### Statistical Analysis

Due to the lack of normal distribution of the antibody titer data raised from the IBV infection, data were transformed to log($e$), which gave an almost normally distributed dataset. Group 1 included birds bled on Days 5, 9, 14, and 18 PI in both experiments, and Group 2 included birds bled on Days 7, 11, 16, and 21 PI in both experiments. The two experiments started at different times, which might have influenced the effect of the challenge with IBV. In order to analyze the difference in the antibody response titer due to infection time, the following model was used:

$$y_{ijl} = \mu + T_i + G_{ij} + Ex_i + T \times Ex_{ik} + D_l + e_{ijl} \quad \text{[Model 1]}$$

where $\mu$ = overall means, $T_i$ = fixed effect of infection time i, $G_{ij}$ = fixed effect of grouping j of birds within time of infection, $Ex_k$ = fixed effect of experiments k, $D_l$ = fixed effect of the first day after infection, and $y_{ijl}$ and $e_{ijl}$ are expected to be normally distributed.

Furthermore, a statistical analysis was done on the basis of the MBL concentration before the IBV challenge according to the grouping and the age of sampling as given for Model 2:

$$y_i = \mu + T_i + b \cdot age_i + e_i \quad \text{[Model 2]}$$

where $T_i$ = fixed effect of the grouping i used in the subsequent experiments, $b$ = regression on age, $age$ = age at drawing the sample in group i, and $\mu$, $y_i$, and $e_i$ were the same as for Model 1.

Finally, the MBL concentration as a consequence of the IBV infection was statistically analyzed, omitting the data of the control. The time factor was included in the model as second-degree polynomium as a covariable within treatment. Also included in the model was the probable effect of the repeated experiments. Thus the model becomes

$$y_{ik} = \mu + T_i + Ex_k + b_1 D + b_2 D^2 + (b_{1i} D + b_{2i} D^2)_i + e_{ik} \quad \text{[Model 3]}$$

where $\mu$, $T_i$, $Ex_k$, $y_{ik}$, and $e_{ik}$ were the same as for Model 1, $b_1$,$b_2$ = regression coefficients on first and second degrees generally, $b_{1i}$,$b_{2i}$ = regression coefficients on first and second degrees specifically for trait i, $D$ = time in days, and $D^2$ = time in days squared.

## RESULTS

Because the basic level of MBL in individual chickens varies from 0.4 $\mu$g/mL to 37.8 $\mu$g/mL (Laursen and Nielsen, 2000), the MBL concentrations in all birds in both experiments were measured weekly prior to the challenge

[5]Catalog no. 54-82-01, Kirkegaard and Perry Laboratories, Gaithersburg, MD.
[6]Statens Serum Institut, Copenhagen, Denmark.
[7]KemEnTec A/S, Copenhagen, Denmark.
[8]Mab 162.2 and Mab 162.4, Statens Serum Institut, Copenhagen, Denmark.
[9]HRP P0397, DAKO A/S, Glostrup, Denmark.

