Correlation of Hematological Changes and Serum and Monocyte Inhibition with the Early Suppression of Phytohemagglutinin Stimulation of Lymphocytes in Experimental Infectious Bursal Disease

A.W. Confer and P.S. MacWilliams*

ABSTRACT

Several experiments were conducted to study the mechanism of infectious bursal disease virus induced suppression of phytohemagglutinin stimulation of peripheral blood lymphocytes. Infectious bursal disease virus inoculation of one week old chicks resulted in significant suppression of phytohemagglutinin stimulation during the first three days after inoculation as demonstrated by whole blood assay. Mild thymic necrosis was seen on day 3. Hematological changes during this time consisted of increased numbers of circulating lymphocytes and monocytes in infected chickens. Absolute monocyte counts remained elevated even after phytohemagglutinin stimulation had returned to normal. Furthermore, even after a 72.3% reduction in the monocyte population in leukocyte preparations, there was still marked viral induced suppression of phytohemagglutinin stimulation. An elevation in the absolute number of circulating large immature lymphocytes correlated with suppression of phytohemagglutinin stimulation. Sera from infected and control chickens depressed phytohemagglutinin stimulation of lymphocytes from control chickens at the 5 and 10% concentration. At the 1% concentration, inhibition by control sera was considerably less than the inhibition by infected sera. The relationship between these findings and the mechanism of viral induced suppression of T-lymphocyte function is discussed.

RÉSUMÉ

Les auteurs ont réalisé plusieurs expériences destinées à étudier le mécanisme de la suppression, par le virus de la maladie de la bourse de Fabricius, de la stimulation des lymphocytes du sang en circulation, par la phytohémagglutinine. L'inoculation du virus à des poussins âgés d'une semaine se traduisit par une suppression significative de la stimulation par la phytohémagglutinine, durant les trois premiers jours suivants, comme le démontra une épreuve avec du sang entier. Une légère nécrose du thymus se produisit, le troisième jour. Au cours de cette période, les changements hématologiques des poussins infectés se traduisirent par une augmentation du nombre des lymphocytes et des monocytes. La numération absolue des monocytes demeura élevée, même après un retour à la normale de la stimulation par la phytohémagglutinine. De plus, même après une diminution de 72,3% des monocytes, dans les préparations de leucocytes, la suppression de la stimulation des lymphocytes par la phytohémagglutinine se révélait marquée. Une augmentation du nombre absolu des gros lymphocytes immatures du sang en circulation correspondait à la suppression de la stimulation des lymphocytes par la phytohémagglutinine. Le sérum des poussins expérimentaux et témoins déprime la stimulation des lymphocytes des témoins par la phytohémagglutinine, aux concentrations de 5 et 10%. A la concentration de 1%, l'inhibition par le sérum des témoins se révélait beaucoup moins importante que celle du sérum des poussins infectés. Les auteurs commentent la relation entre leurs observations et le mécanisme de la suppression de la fonction des lymphocytes T, imputable à un virus.

INTRODUCTION

Infectious bursal disease (IBD) is a viral disease of chickens in which the virus causes destruction of immature B-lymphocytes in the

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This research supported in part by the Organized Research Funds of the School of Veterinary Medicine, Louisiana State University, SVM-164.

Submitted June 23, 1981.
bursa of Fabricius (3,14). In chickens three weeks of age or less, hypogammaglobulinemia and reduced antibody production result from bursal damage and are associated with increased susceptibility to a number of infectious diseases (9,11,12,13,17,18,27,28,29). Transient lymphoid necrosis is observed also in thymic tissue and the spleen; however, in vitro studies indicate that infectious bursal disease virus (IBDV) does not replicate in T-lymphocytes (3,14,15,16).

T-lymphocyte function has been studied in IBD because IBDV infection increases susceptibility to Marek’s disease (4,13). Both natural and acquired resistance to Marek’s disease are T-lymphocyte dependent (8,30). Infectious bursal disease virus infected chickens had delayed type hypersensitivity responses and skin graft rejection rates similar to control birds 28 days postinoculation (PI) (11). In another study, phytomitogen stimulation of T-lymphocytes was depressed most severely three to four weeks after IBDV inoculation (33). In this laboratory, a severe suppression in phytohemagglutinin (PHA) stimulation of T-lymphocytes was detected during the first week after exposure to a live IBD vaccine or pathogenic field strain (6). The mechanism of suppression of PHA stimulation is unknown.

