JOURNAL OF VIROLOGY, Oct. 1997, p. 7889–7894 Vol. 71, No. 10
0022-538X/97/$04.00+0

The Carboxyl-Terminal 120-Residue Polypeptide of Infectious Bronchitis Virus Nucleocapsid Induces Cytotoxic T Lymphocytes and Protects Chickens from Acute Infection

SANG HEUI SEO, LI WANG, ROGER SMITH, AND ELLEN W. COLLISSON*

Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A & M University, College Station, Texas 77843-4467

Received 9 May 1997/Accepted 10 July 1997

Specific cytotoxic T-lymphocyte (CTL) responses to nucleocapsid of infectious bronchitis virus (IBV) were identified by using target cells infected with a Semliki Forest virus (SFV) vector. Effector cells for CTL assays were collected from chickens infected with the Gray strain of IBV or inoculated with a DNA plasmid encoding nucleocapsid proteins. IBV-specific CTL epitopes were mapped within the carboxyl-terminal 120 amino acids of the nucleocapsid protein. CTL lysis of target cells infected with SFV encoding nucleocapsid was major histocompatibility complex restricted and mediated by $CD8^+$ T cells. In addition, splenic T cells collected from chickens inoculated in the breast muscle with a DNA plasmid encoding this CTL epitope(s) recognized target cells infected with wild-type virus or an SFV vector encoding nucleocapsid proteins. CTL activity of splenic T cells collected from chicks immunized with a DNA plasmid encoding CTL epitopes was cross-reactive, in that lysis of target cells infected with serologically distinct strains of IBV was dose responsive in a manner similar to that for lysis of target cells infected with the homologous strain of IBV. Furthermore, chickens immunized with a DNA plasmid encoding a CTL epitope(s) were protected from acute viral infection.

Cytotoxic T-lymphocyte (CTL) responses provide a major defense mechanism for elimination of virus-infected cells, and in some cases CTL have been able to confer complete protection even in the absence of an antibody response (2, 18). CTL recognize endogenously expressed peptide in association with major histocompatibility complex (MHC) class I molecules (4, 5). As CTL often recognize conserved determinants of the virus, CTL epitopes should be valuable in designing effective vaccines (21).

Infectious bronchitis virus (IBV) is a prototype of the *Coronaviridae* family. The virion is composed of a positive-stranded genomic RNA, a lipid-containing membrane, and four structural proteins (17, 25). In addition to the internally localized nucleocapsid protein, the virion is composed of the spike and membrane proteins and a third, smaller envelope protein (3, 7, 9, 10, 19). IBV causes morbidity in all ages of chickens and high mortality in chickens that are less than 6 weeks old (13, 15). IBV continues to cause major economic losses in the poultry industry worldwide, in spite of the widespread use of live attenuated vaccines. Control of IBV is difficult because numerous field isolates differ antigenically from vaccine serotypes (32).

Most studies of IBV immunity have focused on humoral immune responses to spike protein. However, by generating antigenic variants of the S protein, IBV can avoid elimination by neutralizing antibody (8, 14, 16). In a previous study, we reported evidence that IBV-specific CTL were critical in resolving early viral infection in IBV target organs (26). Studies with other viral infections indicate that the internal, nonglycosylated viral antigens contribute to the induction of protective immunity by generating CTL (1, 12, 23). Until now, CTL responses to IBV nucleocapsid protein have not been reported, but CD4 epitopes within the IBV N protein have been identified in the mouse (6). In the present study, we report for the first time that specific CTL epitopes to nucleocapsid of IBV can play a central role in protecting chickens from acute viral challenge.

MATERIALS AND METHODS

Experimental animals. Embryonated eggs of inbred (B^{12}/B^{12}) and outbred (R68C) chickens were obtained from Hy-VAC (Adel, Iowa) and SPAFAS Incorporated (Preston, Conn.), respectively. Eggs were hatched in our laboratory, and chickens were housed in a specific-pathogen-free environment at the Laboratory Animal and Resources Facility, Texas A & M University, College Station.

