Arch Virol (2004) 149: 2413–2426
DOI 10.1007/s00705-004-0350-7

Archives of Virology
Printed in Austria

Infectious bursal disease virus polyprotein expression arrests growth and mitogenic stimulation of B lymphocytes

M. A. Peters, T. L. Lin, and **C. C. Wu**

Department of Veterinary Pathobiology, Purdue University, West Lafayette, Indiana, U.S.A.

Received February 1, 2004; accepted March 30, 2004
Published online June 30, 2004

Summary. Infectious bursal disease virus (IBDV) causes lymphocytolysis and immunosuppression in infected poultry. The IBDV genome encodes a polyprotein VP243 that is post-translationally cleaved by the VP4 protease into the two structural proteins pVP2 and VP3. The objective of the present study was to determine if IBDV polyprotein induced suppression of bursal B lymphocyte growth and their capacity for proliferation. Bursal B cells were examined both for chickens infected with IBDV and for chickens orally inoculated with a DNA construct expressing IBDV VP243 polyprotein. Bursae were collected at 0, 12, 24 and 48 hours after inoculation. Proliferation of bursal B cells (purified AvBu1$^+$ cells) in response to concanavalin A mitogenic stimulation was significantly suppressed by infection at 1 day old with either the classical STC or variant E strains of IBDV. Oral administration of DNA constructs expressing the IBDV VP243 polyprotein from either the classical STC or variant E strains in the pCR3.1 vector resulted in persistent, moderate levels of construct in the bursa until at least 48 hours after inoculation. The VP243 DNA construct similarly induced suppression of proliferation for bursal lymphocytes independently of the virus infection. Expression of VP243 polyprotein in transiently transfected DT40 B lymphocyte culture also suppressed cell growth and proliferative responses to mitogen stimulation. Polyprotein expression did not affect cell viability and suppression of proliferation probably occurred by means of cell cycle arrest. The expression of the mature viral proteins VP2, VP4 or VP3 did not change the rate of cell proliferation or response of B cell cultures to mitogen. The results suggested that IBDV polyprotein is a mediator of immunosuppression.

Introduction

Infectious bursal disease virus (IBDV) is a non-enveloped virus classified as a member of the species *Infectious bursal disease virus*, genus *Avibirnavirus*,

family *Birnaviridae* due to the possession of a bi-segmented double stranded RNA genome [11]. Infectious bursal disease virus infection of chickens 3 weeks of age or older induces clinical disease characterized by severe bursal atrophy and immune suppression [6]. Severe prolonged immune suppression develops with IBDV infection of chickens at an earlier age. Pathogenic serotype 1 IBDV infection is ubiquitous in poultry flocks worldwide. IBDV isolates have been classified in the literature as "classical", "variant" or "very virulent" isolates. This terminology was adopted in 1986 when variant viruses were first described as newly emergent viruses due to a major antigenic shift within serotype 1 [15, 16]. The emergent variant strains could not be cross-neutralized by antiserum against classical strains, and vaccines based on classical strains did not protect chickens against infection with the variant strains. Vaccines based on variant strains are however protective against infection with both classical and variant strains. In addition, a relatively more severe form of acute viral disease has been attributed to the classical strains as opposed to the variant viruses, which are characterized by severe prolonged immune suppression. The classification of IBDV strains in this manner has limitations due to the lack of precise and consistent molecular features to define the strain types. Within the U.S.A. both variant and classical strains are prevalent. Recently in Europe and Asia very virulent isolates have been described for which there is high mortality associated with acute infection.

Infectious bursal disease virus genomic segment B has a single ORF encoding the VP1 protein, an RNA-dependent RNA polymerase. Genomic segment A has two partially overlapping ORFs, the first encoding the smaller VP5 protein and the second ORF encodes a polyprotein VP243, that is subsequently cleaved into the mature capsid proteins VP2 and VP3 by the autoprotease VP4. Infectious bursal disease virus has a tropism for infection of bursal B lymphocytes and depletion of B lymphocytes results in marked suppression of humoral immunity. Peripheral blood lymphocytes (PBL) from chickens infected with IBDV have depressed proliferative responses to stimulation with the mitogens concanavalin A [14] or phorbol myristate acetate [9]. Increased numbers of apoptotic lymphocytes are found in the bursae of IBDV infected chickens in both infected and bystander cells. Apoptosis is induced by IBDV infection of transformed cell lines and in chicken embryo fibroblasts (CEF) and PBL [4, 12, 20–22]. The non-structural protein VP5 has been identified as a virulence factor that can induce apoptosis [23]. Expression of the VP5 gene in transformed cell lines and CEF has been shown to induce apoptosis, and null mutants of VP5 are replication competent but have reduced virulence and reduced rates of bursal apoptosis. There is conflicting evidence as to the role of the structural protein VP2 in the induction of apoptosis. Fernandez-Arias, Mantirez, and Rodriguez [3] found that VP2 expression induced apoptosis only in mammalian cell lines but not in cells of avian origin. However, studies by Yao and Vakharia [23] found VP2 expression induced apoptosis in both avian (CEF and PBL) and mammalian cell lines. Chickens with IBDV-induced immune suppression when exposed to antigen from vaccines or co-infecting pathogens have a marked suppression in specific antibody titers to the antigen. Field exposure or vaccination with IBDV induces high IBDV-specific antibody

titers. Therefore, while infection does induce significant IBDV-specific antibody titers there is concurrently suppression of humoral responses to other antigens.