The studies described herein were undertaken to study possible mechanisms of early IBDV induced suppression of PHA stimulation.

MATERIALS AND METHODS

ANIMALS

Chickens used in these experiments were specific pathogen free white Leghorn chickens.1 Chickens were shipped by the supplier and received within 24 hours of hatching. All experimental chicks were kept in Horsfall-Bauer type isolation units with filtered air under positive pressure.

VIRUS

The virulent Edgar strain of IBDV was used.2 Preparation and storage of viral stocks have been described previously (6). A 0.2 mL volume of viral inoculum containing 10^4.3 EID_50 was given per os to infect chickens in experiments 1 and 2 and trials 1 and 2.

MITOGEN STIMULATION

A microtitration mitogen assay of whole blood was used to sequentially study PHA stimulation of lymphocytes (21). Lyophilized PHA-P^3 was reconstituted in RPMI-1640 tissue culture medium containing penicillin (100 μg/mL) and streptomycin (100 μg/mL). Preliminary studies indicated a 1:80 dilution of PHA to be optimal. Heparinized whole blood was diluted 1:4 with RPMI-1640 medium. Twenty μL of diluted blood was added to each of five wells containing 0.2 mL of diluted PHA or 0.2 mL RPMI-1640 medium with penicillin and streptomycin to serve as nonstimulated control cultures. The cultures were incubated 48 hours at 40°C in a humidified atmosphere of 5% CO_2. After incubation, 0.8 μCi of (H^3)-thymidine (specific activity 6.5 Ci/m mole) was added to each culture and the cultures incubated for an additional 16 hours. Cultures were harvested by washing with distilled water through glass fiber filters using aTitertek cell harvester.3 The filters were desiccated and placed in three mL of toluene base Omniscint.4 Radioactivity was measured in a liquid scintillation counter.5 Mitogen stimulation was expressed for each sample as a stimulation index (SI) according to the following equation:

SI = Counts per minute of PHA stimulated cultures/Counts per minute of non-stimulated cultures.

HEMATOLOGY

Blood was collected in tubes containing ethylenediaminetetra-acetic acid (EDTA). Total white blood cell and differential counts were done using standard techniques (25). In both experiments, the morphology of circulating lymphocytes was examined. Seventy-five to 100 lymphocytes were counted and the percentage of large immature lymphocytes calculated; the absolute number of large lymphocytes was determined.

EXPERIMENTAL DESIGN

A preliminary experiment (experiment 1) was performed using sixty chickens one week of age. Thirty chickens were inoculated with a bursal suspension of IBDV and 30 chickens remained as un inoculated controls. On PI days 3, 7 and 15 blood was collected from 18 chickens by cardiac puncture, five per group for mitogen stimulation and four per group for hematological evaluation. Twenty-one days PI, the birds were killed by cervical dislocation and a necropsy performed.

Experiment 2 utilized 120 chickens. Sixty chickens were inoculated with IBDV at one week of age, and 60 remained as uninoculated controls. On PI days 1, 3, 5, 8, 10 and 15, blood was collected from 40 chickens by cardiac puncture, ten per group for mitogen stimulation and ten per group for the hematology study. At each sampling time, two inoculated and one control chicken were killed by cervical dislocation and a necropsy was performed. Thymic, splenic, and bursal tissues were removed and fixed in 10% neutral buffered formalin. Paraffin embedded sections were cut from each tissue.

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1 SPAFA’s Incorporated, Roanoke, Illinois.
2 Kindly provided by Dr. Phillip Lukert, Athens, Georgia.
3 Difco Laboratories, Detroit, Michigan.
5 Flow Laboratories, Rockville, Maryland.
6 ICN Chemical and Radioisotope Division, Irvine, California.
stained with hematoxylin and eosin, and examined microscopically. Lesions were rated zero to three based on the extent of lymphoid necrosis and/or atrophy.