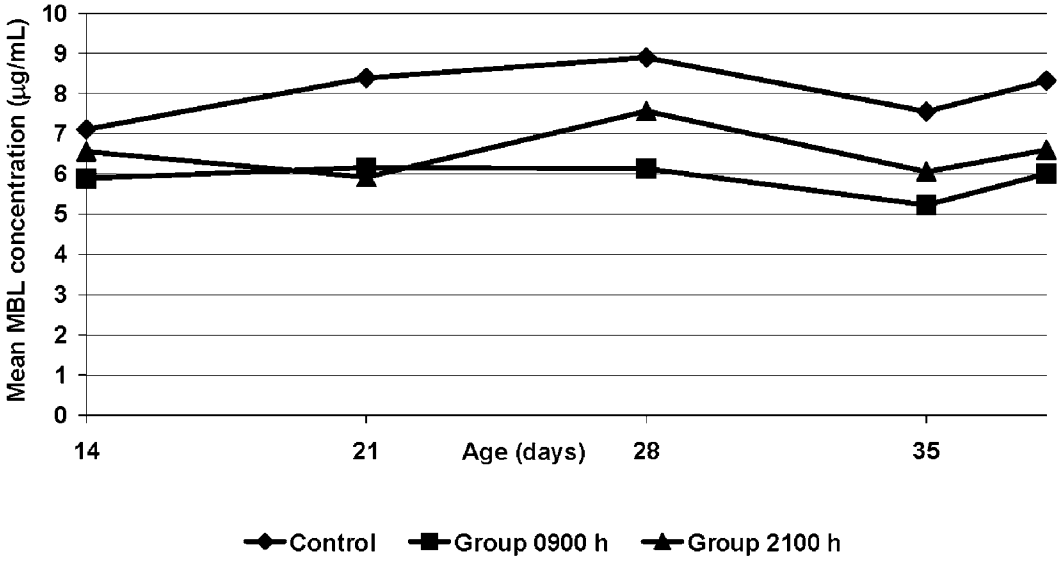


**FIGURE 1.** The mean basic serum mannan-binding lectin (MBL) level in 60 chickens at the indicated ages (30 chickens from each experiment) before challenge. Each curve represents 20 chickens. The control group was later mock inoculated; Group 1 was later inoculated at 0900 h, and Group 2 was inoculated at 2100 h.

with IBV until 4 d before the infection (five samples from each bird) (Figure 1). The F-test of the T-effect (Model 2) showed that the means of the three curves differed ($P < 0.0044$). The control group was significantly higher than the two other groups, which might have been due to the unknown MBL genotypes. The MBL level was fairly stable in 14- to 38-d-old chickens during the sampling period, and a linear effect of time was not found ($P = 0.47$). The mean value of these five samples from each chicken was calculated and used as the basic level of MBL in each chicken (0 percentage according to the acute phase response).

To evaluate serum MBL concentrations during the two experimental infection trials, we inoculated 6-wk-old chickens with IBV. One group of chickens was inoculated with IBV at 0900 h after a long period of darkness, one group of chickens was inoculated with IBV at 2100 h after a long period of light, and one group of chickens was mock inoculated. Serum samples were collected during the acute stage of the infection and analyzed for the concentration of MBL in serum. The MBL concentration was then transformed to the percentage of up-regulation according to the basic level of MBL in each individual chicken. Only days showing the acute stage of the infection are included in Figure 2. At Days 9 to 21, the MBL level returned to basal levels in the two virus-infected groups. The control chickens did not respond to the inoculation, indicating no stress as a result of handling the chickens. The virus-infected chickens responded to the treatment showing a 1.5 to 2-fold increase in the MBL concentration peaking 3 to 4 d PI and returning to normal level on Day 7 PI. However, a discrepancy was observed between chickens inoculated at 0900 h and those inoculated at 2100 h. The statistical analysis according to Model 3, comprising data from serum samples taken during Days 1 to 8 PI, showed differences ($P < 0.0001$) between the two infected groups. The least squares means of the treatments, adjusted for curve-linear effects of time and the effect of experiments, were 23% for chickens challenged at 2100 h and 45% at 0900 h.

The contemporary covariable effects of time after challenge were greater ($P < 0.0001$) for first-degree and ($P < 0.001$) for second-degree coefficients of the polynomial. There was no within-treatment effect of the time after challenge, indicating that the two curves of Figure 2 had the same curvature. The full Model 3 explained 31.2% of the variation in the data set. Taking the first derivative of the polynomial for each of the treatments and equating that to zero and solving for time gave the time of maximum for the curve. This value was 3.6 d for chickens challenged at 0900 h and 4.5 d at 2100 h. When compared with Figure 2, it may seem a bit high due to the statistical model used. However, the importance of the obtained values for the two treatments was the difference of 0.9 d for which the 2100 h curve was delayed compared to the 0900 h curve.

Furthermore, the collected serum samples were analyzed for specific antibodies against IBV with an IgG-specific ELISA. Only samples from Days 5 to 21 PI were analyzed. Figure 3 shows the results. The statistical analyses of the data according to Model 1, comprising data from serum samples taken during Days 11 to 21 PI, showed a difference in titer ($P < 0.0091$). The model explained 15.5% of the variance. The effect of grouping was substantial ($P < 0.0478$), and although the interaction of grouping and time of infection was not significant, the part of the variation explained by the model increased from 14.5 to 15.5%. The least squares means of the transformed data were 8.83 and 8.38, respectively, for infection at 2100 and 0900 h having a standard error of 0.13. Retransforming to titer values gave 6,816 and 4,349, which