Viral source. The nephropathogenic Gray, Arkansas, and Mass41 strains of IBV were propagated by inoculating the allantoic sac of 11-day-old chicken embryos. Allantoic fluid harvested 36 h postinfection (p.i.) was collected as a source for viral inoculation (28). Tissue culture-adapted viruses were propagated in an established chicken kidney (CK) cell line before harvesting 48 h p.i. (26).

Generation of recombinant SFV vectors. IBV genomic RNA was prepared as described by Sneed et al. (28). First-strand cDNA synthesis was carried out as described in the Superscript manual (GIBCO BRL, Grand Island, N.Y.), using a 3′-end primer (5′GTTGGATCCGAGTTCATTCTCACCTAAAGC3′) of IBV genomic RNA before proceeding to amplification. PCR was performed by using the appropriate primers (Table 1) for synthesizing overlapping fragments of the nucleocapsid gene with the following reaction conditions: primary denaturation at 94°C for 5 min, annealing at 55°C for 30 s, extension at 72°C for 45 s, and subsequent denaturation at 94°C for 1 min. A total of 35 cycles were used, with a final extension step of 7 min at 72°C. Resulting PCR products were digested with *Bam*HI before ligation into a Semliki Forest virus (SFV) plasmid pSFV1 also digested with *Bam*HI (GIBCO BRL). After transformation, *Escherichia coli* colonies were screened for inserts by miniscale preparation (24) before checking for correct orientation by ABI PRISM dye terminator cycle sequencing (Perkin-Elmer). Packaging of SFV recombinants was carried out as specified by the manufacturer (GIBCO BRL).

Flow cytometric analysis of infected cells. CK cells (2×10^6) were infected at a multiplicity of infection of 2.0 with recombinant SFV encoding IBV nucleocapsid proteins or transfected by electroporation (250 V, 80 μF) with 20 μg of recombinant DNA plasmids before incubation for 24 or 36 h, respectively. The cells were fixed with 1.8% formaldehyde and permeabilized in 0.1% Nonidet P-40 (Sigma). The cells were then incubated with a 1:50 dilution of mouse anti-IBV polyclonal antibody in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide) on ice for 30 min before being washed with FACS buffer five

* Corresponding author.

TABLE 1. Primers of N fragments

N fragment	Primer sequence	
	Forward	Reverse
N	5′CGCGGATCCGCACCATGGCAAGCGGTAAGG3′	5′GTTTGGATCCGAGTTCATTCTCACCTAAAGC3′
Na	5′CGCGGATCCGCACCATGGCAAGCGGTAAGG3′	5′CGCGGATCCGCATGCTGCTGTTGATCTTCCACTCC3′
Nb	5′CGCGGATCCGCACCATGTTCATCAGCAGCATCTAGT3′	5′GTTTGGATCCGAGTTCATTCTCACCTAAAGC3′
Nc	5′CGCGGATCCGCACCATGTCATCAGCAGCATCTAGT3′	5′CGCGGATCCGCATGTTTGGGCGTCACTCTACTTCCAAA3′
Nd	5′CGCGGATCCGCACCATGCTTCAAACCAGATGGGCTGC3′	5′GTTTGGATCCGAGTTCATTCTCACCTAAAGC3′
Ne	5′CGCGGATCCGCACCATGGCAAGCGGTAAGG3′	5′CGCGGATCCGCATGTTTGGGCGTCACTCTACTTCCAAA3′

times. The cells were then incubated with a 1:50 dilution of fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (IgG) in FACS buffer on ice for 30 min before being washed five times with FACS buffer. The reaction samples were resuspended to 10^6 cells per ml in 1.8% formaldehyde and analyzed with a FACScalibur (Becton Dickinson, San Jose, Calif.).

Generation of target cells. CK cells (2×10^6) were infected either with the Gray strain of IBV (10^6 50% egg infective doses (EID_{50}) for 2 days or with SFV vectors (multiplicity of infection of 2.0) for 12 h before being resuspended in 400 μl of CTL assay medium (RPMI 1640 supplemented with 2% [vol/vol] chicken serum). CK cells were labeled with 300 μCi of $Na_2{}^{51}CrO_4$ for 90 min, washed three times, and resuspended in CTL assay medium at a final concentration of 2×10^5 cells per ml.