Lesion Score
zero — No changes (similar to controls)
one — Focal lymphoid necrosis
two — Moderate multifocal to diffuse lymphoid necrosis
three — Severe diffuse lymphoid necrosis or atrophy

STATISTICAL ANALYSIS

Differences between group (control and inoculated) means at each day postinoculation were analyzed statistically (1).

EFFECT OF ADHERENT CELL REMOVAL ON PHYTOHEMAGGLUTININ STIMULATION

Replicate trials (one and two) were performed with IBDV inoculated chickens. One day 4 PI, five infected and five control chickens were bled in each trial. Peripheral blood lymphocytes were isolated on Ficoll-hypaque as described previously (6). After cells were washed twice in RPMI-1640 medium, the cells were resuspended in RPMI-1640 medium plus 2% fetal bovine serum with penicillin and streptomycin. One-half of this suspension was held at 4°C. Adherent cells were removed from the remaining suspension by incubation at 37°C for 45 minutes in plastic petri dishes as described previously (22, 23). Cells held at 4°C and those incubated at 37°C were each washed and those incubated at 37°C were each washed and those incubated at 37°C were each washed and those incubated at 37°C were each washed at 37°C medium without serum. Phytohemagglutinin stimulation was determined on both cell populations (6). A percent viral induced suppression (% VSup) was calculated by the following equation:

\[ \% \text{ VSup} = (\text{SI control} - \text{SI infected})/\text{SI control} \]

EFFECT OF SERA ON PHYTOHEMAGGLUTINATION STIMULATION

Sera from ten chickens three days PI with IBDV were pooled. Sera from ten hatchmate uninoculated chickens were pooled also. Sera were filtered through a 0.22 μm filter\(^8\) and heat inactivated at 56°C for 30 minutes. In replicate trials, sera were added to PHA stimulated and nonstimulated whole blood cultures from five control chickens at the start of the assay incubation. Final serum dilutions of one, 5 and 10% of the total culture volume were used. A percent inhibition (% Inh.) due to serum was calculated by the following formula:

\[ \% \text{ Inh.} = (\text{SI without serum} - \text{SI with serum})/\text{SI without serum} \]

RESULTS

EXPERIMENT ONE

A significant depression (\(P<0.01\)) in PHA stimulation was present in samples from IBDV inoculated chickens on PI day 3 (Table I). On days seven and 15, differences were insignificant (\(P>0.05\)) in PHA stimulation between infected and control chickens.

Hematologically, a heteropenia (\(P<0.05\)) and monocytosis (\(P<0.0002\)) were seen in infected chickens on day 3 PI. On PI day 7, infected chickens had a heterophilia (\(P<0.02\)). In infected chickens, absolute lymphocyte counts were elevated compared to counts in control chickens on PI days 3-15. Monocyte counts for infected chickens were elevated compared to control counts on day 7. On PI days 3 and 7, there were greater numbers of large lymphocytes in the smears from infected compared to control chickens (Table III). These differences were not significant statistically (\(P>0.05\)). Each IBDV inoculated bird had a severely atrophied bursa at necropsy.

EXPERIMENT TWO

On PI days 1 and 3, PHA stimulation was depressed significantly (\(P<0.02\) and \(P<0.005\) respectively) in IBDV inoculated chickens.

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**TABLE I.** Phytohemagglutinin Stimulation and Hematological Data for Chickens Inoculated with Infectious Bursal Disease Virus (Experiment 1)

<table>
<thead>
<tr>
<th>Days PI</th>
<th>Stimulation Index</th>
<th>Total WBC</th>
<th>Heterophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2.0±0.2*</td>
<td>9.2±2.0</td>
<td>22.850±20.025±</td>
<td>8.608±13.747±</td>
<td>9.269±3.484±</td>
<td>1.147±33.8±</td>
<td>398±53±</td>
</tr>
<tr>
<td>7</td>
<td>16.7±4.1</td>
<td>17.3±3.7</td>
<td>22.825±20.250±</td>
<td>9.286±13.238±</td>
<td>9.140±2.556±</td>
<td>1.78±20±</td>
<td>393±57±</td>
</tr>
<tr>
<td>15</td>
<td>29.8±6.7</td>
<td>22.3±7.9</td>
<td>22.775±20.750±</td>
<td>8.413±10.115±</td>
<td>8.284±7.56±</td>
<td>996±722±</td>
<td>556±287±</td>
</tr>
</tbody>
</table>