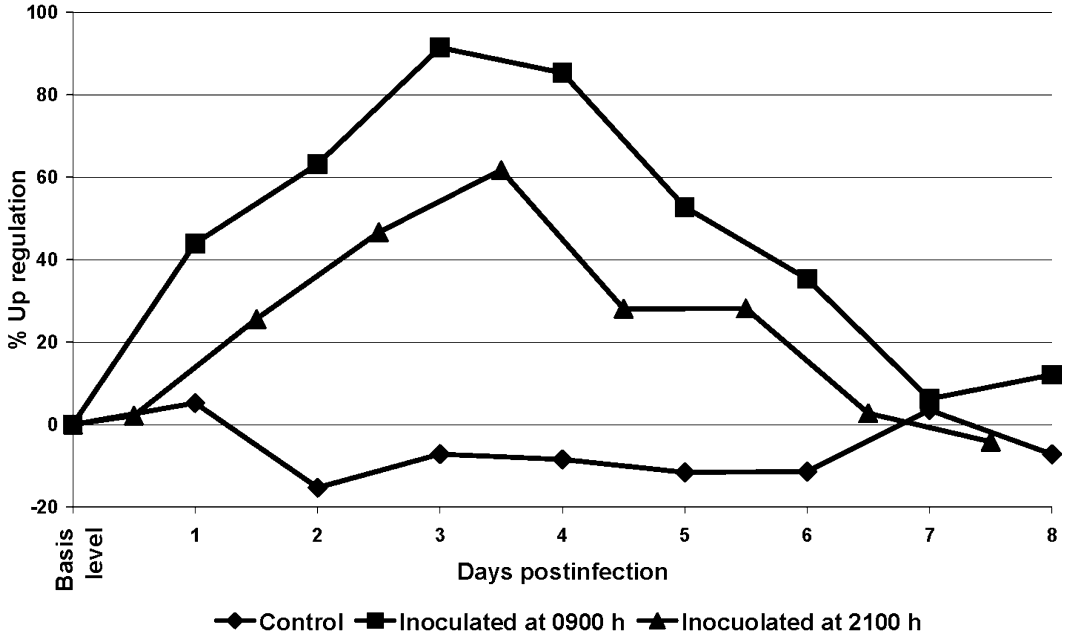


**FIGURE 2.** The mean mannan-binding lectin (MBL) acute phase response in chickens inoculated with infectious bronchitis virus (IBV) at different times. One group was mock infected at 0900 h, one group was infected with IBV at 0900 h, and one group was infected with IBV at 2100 h. The acute phase response was present as a percentage of up-regulation in relation to a determined basic MBL level in each chicken. The curves were built as follows: five chickens from each experiment were bled on Days 1, 3, 5, 9, 14, and 18 postinfection (PI) and five chickens were bled on Days 2, 4, 7, 11, 16, and 21. The values from those 10 chickens from each experiment were gathered in one curve, and the mean value of chickens bled on the same day was calculated.

were the geometric means of the data adjusting for grouping effect, day of bleeding, and experimental series.

Finally, the ability of chicken serum MBL to activate the MBL pathway of the complement system was tested in two randomly chosen birds from each of the three experimental groups in a heterologous complement activation assay by means of deposition of human C4 on the chicken MBL/MASP complex (Figure 4). When bound to