In our laboratory a DNA vaccine encoding the VP243 polyprotein from IBDV has been successfully developed for administration to chickens [1]. The DNA vaccine was engineered into the plasmid pCR3.1 background by cloning of the large segment gene of either IBDV variant E (VP243.pCR3.1VE) or IBDV STC (VP243.pCR3.1 STC) strains. Chickens were inoculated three times at 1-, 7- and 14-days-old with the VP243.pCR3.1VE or the VP243.pCR3.1 STC construct, then challenged with either the variant E or STC IBDV isolates. Vaccination with a DNA vaccine expressing the variant E polyprotein provided 90% protection against homologous challenge with variant E virus. Vaccination with a DNA vaccine expressing the STC polyprotein provided 100% protection against homologous challenge with STC virus [1]. The DNA constructs were therefore considered to have high efficacy in protection against the development of clinical disease and severe bursal atrophy. However the DNA vaccine did not induce significant IBDV-specific antibody titers [1]. The immunological basis for the protection observed is currently unclear and is the subject of further experimental investigation.

The objective of the present study was to determine if IBDV polyprotein expression induced suppression of bursal lymphocyte function. Studies were conducted to investigate the function of B lymphocytes from chickens orally infected at 1-day-old with either variant E or STC strains, or orally inoculated with either the VP243.pCR3.1VE or VP243.pCR3.1 STC DNA constructs. B lymphocyte growth and response to mitogenic stimulation was investigated using proliferation assays. Viral proteins were transiently expressed in the avian B cell line DT40, to differentiate the contribution of the precursor polyprotein VP243 from that of the processed viral proteins VP2, VP4 and VP3. This study is the first to specifically examine the effects of the viral polyprotien precursor on bursal B cell growth and capacity for proliferation.

Methods

Viruses and DNA constructs

Infectious bursal disease virus variant strain E and classical strain STC were collected from infected bursae. Viruses were propagated by intramuscular inoculation of 3-week-old, specific-pathogen-free (SPF) chickens and harvested 3 days later. Serial dilutions of virus stocks were titered by chorioallantoic inoculation of 9-day-old SPF chicken embryos (Charles River SPAFAS, North Franklin, Connecticut, U.S.A.). Variant E and STC IBDV inocula were prepared at a final concentration of 10^6 EID_{50} in 200 µl Dulbecco's Modified Eagles Medium (DMEM, Sigma, St Louis, MO, U.S.A.).

The engineering of the large segment gene from either IBDV variant E (construct VP243.pCR3.1VE) or IBDV STC strain (construct VP243.pCR3.1 STC) into the plasmid pCR3.1 (Invitrogen, Carlsbad, CA, U.S.A.) has been described previously [1]. The VP2 gene was amplified from IBDV VE cDNA with the forward primer 5′ ACGATCGCAGC GATGACAAA 3′ and reverse primer 5′ TCACCTTATGGCCCGGATT 3′. The VP4 gene was amplified using the forward primer 5′ AACGATGGCACGTTTCCCTCACAATCCG 3′

and the reverse primer 5′ *TCAGTGGTGGTGGTGGTGGTG*TTTGATGAACGTTGCCC 3′. The VP3 gene was amplified with the forward primer 5′ AACGATGGCACGTTTCCCTCACA ATCCG 3′ and reverse primer 5′ *TCAGTGGTGGTGGTGGTGGTG*CTCAAGGTCCTCATC 3′. The underlined sequences encode introduced Kozac sequences and the start codon ATG, and the italicized sequence encodes polyhis peptide fusions. The VP243.pCR3.1VE, VP243. pCR3.1 STC, VP2.pCR3.1, VP4.pCR3.1, VP3.pCR3.1 and pCR3.1 plasmids were propagated in transformed *Eschericia coli* strain DH5α at 37 °C in Luria-Bertani (LB) broth using standard molecular techniques [13]. The Qiagen Plasmid Midi kit (Qiagen, Valencia, CA, U.S.A.) was used for the large scale purification of VP243.pCR3.1VE, VP243.pCR3.1 STC and pCR3.1 plasmid DNA.

Experimental design

Embryonated eggs were obtained from a SPF white leghorn chicken flock (Charles River SPAFAS). One-day-old chickens were hatched from the embryonated eggs and transferred to pressure and temperature-controlled, bio-containment units.

An experimental study was performed using 6 treatment groups each with 15 SPF 1-day-old white leghorn chickens. The chickens were inoculated orally with either (i) 10^6 EID_{50} variant E IBDV in 200 μl DMEM, (ii) 10^6 EID_{50} IBDV strain STC in 200 μl DMEM, (iii) 0.5 mg pCR3.1 DNA in 200 μl DMEM, (iv) 0.5 mg VP243.pCR3.1VE DNA in 200 μl DMEM, (v) 0.5 mg VP243.pCR3.1 STC DNA in 200 μl DMEM, or with (vi) 200 μl DMEM. For each treatment group, 5 chickens were euthanased at 12 h, 24 h and 48 h after inoculation and the bursae and spleens were removed and placed into chilled PBS containing 1% BSA and 1% NaN_3 (PBS wash buffer). Lymphocytes prepared from the spleens and bursae were used both for proliferation assays and for determination of the copy number of either the viral RNA genome or of the DNA construct.

Lymphocyte proliferation assay

The bursal tissue was macerated and filtered through a 100 μm pore nylon mesh (PGC Scientific, Frederick, MD, U.S.A.) to form a single cell suspension. Extracted cells were pelleted by centrifugation at 2000 g for 7 min and re-suspended in 4 ml of PBS wash buffer. The cell suspension was purified by centrifugation for 5 min at 1000 g over a Ficoll-Paque gradient (Amersham Biosciences, Piscataway, NJ, U.S.A.) and the collected cells were washed twice in PBS wash buffer. Triplicate haemocytometer readings were taken and the cells suspended in PBS wash buffer at a final concentration of 10^8 lymphocytes/ml.