*Mean ± standard error for five chickens
*Mean ± standard error for four chickens
*\(P<0.01\)
*\(P<0.05\)
*\(P<0.0002\)
*\(P<0.02\)

\(^8\)Falcon 7103 filter, Falcon Plastics, Oxnard, California.
(Table II). Differences observed in PHA stimulation between infected and control birds on days 8, 10 and 15 PI were insignificant (P > 0.05). Hematological differences were noted between infected and control birds. Primarily affected were absolute lymphocyte and monocyte counts. On days 1, 5 and 15, differences in lymphocyte counts between infected and control birds were insignificant (P > 0.05). Infected birds had a significant lymphocytosis (P < 0.05 or less) on days 3, 8 and 10 PI. On each sampling day, an elevated absolute monocyte count was seen in the blood of infected chickens compared to control. This monocytosis was statistically significant (P < 0.02 or less) on PI days 3, 5 and 15. A heteropenia (P < 0.05) was seen on PI day 3 in blood of infected chickens. Compared to controls, there was a decrease in absolute numbers of large lymphocytes in infected chickens on day 1 PI (P < 0.05), and an increase in absolute numbers of large lymphocytes on day 3 PI (P < 0.001) (Table III).

At necropsy, bursal atrophy was obvious in chickens by PI day 5. Mild splenic enlargement was noted in both birds killed at PI day 3 and in one of two birds killed on PI days 5 and 8. Histologically, severe bursal lesions including lymphoid necrosis and atrophy were evident in infected chickens by PI day 3. Thymic changes were present in infected birds on PI day 3 and consisted of mild multifocal cortical necrosis. Mild diffuse medullary cellular hyperplasia was present on PI days 5 and 8. Mild multifocal to diffuse lymphoid necrosis was present in the spleen on PI day 3. Mild multifocal lymphoid necrosis with hyperplasia of lymphoid follicles and red pulp were present in spleens from infected birds on PI days 5 and 8.

EFFECT OF ADHESIVE CELL REMOVAL ON PHYTOHEMAGGLUTININ STIMULATION

The treatment of leukocyte preparations to remove adherent cells resulted in a 72.3% and 88.0% reduction in monocytes and lymphophils respectively. This treatment also resulted in enhanced SI's in both IBDV infected and control chickens (Table IV). In both replicate trials 1 and 2, the percent IBDV induced suppression of PHA stimulation was reduced slightly by the adherent cell reduction treatment (from 69.1 to 57.7% and 77.1 and 64.1%).

EFFECT OF PHYTEOMAGGLUTININ STIMULATION

Both sera from infected and control chickens caused marked inhibition (greater than 68%) of PHA stimulation when used at 5 and

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**TABLE II. Phytohemagglutinin Stimulation, Hematology and Lesions for Chickens Inoculated with Infectious Bursal Disease Virus (Experiment 2)**

<table>
<thead>
<tr>
<th>Stimulation*</th>
<th>Total WBC</th>
<th>Heterophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Lesions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.6±1.1</td>
<td>15.7±4.1</td>
<td>18.6±5.0</td>
<td>19.7±7.8</td>
<td>8.7±0.7</td>
<td>81.3±3.8</td>
<td>599±2.4</td>
</tr>
<tr>
<td>2</td>
<td>3.1±0.4</td>
<td>6.8±0.2</td>
<td>22.2±0.3</td>
<td>20.3±0.5</td>
<td>5.0±0.5</td>
<td>749±1.6</td>
<td>13.6±6.2</td>
</tr>
<tr>
<td>3</td>
<td>20.8±5.9</td>
<td>11.1±2.1</td>
<td>31.9±5.0</td>
<td>31.0±5.0</td>
<td>8.5±3.4</td>
<td>687±6.2</td>
<td>16.1±6.2</td>
</tr>
<tr>
<td>4</td>
<td>21.5±3.0</td>
<td>29.0±2.5</td>
<td>28.8±5.0</td>
<td>25.4±0.5</td>
<td>7.4±3.8</td>
<td>978±8.8</td>
<td>14.9±4.2</td>
</tr>
<tr>
<td>5</td>
<td>31.8±5.6</td>
<td>26.9±2.8</td>
<td>26.3±9.2</td>
<td>21.2±7.8</td>
<td>6.9±2.1</td>
<td>784±12.2</td>
<td>12.2±4.0</td>
</tr>
<tr>
<td>6</td>
<td>14.2±2.2</td>
<td>21.2±2.6</td>
<td>21.4±5.0</td>
<td>20.3±0.0</td>
<td>6.4±7.0</td>
<td>667±3.8</td>
<td>10.8±4.8</td>
</tr>
</tbody>
</table>