Preparation of effector cells. Splenocytes from IBV-infected or uninfected chickens or splenocytes from chickens inoculated with a recombinant plasmid encoding IBV nucleocapsid were used as the source of effector cells. Spleens from six chickens at 10 or 13 days p.i. were collected and pooled as described previously (26) and then stimulated in vitro with irradiated IBV-infected syngeneic CK cells or irradiated N-SFV-infected syngeneic CK cells for 8 or 10 days (3,000 rads) before use as CTL effector cells. The $CD4^+$ and $CD8^+$ lymphocytes were also separated before use as CTL effector cells (26).

CTL assay. Splenic lymphocytes were tested for cytotoxic activity against syngeneic and allogeneic target cells. To achieve various effector-to-target cell ratios, effector cells were diluted in 96-well round-bottom microtiter plates in 100-μl volumes per well, and 100 μl of 2×10^4 target cells infected with IBV or recombinant SFV encoding nucleocapsid genes was added to each well. Microtiter plates were centrifuged at $100 \times g$ for 5 min before incubation for 4 h at 40°C. After centrifugation at $600 \times g$ for 10 min, 100-μl aliquots of supernatants in triplicate samples were collected for determining ^{51}Cr release, using a gamma radiation counter (Cobra Autogamma; Canberra Packard). Percent specific lysis equaled $(E - S/M - S) \times 100$, where E is the mean of three test wells, S is the mean of spontaneous release from the three target cells without effector cells, and M is the mean maximal release from the three target cells with 3% Triton X-100. The spontaneous chromium release of target cells was less than 20% of the total chromium release in all experiments.

Construction of a recombinant DNA plasmid. The pTargeT mammalian expression vector (Promega, Madison, Wis.) was digested with restriction enzyme *Bam*HI and mixed with the Nd or Ne fragment of the IBV nucleocapsid gene (Table 1) cut from the SFV plasmid with BamHI at a vector/insert molar ratio of 1:3 before ligation with T4 DNA ligase (Promega). After transformation by heat shock (Invitrogen, San Diego, Calif.), *E. coli* colonies were screened for inserts by miniscale preparation, and then the correct orientation was determined by ABI PRISM dye terminator cycle sequencing (Perkin-Elmer).

DNA inoculation. The breast muscles of 5-day-old chickens were injected with 200 μl of a solution containing 0.25% bupivacaine-HCl (Sigma Chemical Co., St. Louis, Mo.), using a 25-gauge needle (32), 2 days before 150 μg of each DNA construct in phosphate-buffered saline was injected into the same location. Four days after the first DNA inoculation, the second inoculation was administered to the same site. Four days later, the chickens were challenged with 10^6 EID_{50} of IBV Gray strain and examined for signs of clinical illness such as coughing, sneezing, dyspnea, ataxia, or death. Lungs and kidneys were collected from the chickens immunized with plasmid pTargeT encoding the Nd or Ne nucleocapsid fragment and challenged with the Gray strain of IBV at 5 days p.i. Viral titers were determined as described previously (26).

RESULTS

IBV-specific CTL lysed target cells expressing nucleocapsid proteins. SFV vectors were used to generate target cells expressing individual viral polypeptides. Reverse transcription-PCR cDNA products generated for each fragment as shown in Fig. 1 were ligated into the vector. The corresponding constructs were designated N-SFV, Na-SFV, Nb-SFV, Nc-SFV, Nd-SFV and Ne-SFV. CK cells infected with SFV vectors encoding nucleocapsid polypeptides were analyzed for expression of individual proteins by flow cytometry. More than 90% of the CK cells expressed IBV nucleocapsid polypeptides (Fig. 2A).

Effector cells collected from spleens of chicks infected with the Gray strain of IBV 10 days p.i. were stimulated in vitro for 10 days with irradiated IBV-infected syngeneic CK cells before use in CTL assays. CTL from infected chickens induced lysis of target cells infected with wild-type virus or recombinant SFV vectors encoding the whole nucleocapsid protein in a dose-responsive manner. However, the lysis of target cells infected with SFV alone by CTL from infected chicks was minimal (Fig. 3A). Effector cells from spleens of uninfected chickens caused minimal lysis of target cells expressing the entire nucleocapsid protein. These results indicate that at least one IBV-specific CTL epitope exists in the nucleocapsid protein.

