Early and transient induction of nitric oxide (NO) in infectious bursal disease virus infection is T-cell dependent: A study in cyclosporin-A treated chicken-model

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Received 27 April 2004; revised 19 November 2004

The level of nitric oxide (NO) in the supernatants of mitogen (PHA) stimulated lymphocyte cultures from infectious bursal disease (IBD) virus infected T-cell suppressed and immune competent chickens was monitored. The immune competent chickens when infected with IBD virus showed 4-6 folds increased levels of NO as compared to uninfected chickens. The levels of NO in T-cell suppressed chickens were comparable to uninfected control chickens, in spite of markedly increased hemorrhage suggesting that the muscular hemorrhage observed in IBD in not solely and directly related with NO production. The immune suppressed chickens that did not induce NO production after IBD virus infection showed more severe lesions and supported enhanced virus replication. Taken together it may be suggested that NO production after IBD virus infection, may exert antiviral effect since the immune-suppressed chickens that failed to induce NO showed more severe disease and higher magnitude of virus replication, but does not seem to correlate with the hemorrhagic lesions which in fact may be as a result of the net outcome of various host-factors and the determinants responsible for virus virulence and virus clearance.

Keywords: Infectious bursal disease virus, Immunity, Nitric oxide, T-cell suppression

Nitric oxide (NO) exhibits an enormous range of important functions in the host. It is synthesized from the substrate L-arginine by the enzymatic reaction of the NO synthases (NOS) leading to the production of L-citrulline. NO plays an important role as a signal molecule in many physiological functions of the host viz. as neurotransmitter, vasodilator as well as a cytotoxic effector molecule of the non-specific immune response. NO synthesized by the inducible nitric oxide synthase (iNOS) is a multi-functional mediator in immune defenses and has been reported to exert protective functions against a number of viruses, many intracellular bacteria, parasites and has been implicated in the control of cancers including autoimmunity. As a part of host defense mechanism NO has been shown to be cytotoxic or cytostatic for tumor cells or invasive organisms. An immunoregulatory role for NO has been proposed since it markedly inhibits T cell proliferation, in particular the response of Th1 cells, thus serving as a potential co-regulator of the Th1/Th2 balance. Alijone et al., identified NO as one of the environmental factors that critically govern the response of T cells to IFN-γ. By inducing the over expression of IFN-γ R chains, NO decides whether IFN-γ promotes cell proliferation or the induction of apoptosis.

Recently, investigators have focused on studying the importance of this molecule in viral infections including infectious bursal disease virus (IBDV) infection in chickens. IBDV infection is characterized by hemorrhage in thigh, pectoral muscle and bursa of Fabricius (BF). Since the virus replication takes place in BF, immuno-suppression is the hallmark of infection. The exact cause of clinical disease is, however, not clearly understood but does not seem to be related only to the severity of the lesions and the bursal damage. Indeed, after infection, some birds with a fewer lesions can be found dead, while others despite extensive bursal damage can survive. In fact, Kim et al., demonstrated that IBDV infection resulted in enhanced levels of NO in splenic macrophages. This up-regulation of NO was although very transient but believed to be very important feature of NO induced regulation of anti-IBDV immune response.

While underlining the role of T-cells in IBD pathogenesis, Poonia and Charan have observed that Cyclosporin-A mediated T-cells suppression in
chickens does result in enhancement of disease with conspicuous hemorrhagic lesions than the immune competent chickens.

The aim of this study is to investigate, if the elevation of NO level during IBDV infection has any association with the muscular hemorrhage seen in this disease (since NO is known for vasodilator activity) and secondly if the NO induced after IBDV infection has antiviral activity against IBDV in vivo.

Materials and Methods

Chickens—One-day old broiler chickens (90), unvaccinated against IBD, were procured from a farm with no history of IBDV outbreak. The chicks were screened for the presence of anti-IBDV neutralizing antibodies before inclusion in the experiments. At 3-weeks of age chickens were divided into five experimental groups of 18 each and were kept in separate locations.

The chickens were maintained in the well ventilated houses and provided with feed obtained from the Department of Animal Feed Technology of the university. The poultry feed was regularly monitored for the level of mycotoxins, particularly aflatoxin and was found below 50 ppb (parts per billions). These chickens were not vaccinated against any disease and were apparently healthy throughout the experiment.

Viruses—The strains of IBDV employed in the present investigation were Georgia strain (an intermediate vaccine strain procured from M/S Indovax Pvt. Ltd., Hisar) and a filed isolate of IBDV (FIV) isolated from an outbreak and was kindly gifted by Prof. M.S. Oberoi, Department of Veterinary Microbiology, Punjab Agricultural University, Ludhiana, Punjab.

T cell suppression—T-cell suppression was induced by inoculating chickens with cyclosporin-A (CS-A) (generously gifted from M/S Novartis Pharma AG, Basel, Switzerland). CS-A was dissolved in ethanol and sterile castor oil and the suspension was injected @ 100 mg/kg body weight by im route15.

Experimental design—Three-weeks-old broiler chickens were divided into 5 groups (groups I through V) and were infected with Georgia and FIV strains of IBDV using 10^3 TCID90 per chicken by oculo-oral route. Group II and group IV chickens were injected with CS-A at three days intervals starting at 3 days prior to infection (at -3, 0, 3, 6, 9, 12, 15 and 18 d.p.i.) with 10^5 TCID90 Georgia and FIV strains of IBDV by oculooral route. The control chickens were similarly treated with PBS.

Blood for NO estimation was collected from three chickens per group on days 1, 3, 7, 15, 21 and 28 days post infection.

Separation of peripheral blood mononuclear cells (PBMC)—Mononuclear cells were separated from peripheral blood collected in heparinized tubes with 20 units of heparin per ml of blood and diluted in equal part of serum free RPMI medium. The diluted blood was layered over Histopaque-1077 and centrifuged at 200 g for 20 min. The white band at the inter-phase of mononuclear cells was carefully removed and washed two times with cold PBS and finally in RPMI at the desired concentration14, 15.

NO assay—The levels of NO in the culture supernatants obtained from PBMC were determined by NO-induction assay16. PBMC from the individual chickens were suspended at a concentration of 5x10^9 cells/ml in RPMI-1640 complete medium. PBMC suspension (100 μl) was dispensed into triplicate wells of flat-bottomed 96-well micro-titer plates containing 4 μl PHA in 50 μl RPMI medium (predetermined optimal concentration) and 50 μl medium was added to each well in the end making a total volume of 200 μl per well. The plates were incubated at 37 °C for 72 hr in presence of 5% CO2. NO in culture is readily oxidized to nitrite and nitrate and this was quantified by mixing equal volumes (50 μl) of cell-free supernatants with Griess-reagent in 96-well micro-titer plates. The mixtures were incubated for 10 min at room temperature and absorbance measured at 540 nm. Concentration of nitrite was determined from a standard curve generated by using concentrations of NaNO2 ranging from 0.244 to 500 μM. Equal volumes of these concentrations were incubated with the same volume of Griess-reagent for 10 min and OD540 was taken for the generation of standard curve, which depicted the concentrations of nitrite against their particular ODs. Nitrite accumulation in supernatants of cultures gave an estimate of NO activity of PBMC stimulated with the mitogen. The results were expressed as micromoles (μM) of nitrate, a measure of NO-inducing factor(s) in the culture supernatants.

Virus titration in the target organ—Procedure for monitoring the virus replication in the target organ has been described earlier14. Briefly, bursa of Fabricius of the individual bird was removed carefully
and 10% suspension was made in a suitable cell culture medium. Serial dilutions of the suspension in triplicates were cultured over chicken embryo fibroblast cells. Finally the quantum of the virus replication was expressed as per gram of the tissue based on the highest dilution of the suspension showing the cyto-pathic effect.

Statistical analysis—Duncan's multiple range tests of variable was used to assess the significance of difference between treatment groups.

Results and Discussion

Nitric oxide production following infection with IBDV—A low level of nitrite (values ranging from 2.5 to 5.6 μM) was present in lymphocyte culture supernatants from control birds when stimulated with PHA, whereas, culture supernatants from chickens infected with Georgia or FIV strains showed a 4-6 fold increase in NO production on 1 d.p.i. (Table 1). Nitrite (18.3 and 16.5 μM) was detected in cultures of chickens inoculated with Georgia strain and FIV strain of IBDV respectively. This up-regulation was transient. Interestingly, no such elevation in nitrite levels was observed in chickens treated with CS-A and subsequently infected with IBDV where levels of NO production was found 7.8 and 6.2 μM on 1 d.p.i. (Table 1). Although chickens treated with CS-A (immune suppressed) had relatively more severe disease.

IBD in T-cell suppressed chickens—CS-A treated immune suppressed chickens, which did not have enhanced level of NO production, showed markedly enhanced disease in terms of gross lesions as well as histo-pathologically with up to 100-fold more virus replication in the target organ, than the immune competent control chickens that induced a significantly elevated NO levels. The detailed results were reported earlier.

NO is a free radical gaseous molecule, which is reported to be a mediator of a variety of physiological functions including regulation of nervous system and vascular permeability. It also has important antimicrobial and cytotoxic functions in some animals.

Elevated levels of NO were observed in supernatants from PBMC of chickens vaccinated or infected with Georgia or FIV strains of IBDV on 1 d.p.i. after in vitro stimulation with PHA. Interestingly, the elevated levels of NO as observed at day 1, was drastically reduced as early as at day 3 when the virus replication is normally at peak. It appears that certain T-cell cytokines induced during early stage may have a crucial role in the induction of NO. Accordingly, no such elevation of NO was observed in T-cell suppressed chickens (using CS-A). This indicated that NO production was restricted to proliferating cultures. This short duration of NO production after IBDV infection may in fact reflect and corroborate with the transient but moderate degree of suppression of T-cell proliferative responses observed using a number of strains of varying virulence (unpublished data). Earlier, Kirk et al., were not able to detect NO in unstimulated T-cells but when cells were stimulated with IL-2 for 20 hrs, de novo induction synthesis was observed. It is well established that T-cell derived cytokines such as IFN-γ, IL-2 or TNF-α induce NO production by macrophages. Therefore in the present study immune suppressed chickens were refractory to induction of NO. Similarly, effects of macrophage derived NO on T-cell mitogenic response has been demonstrated in rats. They showed that addition of NG-monomethyl-L-arginine (NMMA) a potent inhibitor of NO production, to the cultures resulted in reversal of the suppressor effect of supernatants of rat splenocyte

<p>| Table I—Nitrite accumulation in supernatants of PBMC from IBDV infected chickens cultured in presence of PHA |
|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>DPI</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.3±2.6A</td>
<td>7.8±2.2B</td>
<td>16.5±3.2A</td>
<td>6.2±1.4B</td>
<td>5.5±1.6A</td>
</tr>
<tr>
<td>3</td>
<td>4.6±1.4A</td>
<td>5.1±0.9A</td>
<td>2.5±0.8B</td>
<td>5.1±1.1A</td>
<td>5.2±2.1A</td>
</tr>
<tr>
<td>7</td>
<td>4.4±0.8B</td>
<td>2.6±0.6A</td>
<td>4.4±1.0B</td>
<td>2.6±0.5A</td>
<td>2.5±0.7A</td>
</tr>
<tr>
<td>15</td>
<td>5.3±1.1A</td>
<td>2.5±1.0A</td>
<td>10.2±2.5A</td>
<td>2.6±0.5A</td>
<td>5.2±1.2A</td>
</tr>
<tr>
<td>21</td>
<td>4.5±1.3A</td>
<td>4.1±1.5A</td>
<td>4.3±2.3A</td>
<td>4.4±1.1A</td>
<td>4.0±0.9A</td>
</tr>
<tr>
<td>28</td>
<td>8.4±2.2A</td>
<td>6.8±1.8A</td>
<td>9.0±2.6A</td>
<td>7.0±2.8A</td>
<td>5.6±1.7A</td>
</tr>
</tbody>
</table>

Values with different superscripts on a day are significantly different.
cultures. Pertile et al. showed that co-culture of normal splenocytes with adherent cells from reo virus infected chickens resulted in inhibition of mitogen induced proliferation of normal splenocytes. Thus, the suppression of T-cells may have failed to induce cytokines which could be responsible for the suppression of NO production.

Studies on the role of T-cell in IBD pathogenesis have shown that chickens treated with T-cell suppressant viz. CS-A have more severe hemorrhage with nearly 100-folds more virus replication than immune competent chickens. But CS-A treated T-cell suppressed chickens did not show elevated NO level suggesting that the levels of NO is not directly related with the hemorrhage and rather it may be the net outcome of several other factors. However, in a study in which chickens were treated with Na-nitro-L-arginine methyl ester (NAME; a nitric oxide synthase inhibitor) before exposure to IBDV much less bursal necrosis and lower levels of viral antigen were detected than the untreated virus infected chickens. It may be possibly argued that the kinetics or the quantitative differences in NO induction post-infection or its inhibition prior to infection, as observed after treatment with NAME, may play a crucial role in determining the outcome of the disease. Another trivial reason for this could be that NAME may have a direct antiviral activity.

On the other hand the results of the present study that immune suppressed chickens that did not show elevated level of NO supported virus replication to nearly 100-folds whereas the chickens that induced NO production (as in case of immune competent chickens) eliminated virus more efficiently. Thus NO produced during IBD virus infection may appears to exert antiviral effect in vivo, however it may have only a limited role in resulting the hemorrhagic lesions observed in IBD.

It would be further interesting to monitor the levels of NO induced by the different lines of chickens vis-a-vis their susceptibility to IBDV infection as well as to study the levels of NO after infection with strains of IBDV of different virulence which could be of significant importance in understanding the novel basis of IBD pathogenesis in chickens.

Acknowledgement

The first author (BP) was a recipient of Senior Research Fellowship of CSIR, New Delhi, India and SC was supported through a non-plan scheme of the university.

References