Lymphocyte populations isolated from the bursa of each chicken were enriched for AvBu1 positive B lymphocytes. Bursal cells were incubated for 30 min at 4 °C with mouse anti-chicken AvBu-1 monoclonal antibody (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) at a 1/200 dilution in PBS wash buffer, then washed in PBS wash buffer. Lymphocytes conjugated with the mouse anti-chicken AvBu-1 monoclonal antibody were purified using anti-mouse IgG Biomag beads (Qiagen) according to the manufacturer's instructions. Briefly, stained cells were incubated for 10 min at room temperature with 400 μl of anti-mouse IgG beads. The cells and beads were mounted in a magnetic field and washed twice in 10 ml DMEM. The column was removed from the magnetic field and the cells were resuspended in 10 ml DMEM. Haemocytometer readings were taken and Av-$Bu1^+$ lymphocytes suspended in DMEM at a final concentration of 10^7 lymphocytes/ml. Aliquots of 10^7 $AvBu1^+$ bursal lymphocytes were stored for extraction of cellular DNA.

Proliferation assays were performed for the Av-$Bu1^+$ bursal lymphocytes, using five duplicates of 100 μl/well for each sample, in 96-well microtiter plates. Lymphocytes were unstimulated or stimulated by 12.5 μg concanavalin A/ml (ConA, Sigma) and incubated for

24 or 48 h at 41 °C in 5% CO_2. Samples were pulsed 6 h prior to collection with 4 μCi ^3H thymidine (Amersham Biosciences), were harvested using a Skatron cell harvester (Packard Bioscience, Meriton, CT, U.S.A.) onto an Optiplate-96 filter plate (Packard Bioscience) and air-dried. Filter plates were treated with 30 μl Microscint 20 scintillation liquid (Packard Bioscience) and counted in a 1205 Betaplate counter (Packard Bioscience). The mean counts per minute (cpm) were determined for all five duplicates of stimulated and unstimulated cells, and the stimulation indices (S.I.) were calculated from the ratio mean cpm stimulated/mean cpm unstimulated. The Mann-Whitney test was used to statistically analyse the differences in stimulation between treatment groups.

Real-time PCR quantitation of DNA construct in spleen and AvBu1$^+$ bursal lymphocytes

Cellular DNA was prepared from the spleens and AvBu1$^+$ bursal lymphocytes of each chicken in groups (iii), (iv), (v) and (vi) in experiment 1. The spleen was dissected from the chickens and homogenised in chilled sterile PBS wash buffer. The homogenate was clarified by centrifugation at 2000 g for 5 min, and the cellular debris was resuspended in PBS wash buffer, homogenized a second time and clarified by centrifugation at 2000 g for 5 min. The clarified homogenates were combined and the splenocytes were lysed in 0.2 M NaOH and 1% SDS, then the DNA was purified by phenol/chloroform extraction and ethanol precipitation [13]. Cellular DNA was similarly prepared from aliquots of 10^7 bursal lymphocytes enriched for AvBu1$^+$ cells.

The VP243.pCR3.1VE and VP243.pCR3.1STC plasmid DNA copy number was quantified in both the spleen and AvBu1$^+$ bursal lymphocytes using a real time PCR assay that detects IBDV VP243 sequence and differentiates between variant E and classical STC strains. Briefly, the assay employed IBDV universal forward primer 5′ GTCGAGTGGATATTGGCCCC 3′ and reverse primer 5′ GGCTCCTGCGTTATTCTTGC 3′ (Fischer Scientific, Itasca, IL, U.S.A.) to amplify the IBDV amplicon between bases 2026 and 2161. The strain-specific, dual-labeled, fluorescent probes JOE -5′ CAATGCTTGTGGCGAGATTGAGAAAGTAAG 3′-BHQ1 (detects classical STC IBDV strain) and FAM-5′ CAACGCTTGTGGCGAGATTG AGAAAATAAG 3′-BHQ1 (detects variant E IBDV strain) (Biosearch Technologies, Novato, CA, U.S.A.) were used to differentiate strain sequences. A 25 μl reaction mixture was prepared containing 250 μM each of dATP, dCTP, dGTP and dTTP (Promega, Madison, MA, U.S.A.), 3 mM $MgCl_2$, 2.5 μl of 10× Taq DNA polymerase buffer (Promega), 1 U of Taq DNA polymerase (Promega), 2.5 μM of each primer, either 3 μM JOE-labeled classical STC IBDV probe or 3 μM FAM-labeled variant E IBDV probe, and 4 μl of template DNA. The reduction in quenching of the fluorophore by the BHQ1 dye due to the polymerase 5′ nuclease activity during PCR amplification was measured in the Rotor Gene, real-time, thermal cycling system (Corbett Research, Mortlake, NSW, Australia). The cycle profile was 1 cycle of 60 °C for 3 min, 95 °C for 3 min, and 50 cycles of 95 °C for 20 sec, 60 °C for 1 min.

Standard curves were established for the variant E and classical STC PCR assays using 10-fold dilution series of 2 μg/ml of either variant E or STC IBDV template cloned into TopoII vector (Invitrogen). Cycle time (C_t) was measured for each assay. Sample C_t values were normalized against C_t values for amplification of the 28S internal standard. The 28S gene was amplified using forward primer 5′ GGCGAAGCCAGAGGAAACT 3′, reverse primer 5′ GACGACCGATTTGCACTGC 3′, and the probe FAM-5′ AGGACCGCTACGGACCTC CACCA 3′-BHQ1. Plasmid copy number was calculated from the cycle times by linear regression using the standard curve for each bursal sample of 10^7 cells and for total spleen homogenate.