CTL lysis of target cells expressing IBV nucleocapsid protein was MHC restricted and mediated by $CD8^+$ T cells. The in vitro-stimulated T cells induced lysis of syngeneic target cells expressing whole nucleocapsid with maximum chromium release of 42% at an effector/target cell ratio of 45:1. In contrast, effector cells did not lyse the allogeneic CK cells expressing whole nucleocapsid or the uninfected syngeneic target cells (Fig. 3B).

Depletion of $CD4^+$ T cells from in vitro-stimulated T cells lysed syngeneic target cells expressing nucleocapsid proteins in a dose-responsive manner similar to that for the undepleted effector cell population, whereas depletion of $CD8^+$ T cells reduced CTL activity as much as 75%. The residual CTL activity could be caused by the presence of natural killer cells or residual $CD8^+$ T cells (Fig. 3C). Depletion of T-cell subpopulations was confirmed by flow cytometry (data not shown).

The IBV-specific CTL epitope mapped to the carboxyl-terminal 120 amino acids of nucleocapsid. To locate IBV-specific CTL epitopes in the nucleocapsid, the amino-terminal 172 amino acids and carboxyl-terminal 238 amino acids were ex-

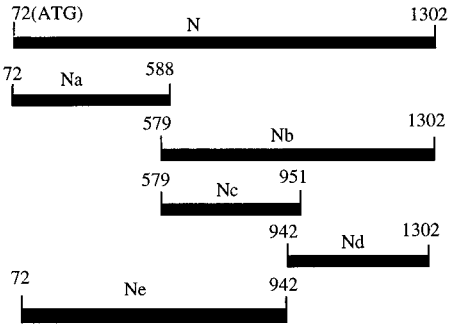

FIG. 1. Diagram of nucleocapsid constructs. Each labeled fragment was amplified from cDNA of genomic RNA by using appropriate primers (Table 1). The resulting PCR products were cloned into an SFV vector or pTargeT DNA vector, using *Bam*HI cloning sites.

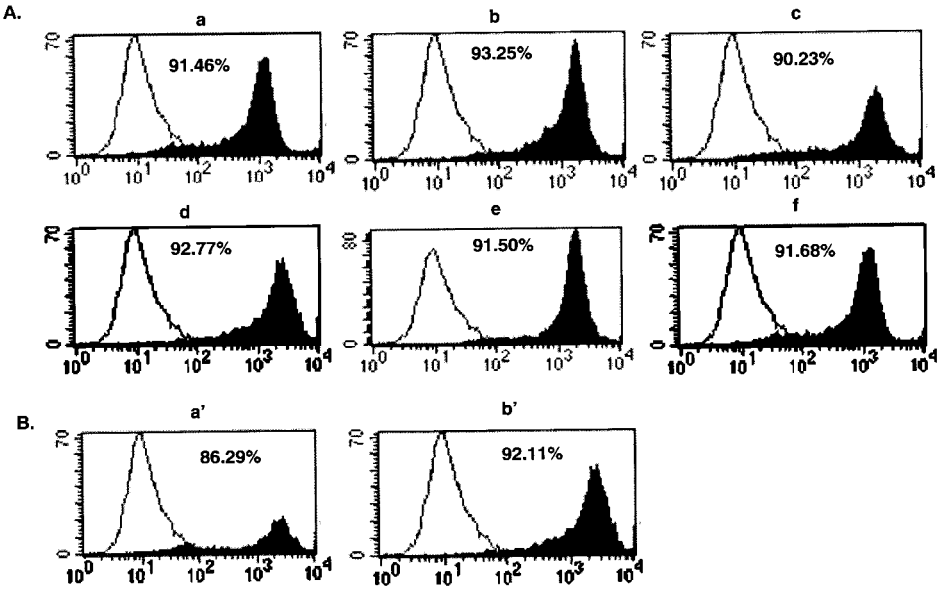

FIG. 2. (A) Detection by flow cytometry of viral protein expression in CK cells infected with SFV recombinants. CK cells were infected with SFV recombinants N-SFV (a), