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Immune system dysfunction in broiler chickens experimentally inoculated with fowl adenovirus serotype-4 associated with inclusion body hepatitis hydropericardium syndrome

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Abstract: Inclusion body hepatitis-hydropericardium syndrome (IBH-HPS) in broilers is caused by an adenovirus, here referred to as inclusion body hepatitis-hydropericardium syndrome virus (IBH-HPSV). This study describes the immune status of broiler chickens experimentally inoculated with an isolate of fowl adenovirus-4 serotype-1, involved in IBH-HPS in various trials. When compared with the unchallenged controls, IBH-HPSV-inoculated broilers had significantly higher atrophy of the bursa of Fabricius (BF) by up to 2-fold, as well as of the thymus (up to 9-fold) and spleen (up to 1.5-fold) ($P < 0.05$), with up to 60% mortality. Upon challenge with sheep red blood cells (SRBCs), IBH-HPSV-inoculated birds had from 2- to 3-log lower anti-SRBC antibody titers than the control. Response to phytohemagglutinin-P (PHA-P) was significantly reduced in IBH-HPSV-inoculated birds ($P < 0.05$) as compared to the control. Results of the studies revealed that the immune status of broilers is compromised significantly during IBH-HPSV infection in terms of lymphoid organ integrity, humoral, and cell-mediated immune responses, respectively. These findings suggest that immune dysfunction could be a contributing factor in the increased mortality of birds affected by IBH-HPS, which renders the birds immunosuppressed for the rest of their lives.

Key words: Immune system dysfunction, inclusion body hepatitis-hydropericardium syndrome, broilers, immune response, sheep red blood cells, phytohemagglutinin-P

Introduction

Adenoviruses have been incriminated as the etiological agents for various diseases in poultry (1). In addition to inclusion body hepatitis (IBH), various serotypes of fowl adenovirus (FAV) are reported to be the causative agents of hepatitis-hydropericardium syndrome (IBH-HPS), which may also be called

Leechi disease (2). The disease was first reported in Pakistan in 1988 (3). It has also been reported in other areas including Iraq, South and Central America, Slovakia, Russia, and Japan (4-7).

IBH-HPS is an emerging disease, particularly affecting broiler chickens 2-6 weeks old (8), and it causes severe economic loss to the broiler industry.

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The disease is caused by FAV serotype-4 (FAV-4), a nonenveloped icosahedral virus belonging to the adenovirus species of the family *Adenoviridae*, and it is characterized by hydropericardium and hepatitis with intranuclear inclusion bodies in hepatocytes (8). The course of the disease under natural conditions following oral inoculation ranges from 7 to 15 days (4,8); after subcutaneous (SC) inoculation of the infected liver homogenate, the course of the disease ranges from 2 to 5 days (9), which may result in 10%-60% mortality in fast-growing broiler chickens (10).

Initially for IBH-HPS, nutritional disorders, including rancid fat and fishmeal or vitamin and mineral imbalances in the feed, were implicated as the causative factors (3). However, various researchers successfully induced the disease with SC inoculation of liver homogenates from affected birds. A viral etiology of IBH-HPS has been suggested based on basophilic intranuclear inclusion bodies in the hepatocytes (8) and the presence of icosahedral adenoviral particles in purified liver homogenates observed by electron microscopy (11). The etiological agent has been further characterized as an adenovirus on the basis of polypeptide and nucleic acid identification (12), by studying protein profiles by SDS-PAGE and immunoblotting (13), and by nucleotide sequence analysis of the hexon gene of the isolated virus (14).

Inclusion body hepatitis-hydropericardium syndrome virus (IBH-HPSV) has a predilection for lymphoid organs. Naeem et al. (15) demonstrated IBH-HPSV at different days postinoculation (PI) in the bursa of Fabricius (BF) and thymus of broilers and also observed that homogenates from the affected lymphoid organs reproduced the disease in healthy broilers. Asrani et al. (10) observed lymphocytosis and cyst formation in the BF (3), which may lead to depletion of lymphocytes in the medullae of the follicles in the BF (16).

There is scanty information regarding the interaction of IBH-HPSV with the immune system of broiler chickens. Therefore, the present study was planned to evaluate the interaction between IBH-HPSV and the immune system of broilers.

Materials and methods

Animals

One-day-old broiler chicks procured from a commercial hatchery were housed in the Laboratory Animal House of the Institute of Microbiology of the University of Agriculture, Faisalabad, Pakistan, in isolation rooms. A broiler starter feed and water were available to them ad libitum and no vaccinations were given to the experimental birds.

Virus isolation and propagation

Liver samples from outbreaks of IBH-HPS experiencing 100% morbidity and 45% mortality were collected (August-September 2005) from Faisalabad. IBH-HPSV was purified and characterized for use in the experiments of the present study. For this purpose, infected livers were homogenized as 20% (w/v) liver homogenate in sterile phosphate-buffered saline (PBS) followed by centrifugation at $800 \times g$ for 15 min. The supernatant thus collected was passed through 0.45- and 0.22- μm filters. Streptomycin (200 $\mu\text{g}/\mu\text{L}$) and gentamicin (100 $\mu\text{g}/\mu\text{L}$) were added to the filtrate and 0.4 mL/bird was then inoculated subcutaneously in 17-day-old birds.

Livers were collected from infected broiler chickens showing typical signs of hydropericardium within 36-48 h PI and were again homogenized, clarified, and filtered as above. The filtrate was then pelleted at $80,000 \times g$ for 2 h (SW-28 Rotor, Beckman Instruments Inc., Palo Alto, CA, USA). The pellet was resuspended in sterilized PBS and further purified by centrifugation through a 35% sucrose cushion at $90,000 \times g$ for 90 min. The material was again run at $90,000 \times g$ for 4 h on sucrose gradient ranging from 15% to 55%. A distinct band was collected at the junction of 25% and 35% sucrose preparations and the virus was pelleted again by centrifugation. The embryo infective dose₅₀ (EID₅₀) was calculated following the method of Reed and Muench (17). A virus titer of $10^{4.5}$ EID₅₀/mL was used for SC inoculation of chickens throughout the study.

Characterization of virus

The hypervariable region (hexon gene) of the virus was amplified by polymerase chain reaction (PCR) using specific primers. The resultant PCR product of 730 kb was analyzed by nucleotide sequencing, which was submitted to GenBank (Accession No.

DQ 264728). Sequence BLAST analysis of the gene confirmed that livers from the birds in the IBH-HPS outbreak contained FAV-4, and a phylogenetic tree was constructed and published (18). The same characterized IBH-HPSV was used for the present study.

Effect of HPSV on the lymphoid organs

Two equal groups (group 1 and group 2) were formed from the division of 120 broiler chickens. The birds in the group 1 were inoculated subcutaneously with 0.4 mL of $10^{4.5}$ EID₅₀ of IBH-HPSV at 15 days of age, while the birds of group 2 served as the uninoculated control group and were injected with 0.4 mL of plain PBS. Birds in both groups were weighed before slaughter and at days 2, 4, 6, 8, 10, and 12 PI. The BF, thymus (all lobes from the left side of the neck), and spleen were removed and weighed to the nearest milligram, and the results were expressed as percentage organ-to-body weight ratios.

Histopathology

The thymus, BF, and spleen tissue samples collected on the days mentioned above were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections were then examined under a light microscope. Lesions of the lymphoid organs (spleen, BF, and thymus) were scored following the scoring criteria described by Nakamura et al. (19) based on lymphoid necrosis and/or lymphocyte depletion. Briefly, for BF and splenic lesions, 0 = less than 5% of the lymphoid follicles affected, 1 = 5% to 25% of the lymphoid follicles affected, 2 = 26% to 50% of the lymphoid follicles affected, 3 = 51% to 75% of the lymphoid follicles affected, and 4 = more than 75% of the lymphoid follicles affected. Thymic lesions were scored on the basis of cortical lymphocyte necrosis and atrophy as follows: 0 = no detectable lesions, 1 = mild multifocal cortical lymphocyte necrosis, 2 = moderate multifocal cortical lymphocyte necrosis or mild diffused cortical atrophy, 3 = moderate diffused cortical atrophy, and 4 = severe diffused cortical atrophy.

Antibody response

At 15 days of age, anti-IBH-HPSV antibody-negative broiler chickens (n = 80) were divided into 2 equal groups. Birds in group 1 were inoculated

subcutaneously with 0.4 mL of $10^{4.5}$ EID₅₀ of IBH-HPSV, and those in group 2 served as the uninoculated controls and were inoculated subcutaneously with 0.4 mL of plain PBS. At 1 week PI, at 3 weeks of age, chickens in both groups were intravenously given a single injection of 1 mL of a 5% saline suspension of sheep red blood cells (SRBCs). Blood samples were drawn from 10 birds at random in each group on days 7, 12, 17, and 22 PI. The collected serum was heat-inactivated at 56 °C for 30 min, divided into 2 aliquots, and stored at -20 °C until used for titration of anti-SRBC antibody levels. One aliquot from each sample was used for the determination of total anti-SRBC antibodies. The other aliquot was treated with mercaptoethanol to quantify mercaptoethanol-resistant (MER, presumably IgG) and mercaptoethanol-sensitive (MES, presumably IgM) responses using a microhemagglutination technique as described by Yamamoto and Glick (20) and LePage et al. (21). The titers were expressed as the log of the reciprocal of the last dilution in which visible agglutination was observed.

Cell-mediated immunity

Thirty broilers were divided into 2 groups at 15 days of age. Birds in group 1 were challenged with IBH-HPSV and the birds in the other group served as controls. The *in vivo* lymphoproliferation was quantified by injecting phytohemagglutinin-P (PHA-P; Sigma, St. Louis, MO, USA) into the broilers of both groups at 3 weeks of age (1 week after IBH-HPSV inoculation) as described previously by Kidd et al. (22) and Mahmood et al. (23,24). The toe web between the third and fourth digits of the left foot was injected with 100 µg of PHA-P dissolved in 100 µL of sterile PBS. The right foot, which served as a control, was injected in an identical manner to that of the left foot with 100 µL of sterile saline. The toe web swelling was measured with a constant tension caliper before PHA-P injection and at 24, 48, and 72 h after injection. The data were expressed as the PHA-P-mediated swelling minus the sterile saline-injected control swelling (mm) in both treatment groups.

Statistical analysis

Data were analyzed by using the general linear model procedure of SAS (SAS Institute, Cary, NC, USA), and the treatment means were separated using Duncan's multiple range test.

Results

The effects of IBH-HPSV infection on the BF, thymus, and spleen of broiler chickens inoculated with $10^{4.5}$ EID₅₀ of IBH-HPSV at 15 days old are presented in Table 1. Atrophy of the BF, spleen, and thymus occurred soon after IBH-HPSV exposure when compared with the unexposed birds. The lymphoid organ-to-body weight ratio was reduced significantly ($P < 0.05$) at 6 days PI and remained significantly lower than that of the control group throughout the experiment. The organ-to-body weight ratio of the spleen was significantly lower ($P < 0.05$) from day 4 PI and remained that way throughout the experiment. Briefly, the BF exhibited from 1.2- to 2.3-fold reductions, the thymus exhibited from 1.2- to 11-fold reductions, and the spleen exhibited from 1.2- to 2-fold reductions in weight compared to those of uninoculated control broiler chickens.

The birds exhibited typical signs and pathognomonic lesions of IBH-HPS, including enlarged and friable livers and accrual of a transparent, straw-colored watery/jelly-like fluid that

was observed in the pericardial sac. Small multifocal areas of necrosis, mononuclear cell infiltration in portal areas, and basophilic intranuclear inclusion bodies in the hepatocytes were also observed.

The data on mortality and histological lesion scores are presented in Table 2. Mortality was recorded from day 2 through day 10 PI, being maximum (60%) on day 6 PI. Lymphoid follicle necrosis and lymphocyte depletion were observed in the BF and spleen from day 2 through day 12 PI. About 70% of the lymphocytes in the BF were depleted on days 4-6 PI. Depletion of lymphocytes in the spleen was milder but consistent (Table 2). Lymphoid necrosis in the thymus started later (day 6 PI) but persisted up to the termination of the experiment (day 12 PI).

Data for the production of antibodies against SRBCs are given in Table 3 and 4. The birds in the IBH-HPSV-infected group had a 1-log and 3-log decrease compared to the control birds in total anti-SRBC antibody levels ($P < 0.05$) by day 7 and day 17 after SRBC injection, respectively, and these values continued to be lower (but not significant) at

Table 1. Organ-to-body weight ratio of the lymphoid organs collected from broiler chickens during IBH-HPSV infection (birds were inoculated at 15 days of age).

Days PI	Organ weight-to-body weight ratio ¹					
	Bursa of Fabricius		Thymus ²		Spleen	
	IBH-HPSV	Control	IBH-HPSV	Control	IBH-HPSV	Control
2	0.06	0.08	0.06	0.09	0.10	0.11
4	0.07 ^a	0.12 ^b	0.08	0.11	0.12 ^a	0.20 ^b
6	0.08 ^a	0.16 ^b	0.09 ^a	0.14 ^b	0.13 ^a	0.25 ^b
8	0.11 ^a	0.20 ^b	0.10 ^a	0.21 ^b	0.15 ^a	0.30 ^b
10	0.20 ^a	0.36 ^b	0.12 ^a	0.30 ^b	0.26 ^a	0.48 ^b
12	0.32	0.38	0.21 ^a	0.30 ^b	0.35 ^a	0.57 ^b

($P < 0.05$)

^{a,b}The means for a given organ within a row with no common superscript differ significantly.

¹Broilers were inoculated subcutaneously with $10^{4.5}$ EID₅₀ of IBH-HPSV at 15 days of age. Organs from birds from each group were collected on the day described PI. The data are means of percentage organ weight relative to body weight from 10 birds.

²All thymus lobes from the left side of the neck were collected from each bird.

Table 2. Mortality and histopathological lesion scores of the BF, thymus, and spleen of broiler chickens inoculated subcutaneously with $10^{4.5}$ EID₅₀ of IBH-HPSV at 15 days of age.

Inoculant	Days PI	No. of dead birds/total inoculated	Mean lesion score ^a		
			Thymus	Spleen	Bursa of Fabricius
IBH-HPSV	2	3/10	0.0	0.1	0.5
	4	6/10	0.0	2.6	3.1
	6	3/10	0.9	2.9	3.3
	8	2/10	1.3	2.5	2.8
	10	1/10	2.5	0.9	1.5
	12	0/10	1.0	0.0	0.5
None	2	0/10	NT	NT	NT
	4	0/10	0.0	0.0	0.0
	6	0/10	0.0	0.0	0.0
	8	0/10	NT	NT	NT
	10	0/10	NT	NT	NT
	12	0/10	NT	NT	NT

^aBursa of Fabricius and splenic lesion scores: 0 = less than 5%, 1 = 5% to 25%, 2 = 26% to 50%, 3 = 51% to 75%, and 4 = more than 75% of lymphoid follicles affected. Thymic lesion scores on the basis of cortical lymphocyte necrosis and atrophy: 0 = no detectable lesions, 1 = mild multifocal cortical lymphocyte necrosis, 2 = moderate multifocal cortical lymphocyte necrosis or mild diffused cortical atrophy, 3 = moderate diffused cortical atrophy, and 4 = severe diffused cortical atrophy.

Table 3. Anti-SRBC response of broilers inoculated with $10^{4.5}$ EID₅₀ of IBH-HPSV.¹

Days post-SRBC	IBH-HPSV mean/log ₂	Control mean/log ₂
7	3.5 ^a	6.0 ^b
12	4.3 ^a	7.5 ^b
17	4.5 ^a	8.3 ^a
22	4.7	5.6

(P < 0.05)

^{a,b}Means within a row with no common superscript differ significantly.

¹Broilers were inoculated with IBH-HPSV at 15 days of age. Ten birds per group were injected intravenously with a 7% saline suspension of SRBCs at 1 mL/bird at 3 weeks of age for antibody response.

day 22 post-SRBC injection. Table 4 presents IgM and IgG anti-SRBC antibody responses after IBH-HPSV exposure. Birds in the IBH-HPSV group had significantly reduced IgM anti-SRBC antibodies at days 7 to 17 post-SRBC injection (P < 0.05). Anti-SRBC IgG levels did not differ between the IBH-HPSV-inoculated and uninoculated groups on days 7 and 12. However, on days 17 and 22 after SRBC injection, birds in the IBH-HPSV-inoculated group had significantly lower IgG anti-SRBC antibody responses compared with the uninoculated control broiler chickens (P < 0.05).

The response of broilers to PHA-P is presented in Table 5. Toe web swelling measured at 24, 48, and 72 h after the PHA-P injection was significantly lower in the IBH-HPSV-inoculated group (P < 0.05) than in the control.

Table 4. Anti-SRBC IgM and IgG response of broilers inoculated with $10^{4.5}$ EID₅₀ of IBH-HPSV.¹

Group	Days post-SRBC and challenge immunoglobulin type (log ₂)							
	7 days		12 days		17 days		22 days	
	MES ²	MER ³	MES	MER	MES	MER	MES	MER
Control	5.1 ^b	0.9	5.9 ^b	1.6	6.5 ^b	1.8	5.0	0.6
IBH-HPSV	3.0 ^a	0.5	3.6 ^a	0.9	3.5 ^a	1.0	4.2	0.5

(P < 0.05)

¹See text for details.

²MES: mercaptoethanol-sensitive immunoglobulin, presumably IgM.

³MER: mercaptoethanol-resistant immunoglobulin, presumably IgG.

^{a,b}Means within a column with no common superscript differ significantly.

Table 5. The responses of broiler chickens inoculated with $10^{4.5}$ EID₅₀ of IBH-HPSV and uninoculated control broiler chickens to PHA-P injection.^A

Group	Mean thickness (mm) following PHA-P injection ^B		
	24 h	48 h	72 h
Control	7.6 ^{1,a}	6.2 ^a	6.6 ^a
IBH-HPSV	5.7 ^b	4.4 ^b	4.5 ^b

(P < 0.05)

^{a,b}Means within a column with no common superscript differ significantly.

^AFor the experimental results, 100 µg of PHA-P dissolved in 100 µL of sterile saline was injected into the right toe web; the left toe web was injected with 100 µL of sterile saline in an identical manner as the control.

^BThe toe webs were measured with a constant tension caliper before injection and at 24, 48, and 72 h afterwards. The data were expressed as the PHA-P-mediated swelling minus the sterile saline-injected control swelling (mm) in both treatment groups.

Discussion

Immunosuppressive viral diseases threaten the poultry industry by causing heavy mortality and economic loss of production, often because of increased chicken susceptibility to secondary infections and suboptimal responses to vaccination. FAV-4 causes immunosuppression by damaging lymphoid tissues. The presence of infectious bursal disease virus and chicken anemia virus may predispose to IBH-HPSV infection, which may predispose to other viral infections (25).

In the current study, the pathogenicity and immune status of broiler chickens experimentally injected subcutaneously with IBH-HPSV and housed

in isolation rooms were examined and compared with those of an uninoculated control group housed in similar isolation rooms. The immune assessment was carried out by using assays described by Dietert et al. (26) for avian immune assessment. These included BF, thymus, and spleen weight-to-body weight ratios as a measure of lymphoid organ integrity, the pathological lesion scores for these organs, response against SRBCs as a measure of antibody-mediated immune response, and PHA-P toe web swelling assays as a measure of cell-mediated immune response.

The IBH-HPSV-inoculated birds showed significantly more (P < 0.05) depression in immunological parameters when compared with the

uninoculated controls. The lesion scores in the IBH-HPSV-inoculated birds were also significantly higher ($P < 0.05$) compared with the uninoculated control, which indicates that IBH-HPSV replicates in the lymphoid organs and is responsible for cytopathic effects in these organs. Naeem et al. (15) isolated IBH-HPSV antigens in the lymphoid organs at up to 21 days after experimental inoculation and reproduced the disease with homogenates of these organs, which supports our findings.

The lymphoid organ data indicate that the growth of both primary and secondary lymphoid organs was depressed significantly ($P < 0.05$). Thymic atrophy started later than BF atrophy in IBH-HPSV-inoculated birds and was of a greater magnitude than that for the BF and spleen. The BF (27) and thymus (28) serve as primary organs of lymphocyte development, so any alterations in the development of these organs in response to possible lymphotropic agents will result in altered immunological functions associated with both B and T lymphocytes.

This, indeed, was the case when antibody response against SRBCs was quantified; the broilers in the IBH-HPSV-infected group were highly suppressed. Within the first 3 to 4 days after SRBC injection, IBH-HPSV-infected broilers had 1- to 2-log lower antibody levels. This suppression persisted for 17 days after SRBC injections. In the antibody response trial, the observed decline in antibody level was observed in IBH-HPSV-inoculated birds compared to uninoculated birds at the terminal stage of the primary and SRBC antibody response. By this stage, IBH-HPSV exposure induced significant BF, thymic, and spleen atrophy. However, what is not yet known is the integrity of lymphoid components such as lymphocyte number and CD4⁺ and CD8⁺ cells during the progression of lymphoid organ atrophy and disease after exposure to IBH-HPSV. Studies on the correlation among lymphoid cell number, subpopulation ratios, and the observed slower indication of primary antibody response in the IBH-HPSV-infected birds are underway. Antibody levels around days 8 and 9 PI in IBH-HPSV-exposed broilers were lower than those of the unexposed birds. A central feature of humoral immune response requires an organism to possess a vast repertoire of antibodies to protect itself against foreign pathogens. The findings of the current study imply that during IBH-HPSV

infection, broilers cannot mount an effective primary antibody response to fight concurrent bacterial, viral, or fungal infections.

When lectin PHA-P was injected intradermally into broilers, the response primarily involved stimulation of T cell division with minimal effects on B cells (29). Therefore, lymphoproliferation in response to PHA-P is considered a good *in vivo* measure of T lymphocyte function. In the present study, IBH-HPSV-exposed broilers exhibited reduced swelling in response to PHA-P injection, suggesting a suppression of lymphoproliferative ability as compared with the unexposed broilers. It was established that avian cytotoxic T lymphocytes (CD8⁺) are key players in killing virus-infected cells (30) and that T helper cells (CD4⁺) are crucial in expanding the B lymphocyte-mediated antibody repertoire by producing cytotoxin with B lymphocyte proliferation potential. The data from the present study clearly show an alteration in T lymphocyte response in IBH-HPSV-infected broilers causing a possible alteration in the immune protection mechanism involving T lymphocytes.

In conclusion, the findings of the present study suggest that IBH-HPSV induces an immunosuppressive condition in broilers. This condition can be compared with the previously known infectious bursal disease virus and reovirus-induced immunosuppression in chickens. These viruses are known to cause BF atrophy and humoral and cell-mediated immunosuppression. Similarly, chicken anemia virus has been shown to be the cause of atrophy and hypocellularity in the chicken thymus (31). It is not clear whether the mortality observed in broilers is a direct result of infection with IBH-HPSV or whether the infection results in an immune dysfunction, which may then lead to enhanced secondary infections with concurrent bacterial, viral, and/or fungal agents resulting in death. Immune dysfunctions in broilers by IBH-HPSV seem to correlate with the pathogenicity of IBH-HPS.

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