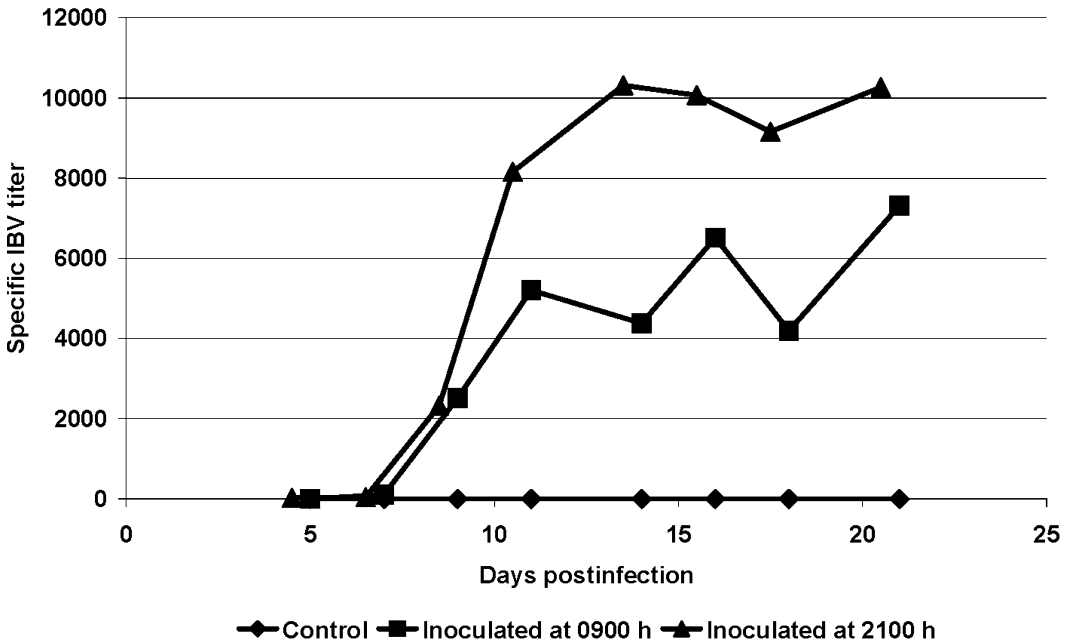


**FIGURE 3.** The mean infectious bronchitis virus (IBV)-specific antibody titer in chickens inoculated with IBV at different times. One group was mock infected at 0900 h, one group was infected with IBV at 0900 h, and one group was infected with IBV at 2100 h. The curves were built up as follows: five chickens from each experiment were bled on Days 5, 9, 14, and 18 postinfection (PI) and five chickens were bled on Days 7, 11, 16, and 21. The values from these 10 chickens from each experiment were gathered in one curve, and the mean value of chickens bled on the same day was calculated.

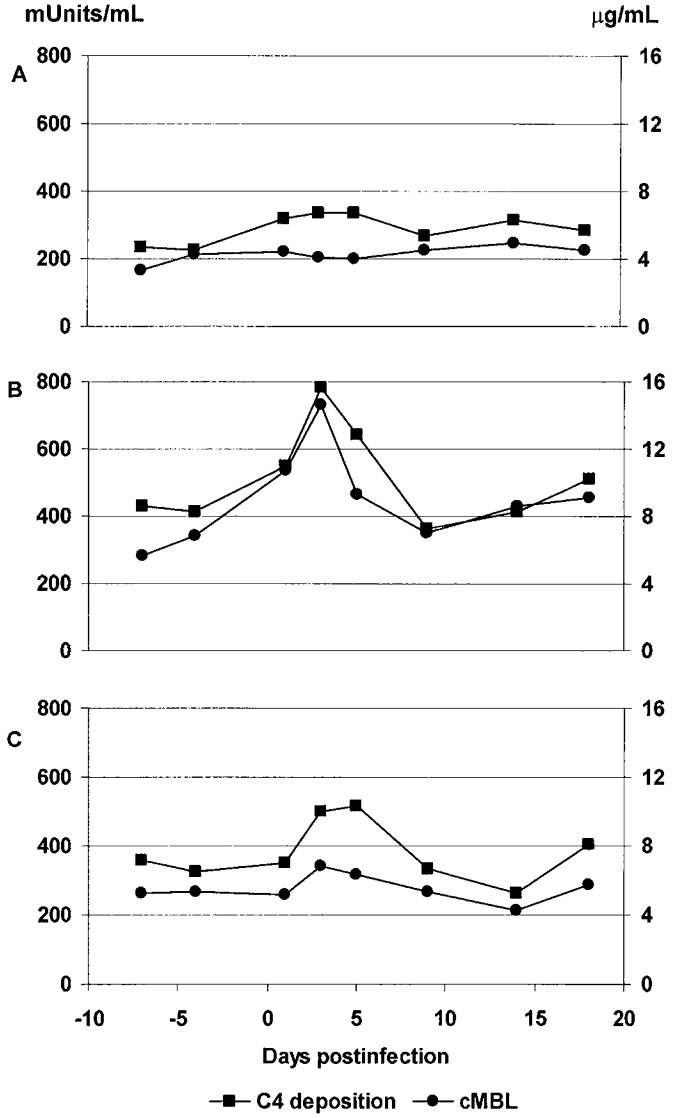


**FIGURE 4.** Complement activation values measured as deposition of human complement factor C4 on the chicken mannan-binding lectin (cMBL)-associated serine protease (MASP) complex. The means of two birds of each indicated experimental group are shown. The MBL concentration was measured in micrograms per milliliter, whereas the C4 deposition was measured as milliunits per milliliter. Panel A: controls, Panel B: inoculated at 0900 h, Panel C: inoculated at 2100 h.