Real-time RT PCR quantitation of virus copy number in AvBu1$^+$ bursal lymphocytes

Cellular RNA was prepared from aliquots of 10^7 AvBu1$^+$ bursal lymphocytes from chickens in groups (i), (ii) and (vi) in experiment 1. Cells were resuspended in RLT buffer (RNeasy mini kit: 4 M guanidine thiocyanate, 15 mM piperazine-N,N′-bis(2-ethane)-sulphonic acid, pH 6.0, Qiagen) and stored at −80 °C. Total cellular RNA was extracted from the lymphocyte samples using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Purified RNA was eluted in a final volume of 50 μl 0.1% diethyl pyrocarbonate (DEPC) treated water. Reverse transcription was performed at 42 °C for 50 min, in a 25 μl reaction mix containing 2.5 μM of each IBDV primer, 250 μM each of dATP, dCTP, dGTP and dTTP (Promega), 1× Superscript RnaseH$^-$ Reverse Transcriptase buffer (Invitrogen), 1 U Superscript RnaseH$^-$ Reverse Transcriptase (Invitrogen), 0.01 M DTT (Invitrogen). The copy number of the viral RNA genome was quantified from cDNA using the IBDV real time PCR assay described for IBDV plasmid DNA. The internal standard 28S cDNA was prepared by reverse transcription of cellular mRNA. Reaction conditions were otherwise as described previously.

Cell culture model for viral protein expression

The B lymphoblastic cell line DT40 (American Tissue Culture Collection) was chemically transfected with 6 μg VP243.pCR3.1VE, VP2.pCR3.1, VP4.pCR3.1, VP3.pCR3.1 or pCR3.1 DNA using 30 μl TransIT-LT1 (1.33 mg/ml) (Mirus Corp., Madison, W.I., U.S.A.) according to the manufacturer's instructions. Positively transfected cells were selected by growth in 500 μg geneticin (Invitrogen)/ml. Transfection efficiency was assessed at 24 h from the proportion of fluorescent cells transfected with the control pEGFP plasmid. A sample of 10^3 transfected cells was fixed, permeabilised and blocked with 5% BSA using standard cytological methods. The expression of either the VP243 polyprotein, or the VP2, VP4 or VP3 viral proteins was determined by immunofluorescence microscopy using polyclonal hyperimmune serum from chickens infected with IBDV variant E diluted 1/100 in PBS wash buffer, then secondary staining with mouse anti-chicken FITC conjugated serum (KPL, Gaitehrsburg, MD, U.S.A.) also diluted 1/100. The proportion of fluorescent VP243.pCR3.1 transfected cells was calculated over 2 passages (passaging every 48 h). In subsequent experiments the proportion of fluorescent cells transiently transfected with VP243.pCR3.1VE, VP2.pCR3.1, VP4.pCR3.1, VP3.pCR3.1 or pCR3.1 plasmid was determined at 48 h after transfection. The proportion of viable cells was assessed by trypan blue staining.

For each plasmid construct ten duplicate samples of 10^6 cells/ml in 100 μl volumes were transferred to microtiter plates 48 h after transfection. Five wells in each treatment group were stimulated with 100 μl of 5 μg LPS/ml (1 mg/ml stock solution) and 100 μl media was added to the five control wells. The cells were pulsed with 4 μCi ^{3}H thymidine at 43 h and the incorporation of label was analyzed at 48 h as described previously for chicken lymphocyte proliferation assays.

Results

Lymphocyte proliferation assays

Proliferation in response to mitogenic stimulation is characterized by S.I. greater than 1. Proliferation of AvBu1$^+$ bursal cells in response to conA stimulation from chickens in treatment groups (i) to (vi) are presented in Fig. 1. ConA stimulated proliferation of purified AvBu1$^+$ bursal cells from control chickens at all time points after inoculation with either DMEM or with plasmid DNA pCR3.1.