*Mean ± standard error for ten chickens
*Mean lesion score for two chickens
P < 0.02
P < 0.005
P < 0.05
P < 0.01
*Mild medullary cellular hyperplasia
*Mild lymphoreticular hyperplasia

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**TABLE III. Absolute Numbers of Large Lymphocytes in the Blood of Chickens After Infectious Bursal Disease Virus Inoculation**

<table>
<thead>
<tr>
<th>Days PI</th>
<th>Absolute Numbers of Large Lymphocytes per μL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1*</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>2225 ± 1400*</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>7 or 8</td>
<td>622 ± 161*</td>
</tr>
<tr>
<td>11</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>224 ± 32</td>
</tr>
</tbody>
</table>

*Average number of large lymphocytes ± standard error from four chickens
*Average number of large lymphocytes ± standard error from ten chickens
P > 0.05
P < 0.05
P > 0.001

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TABLE IV. Phytohemagglutinin Stimulation of Lymphocytes Before and After Adherent Cell Removal

<table>
<thead>
<tr>
<th>Virus Inoculation</th>
<th>Treatment</th>
<th>Stimulation Index</th>
<th>% IBVD Induced Suppression</th>
<th>Stimulation Index</th>
<th>% IBVD Induced Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>19.4 ± 5</td>
<td>—</td>
<td>19.2 ± 4</td>
<td>—</td>
</tr>
<tr>
<td>IBDV</td>
<td>None</td>
<td>6.0 ± 1</td>
<td>69.1</td>
<td>4.4 ± 2</td>
<td>77.1</td>
</tr>
<tr>
<td>None</td>
<td>Adherent Cell Reduction</td>
<td>58.4 ± 16</td>
<td>—</td>
<td>30.1 ± 7</td>
<td>—</td>
</tr>
<tr>
<td>IBDV</td>
<td>Adherent Cell Reduction</td>
<td>39.7 ± 11</td>
<td>57.7</td>
<td>10.8 ± 3</td>
<td>64.1</td>
</tr>
</tbody>
</table>

1 Blood was obtained four days after IBVD inoculation
2 Mean SI ± standard error for five birds
3 Adherent cells reduced by incubating leukocytes for 45 minutes at 37°C in sterile plastic Petri dishes

10% concentrations (Fig. 1). At the 1% concentration, sera from infected birds caused a marked inhibition (59% and 69%) while sera from control birds caused less inhibition (32 and 48%).

DISCUSSION

Infectious bursal disease virus inoculation resulted in a transient suppression of PHA stimulation of lymphocytes in a whole blood assay similar to that reported previously using Ficoll-hypaque separated lymphocytes (6). Phytohemagglutinin is considered to be specific for T-lymphocytes in chickens (18). Histological examination of lymphoid organs during the time of mitogen depression demonstrated mild multifocal thymic cortical necrosis and mild lymphoid necrosis randomly scattered throughout the spleen. The thymic changes were not as severe or of as long a duration as those reported previously by several authors in IBD (3, 14). Henry et al (15) however, found few thymic changes by PI day 5. These differences in the severity of thymic lesions could be due to viral titers in inoculums, strains of viruses, or chickens used by different investigators. In our study, the small number of birds examined on each sampling day could account for the lack of correlation with several of the previous studies. Whether these thymic changes affected the absolute numbers of circulating T-lymphocytes and resulted in suppression of PHA stimulation was not determined. Subpopulations of lymphocytes are present in avian thymic tissue that can either help or suppress immune responses (25). Viral destruction of precursor helper T-lymphocytes in thymic tissue could potentially cause depressed mitogen responses without causing marked effects on circulating T-lymphocytes. The cause of thymic necrosis in IBD is not known because in vitro studies have indicated that IBVD does not replicate in T-lymphocytes (16). This observation may indicate, also, that suppression in PHA stimulation may not be due to direct viral affects on T-cells.