microorganism, the MBL/MASP complex activates the complement components C4 and C2, thereby generating the C3 convertase and leading to opsonization by deposition of C4b and C3b fragments. The contribution from the classical complement pathway was inhibited by means of high ionic strength in the experimental buffer (data not shown; Petersen et al., 2000, 2001). The control group showed no difference in the ability to deposit C4 on MBL/MASP complex before or after inoculation (Figure 4a), whereas the test groups showed a rise in the ability to deposit C4 (Figures 4b,c). This rise in C4 deposition was parallel with a rise in cMBL concentrations on Days 3 to 4 PI.

## DISCUSSION

Previously, it has been reported that the basic serum level of cMBL is stable in chickens during the first 2 wk after hatch, followed by a dramatic decrease to minimum at 4 wk of age (Laursen and Nielsen, 2000). Therefore, we measured the MBL level in each chicken before challenge to avoid low cMBL in serum when performing the experimental infection. However, we were not able to demonstrate reduced cMBL in sera from chickens 4 to 5 wk old (Figure 1). In fact, in our experiments, we analyzed serum samples weekly taken from a total of 60 birds at 14 to 38 d of age prior to inoculation with virus, and none of these birds had a dramatic decrease in serum concentration of cMBL. Laursen and Nielsen (2000) reported the decrease in serum level of cMBL at 4 to 5 wk of age on the basis of samples taken from five chickens only. Therefore, we suggest further studies are needed to investigate the normal serum level of cMBL in chickens up to 6 to 8 wk of age.

Chickens inoculated at 0900 h had a higher MBL acute phase response to the IBV infection than chickens inoculated at 2100 h (Figure 2). This finding could not be due to volume differences in the blood during the day because all blood samples were taken at the same time of the day (0900 h), nor do we think it could be due to feed intake, as water and food were supplied ad libitum. Apparently, the MBL response was correlated with light and darkness or the diurnal rhythm in general. Many neuroendocrine hormones exhibit rhythmicity. Given the close relationship between the neuroendocrine pathways and the immune system, it is expected that some immune parameters exhibit diurnal rhythmicity. As an example, the neural hormone melatonin participates in many important physiological functions, including the control of seasonal reproduction, as well as influencing the immune system (Guerrero and Reiter, 1992). In chickens, the serum concentration of melatonin reaches its maximum level at the midpoint of the dark phase and its minimum level at the midpoint of the light phase (Lynch, 1971; Petrovsky and Harrison, 1997). However, other explanations may be just as possible. In the future, it would be interesting to further investigate the mechanism(s) behind the observed difference in serum cMBL levels.

Chickens inoculated at 0900 h had a lower antibody titer than chickens inoculated at 2100 h (Figure 3). One possible explanation for the difference in specific antibody titer could be that the specific antibody response was subjected to similar light and darkness mechanisms or to diurnal rhythm mechanisms as cMBL. However, another possibility could be an antiviral neutralizing effect of MBL through complement activation and opsonization by MBL receptors on phagocytic cells as the first line of defense removing IBV before the adaptive immune response takes over. The latter possibility was tested in a heterologous complement activation system. As shown in Figure 4, the ability of serum to deposit C4 on the MBL/MASP complex is totally parallel with the level of serum concentrations of MBL. This result suggests that

quick removal of IBV by opsonization is a probable explanation of the lower specific antibody titer in serum samples from chickens inoculated at 0900 h than in those inoculated at 2100 h.

In conclusion, our result implies that the nature of the immune response was modified by the time of the day that the inoculation occurred. From a practical point of view, it seems important to further investigate the interaction between components of the innate immune system and the adaptive immune system when designing new vaccine strategies for chickens. Moreover, it also seems important to investigate the involvement of intermittent lighting to chickens in relation to immunological factors.

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