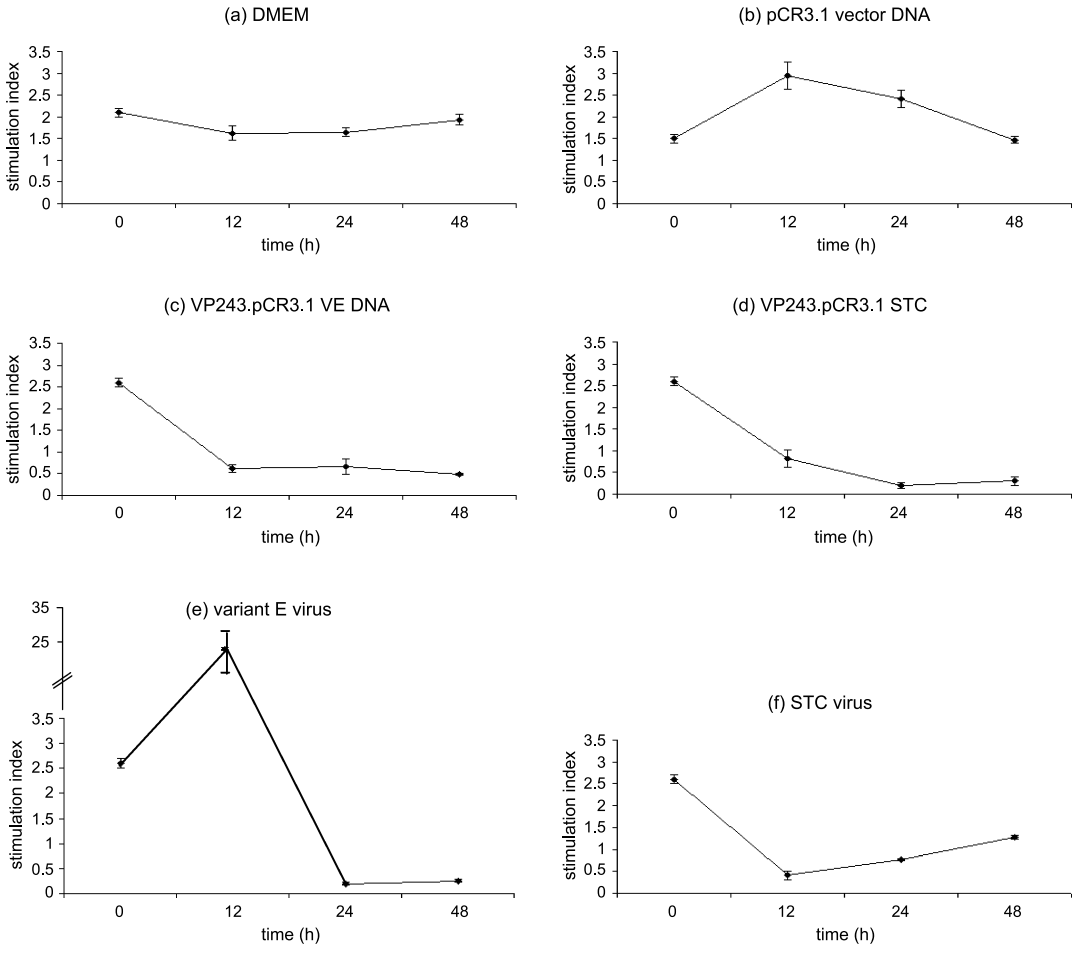

Fig. 1. Chickens were orally inoculated with (**a**) DMEM, (**b**) pCR3.1 vector DNA control, (**c**) VP243.pCR3.1VE DNA, (**d**) VP243.pCR3.1STC DNA, (**e**) IBDV strain variant E virus, or (**f**) IBDV strain STC virus. AvBu1$^+$ bursal lymphocytes were prepared from chickens 12 h, 24 h and 48 h after inoculation. Stimulation indices (S.I.) were calculated from the ratio of the average uptake of ^{3}H thymidine in stimulated and unstimulated samples. Median S.I. are illustrated as filled box symbols

Proliferation was suppressed after inoculation with IBD virus for both classical STC strain and Variant E strain. The S.I. was less than 1 for AvBu1$^+$ bursal cells from chickens infected with IBDV classical strain STC at 12 h and 24 h after infection. The S.I. was significantly lower at 12 h ($p < 0.001$) and 24 h ($p < 0.001$) than that from both control chickens inoculated with either DMEM or plasmid pCR3.1 (Fig. 1f). The S.I. from chickens infected with IBDV variant strain E was significantly suppressed by 24 h after infection relative to control chickens inoculated with DMEM ($p < 0.01$) and also relative to control chickens inoculated with plasmid pCR3.1 ($p < 0.001$) (Fig. 1e). However, S.I. from chickens infected with IBDV variant strain E was strongly positive at 12 h after infection and was significantly greater than for control chickens inoculated with DMEM ($p < 0.01$)

or with pCR3.1 ($p < 0.01$). At 48 h after infection the lymphocyte proliferative responses for chickens infected with either IBDV variant E strain or STC strain were not significantly different from either of the negative control groups ($p < 0.05$).

Proliferation was suppressed for chickens inoculated with either the VP243.pCR3.1STC or the VP243.pCR3.1VE plasmid DNA. For chickens inoculated with VP243.pCR3.1STC plasmid DNA the S.I. was significantly suppressed relative to negative control chickens inoculated with pCR3.1 plasmid at 12 h ($p < 0.01$), 24 h ($P < 0.01$) and 48 h ($p < 0.05$), and was significantly suppressed relative to control chickens receiving DMEM at 12 h ($p < 0.05$), 24 h ($P < 0.01$) and 48 h ($p < 0.05$) (Fig. 1d). The most severe suppression of S.I. occurred at 24 h after inoculation. Likewise S.I. was significantly suppressed at 12 h ($p < 0.05$), 24 h ($p < 0.05$), and 48 h ($p < 0.01$) after inoculation with VP243.pCR3.1VE plasmid DNA relative to negative control chickens inoculated with pCR3.1, and was significantly suppressed relative to control chickens receiving DMEM at 12 h ($p < 0.05$), 24 h ($P < 0.05$) and 48 h ($p < 0.01$) (Fig. 1c).

Real-time PCR quantitation of DNA construct in spleen and AvBu1$^+$ bursal lymphocytes

Real time PCR for quantifying cDNA from IBDV variant E and IBDV STC strains were standardized using tenfold dilution series of the cloned amplicons in the pCR3.1 vector. The coefficient of determination (R^2) for the variant E standard curve was 0.999, the significance threshold (ΔR_n) was 0.01, and the cycle threshold (C_t) range was cycles 5–35. The R^2 value for the STC standard curve was 0.905, the ΔR_n significance threshold was 0.01, and the C_t threshold range was cycles 10–40. Copy number of IBDV template in either total spleen or total AvBu1$^+$ bursal lymphocyte populations was calculated from the C_t values, using the known concentration of the standards.

IBDV sequence was not detected using real time PCR in samples of DNA from the bursae and spleens of control chickens inoculated with either DMEM or pCR3.1 vector. IBDV variant E and STC sequences were detected at 12 h, 24 h and 48 h after inoculation in the spleens and bursae of all chickens orally inoculated with either VP243.pCR3.1VE DNA or VP243.pCR3.1STC DNA (Figs. 2a and b). In general, concentrations were approximately tenfold higher in bursae than in spleens. VP243.pCR3.1VE DNA and VP243.pCR3.1STC DNA levels declined progressively in the spleens with time after inoculation, but remained high in the bursae until 48 h after inoculation.

Real-time RT PCR quantitation of virus in AvBu1$^+$ bursal lymphocytes

Standard curves were derived for the real time RT PCR assays of the 28S gene (internal standard), IBDV variant E and IBDV STC strains. The 28S gene was amplified by real time RT PCR from a tenfold dilution series of sample RNA.