Hematological changes due to IBVD inoculation were observed in both experiments. Of interest in relation to the effects of IBVD on T-lymphocyte functions were the alterations in absolute lymphocyte and monocyte counts in infected chickens. Absolute lymphocyte and monocyte counts were elevated, often significantly, in blood from infected chickens compared to controls. In previous studies with Marek’s disease, it was shown that viral induced suppression of mitogen stimulation of spleen cell preparations was due to macrophages (22, 23). The correlation of monocytoposis and suppression of PHA stimulation on PI day 3 in experiment 1 indicated a possible cause and effect relationship between monocytes and suppression of PHA stimulation in IBD. In experiment 2, a marked monocytoposis persisted even after PHA stimulation returned to normal. Furthermore, after a 72.3% reduction in the monocyte population by incubation in petri dishes, there was still marked viral induced suppression of PHA stimulation. Therefore, monocytoposis does not appear to be the cause of early mitogen suppression in IBD.

A lymphopenia was present in experiment 2 and correlated with suppressed PHA stimulation on PI day 1. A lymphocytosis was present in infected chickens on PI day 3. Paradoxically, this correlated with a depression in PHA stimulation indicating that the intensity of stimulation in the whole blood assay did not reflect merely the

![Fig. 1 Comparison of serum inhibition of phytohemagglutinin stimulation of lymphocytes from control chickens.](image-url)
number of lymphocytes present. Examination of the lymphoid pop-
ulation on day 3 PI indicated a sig-
nificant increase (fourfold) in the absolute number of large (immu-
nective) lymphocytes in infected birds. Immature large lympho-
cytes do not undergo blastogenesis as readily as mature small lympho-
cytes (2, 32). Therefore, the depression of mitogen responsiveness
seen on day 3 may be an indi-
cation of immature lymphocytes released into circulation.

Samples of bovine fetal serum that enhance mitogen stimulation of lymphocytes from cattle may suppress PHA stimulation of chicken lymphocytes at the 2, 5 and 10% concentrations (unpublished data). Chicken lymphocytes do not need sera to undergo blastogenesis in the presence of phytothymogens (5, 20, 24). Sera from both control and infected chickens depressed PHA stimulation of lymphocytes from control chickens. At the 1% concentration, control serum inhibi-
tion was considerably less than the inhibition by the infected
serum. A number of serum factors have been found in diseased ani-
mals that inhibit phytothymogen induced blastogenesis (7, 31).
Chicken lymphocyte blastogenesis is suppressed by high concentra-
tions of normal serum, this infers that there are nonspecific suppressive
factors in serum. At low concentra-
tions, these factors may be diluted where their influence on
blastogenesis is reduced. In infected serum, additional suppressive factors may be present that are in high enough concentra-
tion that they inhibit blastogenesis even at lower dilutions. Virus may
be present in serum at three days PI (34). Also IBDV is known to
induce interferon and interferon may inhibit blastogenesis (10). Additional studies are indicated to examine serum factors in IBD
infection.

In conclusion, we have shown that the suppression of mitogen
responsiveness during IBDV infection corresponded to an increase in immature lymphocytes in circulation but was not related to
numbers of circulating mono-
cytes. We have also demonstrated that serum may suppress mitogen
responsiveness as well. Further studies are needed to examine the
significance of IBDV induced T-
lymphocyte depression as it relates to enhanced disease susceptibility
particularly to T-lymphocyte
dependent diseases such as Marek's
disease.

ACKNOWLEDGMENTS
The authors wish to thank Ms.
Billy Cleghorn and Ms. Judy Don-
ovan for excellent technical assistance.

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ney in gnotobiotic and battery reared
white Leghorns experimentally infec-
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