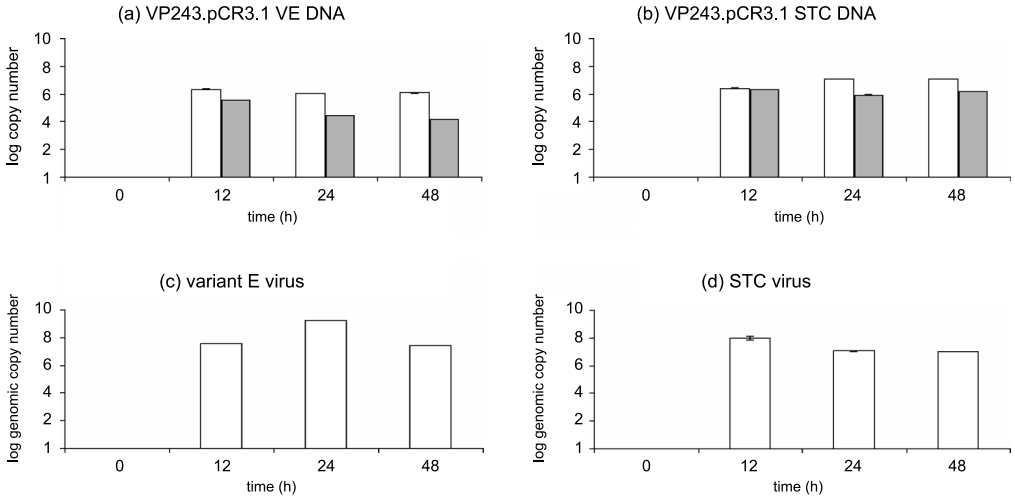

Fig. 2. AvBu1$^+$ bursal lymphocytes and total splenic lymphocytes were purified from 5 chickens at 12 h, 24 h and 48 h after oral inoculation with either (**a**) VP243.pCR3.1 VE DNA or (**b**) VP243.pCR3.1STC DNA. Real time PCR was used to quantify the DNA constructs VP243.pCR3.1 VE and VP243.pCR3.1STC in splenocytes and AvBu1$^+$ bursal lymphocytes. The PCR assays used probes that were specific for either the variant E or classical STC IBDV strains respectively. Assay results are expressed as copy number of DNA template for the total bursal AvBu1$^+$ lymphocyte population or for total splenocytes. Assays for each chicken were the average of triplicates. Real time RT PCR was used to quantify IBDV RNA genome sequence in AvBu1$^+$ bursal lymphocytes. AvBu1$^+$ bursal lymphocytes were purified from 5 chickens at 12 h, 24 h and 48 h after oral inoculation with either (**c**) IBDV variant E strain or (**d**) IBDV STC strain. Assay results are expressed as copy number of genomic template for the total bursal AvBu1$^+$ lymphocyte population. Assays for each chicken were the average of triplicates. Copy number in bursal samples is illustrated as open bars, and in splenocytes as shaded bars

The R^2 value for the standard curve was 0.997, the ΔR_n significance threshold was 0.05, and the C_t threshold range was cycles 10–40.

IBDV sequence was not detected using real time RT PCR in samples of RNA from the bursae of chickens inoculated with either DMEM or pCR3.1 vector. IBDV variant E and STC RNA were detected at 12 h, 24 h and 48 h after inoculation in bursal AvBu1$^+$ lymphocytes of all chickens orally infected with either the variant E or the STC viruses. Mean concentrations were highest at 24 h after infection with variant E IBDV, and were highest at 12 h after infection with STC IBDV (Figs. 2c and d).

Cell culture model for viral protein expression

The expression level of viral polyprotein VP243 (110 kDa), and proteins VP2 (40–48 kDa), VP3 (32 kDa) and VP4 (28 kDa) in DT40 cells was determined by immunofluorescent staining (Table 2).

The baseline rate of growth of DT40 cells was 4 to 6 fold for each 24 h period (Table 1). Transfection of DT40 cells with the VP243.pCR3.1 construct significantly suppressed cell growth by 72 h when compared to cells transfected with pCR3.1 (Table 1). There was no change in cell viability or change in expression of the VP243 polyprotein associated with the suppression of growth. At least 45 to 50% of DT40 cells transfected with either the VP243.pCR3.1VE, VP2.pCR3.1, VP4.pCR3.1, VP3.pCR3.1 or pCR3.1 plasmids expressed viral proteins by 48 h after transfection (Table 2). Proliferation was significantly lower ($p < 0.001$) at

Table 1. The B lymphocyte cell line DT40 was transfected with either the pCR3.1 plasmid or with construct pVP243. Expression of viral proteins at 24 h intervals was measured by immunofluorescence using antiserum against VP2 and the proportion of positive cells calculated. Cellular viability was measured by trypan blue exclusion assay, and cell growth was calculated using a haemocytometer. The fold increase in cell numbers over each 24 h period was normalized against the previous 24 h sample. DT40 cells were passaged at 48 h intervals following transfection

Time[a]	Construct	% Viability (trypan blue assay)	% IFA	Fold increase in cell numbers
24 h	pCR3.1	90	1	4.2
	VP243.pCR3.1	93	90	4.3
48 h	pCR3.1	98	0	4.1
	VP243.pCR3.1	99	75	4.25
72 h	pCR3.1	96	2	6.1
	VP243.pCR3.1	98	75	1.25[b]
96 h	pCR3.1	90	0	5.3
	VP243.pCR3.1	94	22	1.15[b]

[a]Time after transfection

[b]p-value from t-test significantly different at the <0.05 level between pCR3.1 and pVP243 treatments

Table 2. Proliferation of DT40 cells 48 h after transient transfection with pCR3.1, pVP243, pVP2, pVP4 or pVP3. Transiently transfected cells were stimulated with LPS and stimulation indices were calculated at 48 h relative to unstimulated cells

Construct	% Viability (trypan blue assay)	% IFA	Stimulation index		
			mean	S.E.	p value[a]
pCR3.1	90	0	1.69	0.001	N.A.
VP243.pCR3.1	93	50	0.18	0.01	<0.001
VP2.pCR3.1	97	52	1.67	0.05	>0.05
VP4.pCR3.1	90	47	2.2	0.1	>0.05
VP3.pCR3.1	96	45	1.93	0.06	>0.05

[a]p value from one-way t-test of stimulation indices for treatment group relative to pCR3.1 transfected cells

48 h for cells transiently transfected with the VP243.pCR3.1 construct when stimulated with LPS and the mean stimulation index was 0.18 (Table 2). Transient transfection with the VP2.pCR3.1, VP4.pCR3.1, VP3.pCR3.1 or pCR3.1 plasmids did not suppress the proliferation of DT40 cells stimulated with LPS (Table 2).

Discussion

Suppression of proliferative responses to conA mitogenic stimulation in 1-day-old AvBu1$^+$ bursal cells infected with either classical STC or variant E strains of IBDV is consistent with similar findings from experiments examining peripheral lymphocytes from IBDV infected chickens [2]. In this study we demonstrated that the suppression of mitogenic responses in B cells was seen not only in chickens infected with IBDV but also in chickens inoculated with either the VP243pCR3.1VE or VP243pCR3.1STC DNA constructs expressing the IBDV polyprotein. Expression of VP243 polyprotein in DT40 lymphocytes similarly suppressed cell growth and lymphocyte proliferative response to mitogen stimulation. The effects on cell growth were the same for VP243 polyprotein derived from either the variant E or classical STC strains.

VP243.pCR3.1VE and VP243.pCR3.1STC DNA constructs administered orally to 1-day-old chickens resulted in detection of moderate splenic concentrations of the construct at 12 h after inoculation, and clearance from the spleen resulted in a progressive decline in concentration over the next 36 hours. The higher concentrations of DNA construct in the bursa at all time points suggests uptake of DNA from the intestine into the lumen of the bursa and bursal cells. Persistence of DNA in the bursae for the duration of the study following oral administration is preliminary evidence that oral or cloacal inoculation may have advantages for DNA vaccination against IBD. The use of this construct as a DNA vaccine has been shown in experimental trials to protect against the development of severe clinical disease and bursal atrophy [1]. Indeed, different DNA vaccine constructs encompassing VP2 protein have been identified as providing some level of protection against IBD virus challenge [2]. VP2 protein contains major neutralizing conformational epitopes shown in multiple studies to be important to protective immunity. As VP2 protein is central to the development of IBD vaccine strategies, the finding that suppression of cell growth is specifically associated with the VP243 polyprotein intermediate is an important consideration in the future development of vaccines. In one study comparing protection against viral challenge conferred by subunit vaccination with VP2 capsids and polyprotein-derived tubular structures, greater protective efficacy was found for the VP2 capsids [8]. Conversely, the results of a second study comparing DNA vaccines encoding either VP243 polyprotein or VP2 found greater protective efficacy for the polyprotein vaccine [5]. Challenge-protection studies assess protection as the final result of a complex multi-factorial process involving antigen presentation to the immune system and the generation of an immune response. However the results of our study measure a specific aspect of the lymphocyte response to polyprotein antigen presentation, and demonstrate aspects of the interaction

of the vaccine antigen with the immune system that could be targeted for further experimentation as a means to develop methods to enhance the vaccine performance.

Arrest of cell cycle growth and suppression of mitogenic responses due to inoculation of VP243 DNA constructs alone suggest that polyprotein or one of the proteins derived from polyprotein may mediate suppression of bursal function during viral infection. The expression of VP243 polyprotein in transiently transfected DT40 B lymphocyte cultures similarly suppressed cellular proliferation and markedly suppressed proliferation in response to mitogenic stimulation. Quantitation of the DNA construct in bursal B cells shows an inverse association between concentration of the DNA construct and proliferative responses, further validating the relationship between polyprotein and growth suppression. The transfected cells did not have reduced viability, indicating that the suppression of growth was due to cell cycle arrest rather than polyprotein toxicity. Arrest of cell growth was identified by 72 hrs after transfection, however the level of expression of VP243 polyprotein remained constant at 72 h. The number of cells expressing the VP243 polyprotein only began to decline in the arrested cells by 96 h after transfection, indicating that reduced polyprotein expression occurred as an effect of cell cycle arrest. Expression of the mature, processed viral proteins VP2, VP4 and VP3 did not suppress cell proliferation, indicating that the suppression of growth is specific to the polyprotein. To the best of our knowledge there have been no previous studies examining the effect of VP243 polyprotein on cell cycle or cytopathology. Previous studies examining the effect of VP2 expression in cell lines have generated conflicting results as to the role of VP2 in the induction of apoptosis. In the present study there was no evidence of toxicity or reduced growth in cells expressing the VP2 protein.

During virus infection VP243 polyprotein is cleaved by the auto-protease VP4 into pVP2 (a precursor VP2), VP4 and VP3 proteins, and pVP2 is subsequently cleaved by a cellular factor into the mature form VP2 that is found within the inner core of the capsid [10]. The reported stoichiometry of viral proteins in the cytoplasm is 51% 41 kDa pVP2, 40% 32 kDa VP3 and 6% 28 kDa VP4, indicating that VP4 must be degraded during infection. The correct post-translational processing of pVP2 into VP2 is reported to be dependent on association with a scaffold of VP3 that in turn associates with both VP1 and the dsRNA genome [17–19]. The correct expression of the VP243 polyprotein and its processing into the mature viral proteins VP2, VP4 and VP3 has been difficult to model in cell culture expression systems. During VP243 plasmid expression in cells the polyprotein is processed into pVP2, VP4 and VP3, but mature processing of pVP2 to VP2 does not occur to any great extent. This is perhaps due to the absence of VP3 interactions with VP1 and dsRNA, and the subsequent failure of VP3 to form a correct scaffold for capsid assembly [7]. Within cells expressing the construct there is also an aggregation of viral protein into tubules that is not seen during the normal course of cellular infection and the protein aggregates interfere with the correct assembly of structural proteins into capsids [8]. The tubular structures formed by polyprotein have been shown to have

a lower protective efficacy than VP2 capsid structures when used in vaccine-challenge studies [8]. Therefore, one hypothesis with which to interpret the suppression of bursal cells induced by VP243 expression, but not by expression of the mature proteins VP2, VP4 and VP3, is that incorrect processing of polyprotein and the aggregation of viral proteins may induce functional suppression of these cells. Further work is needed to elucidate the mechanism by which IBDV polyprotein VP243 DNA construct contributes to immunosuppression of bursal B lymphocytes.

Acknowledgements

The authors wish to acknowledge the assistance of Dr. Mingkun Hsieh and Ms. Donna Schrader with animal studies.

References

1. Chang HC, Lin TL, Wu CC (2001) DNA-mediated vaccination against infectious bursal disease in chickens. Vaccine 20: 328–335
2. Chang HC, Lin TL, Wu CC (2003) DNA vaccination with plasmids containing various fragments of large segment genome of infectious bursal disease virus. Vaccine 21: 507–513
3. Fernandez-Arias A, Martinez S, Rodriguez JF (1997) The major antigenic protein of infectious bursal disease virus, VP2, is an apoptotic inducer. J Virol 71: 8014–8018
4. Jungmann A, Nieper H, Muller H (2001) Apoptosis is induced by infectious bursal disease virus replication in productively infected cells as well as in antigen-negative cells in their vicinity. J Gen Virol 82: 1107–1115
5. Li J, Huang Y, Liang X, Lu M, Li L, Yu L, Deng R (2003) Plasmid DNA encoding antigens of infectious bursal disease viruses induce protective immune responses in chickens: factors influencing efficacy. Virus Res 98: 63–74
6. Lukert PD, Saif YM (1991) Infectious bursal disease. In: Calnek BW (ed) Diseases of poultry. Iowa State University Press, Ames, pp 648–663
7. Maraver A, Ona A, Abaitua F, Gonzalez D, Clemente R, Ruiz-Diaz JA, Caston JR, Pazos F, Rodriguez JF (2003) The oligomerization domain of VP3, the scaffolding protein of infectious bursal disease virus, plays a critical role in capsid assembly. J Virol 77: 6438–6449
8. Martinez-Torrecuadrada JL, Saubi N, Pages-Mante A, Caston JR, Espuna E, Casal JI (2003) Structure-dependent efficacy of infectious bursal disease virus (IBDV) recombinant vaccines. Vaccine 21: 3342–3350
9. McNeilly F, Walker I, Allan GM, Adair B (1999) Bursal lymphocyte proliferation in the presence of phorbol myristate acetate: effect of IBDV strains on the proliferation response. Avian Pathol 28: 301–303
10. Muller H, Becht H (1982) Biosynthesis of virus-specific proteins in cells infected with infectious bursal disease virus and their significance as structural elements for infectious virus and incomplete particles. J Virol 44: 384–392
11. Murphy F, Gibbs E, Horzinek M, Studdert M (1999) Veterinary virology, 3 edn. Academic Press, California
12. Ojeda F, Skardova I, Guarda MI, Ulloa J, Folch H (1997) Proliferation and apoptosis in infection with infectious bursal disease virus: a flow cytometric study. Avian Dis 41: 312–316

13. Sambrook J, Fritsch E, Maniatis T (1989) Commonly used techniques in molecular cloning, a laboratory manual. vol 3. Cold Spring Harbor Laboratory Press, New York, pp E3–E4
14. Sharma JM, Kim IJ, Rautenschlein S, Yeh HY (2000) Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. Dev Comp Immunol 24: 223–235
15. Snyder DB, Lana DP, Savage PK, Yancey FS, Mengel SA, Marquardt WM (1988) Differentiation of infectious bursal disease viruses directly from infected tissues with neutralizing monoclonal antibodies: evidence for a major antigenic shift in recent field isolates. Avian Dis 32: 535–539
16. Snyder DB, Vakharia VN, Savage PK (1992) Naturally occurring neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease viruses in the United States. Arch Virol 127: 89–101
17. Tacken MG, Rottier PJ, Gielkens AL, Peeters BP (2000) Interactions in vivo between the proteins of infectious bursal disease virus: capsid protein VP3 interacts with the RNA-dependent RNA polymerase, VP1. J Gen Virol 81: 209–218
18. Tacken MG, Peeters BP, Thomas AA, Rottier PJ, Boot HJ (2002) Infectious bursal disease virus capsid protein VP3 interacts both with VP1, the RNA-dependent RNA polymerase, and with viral double-stranded RNA. J Virol 76: 11301–11311
19. Tacken MG, Van Den Beuken PA, Peeters BP, Thomas AA, Rottier PJ, Boot HJ (2003) Homotypic interactions of the infectious bursal disease virus proteins VP3, pVP2, VP4, and VP5: mapping of the interacting domains. Virology 312: 306–319
20. Tanimura N, Sharma JM (1998) In-situ apoptosis in chickens infected with infectious bursal disease virus. J Comp Pathol 118: 15–27
21. Tham KM, Moon CD (1996) Apoptosis in cell cultures induced by infectious bursal disease virus following in vitro infection. Avian Dis 40: 109–113
22. Vasconcelos AC, Lam KM (1995) Apoptosis in chicken embryos induced by the infectious bursal disease virus. J Comp Pathol 112: 327–338
23. Yao K, Vakharia VN (2001) Induction of apoptosis in vitro by the 17-kDa nonstructural protein of infectious bursal disease virus: possible role in viral pathogenesis. Virology 285: 50–58

Author's address: Dr. C. C. Wu, Department of Veterinary Pathobiology, Purdue University, 406 S. University St., West Lafayette, IN 47907-2065, U.S.A.; e-mail: wuc@purdue.edu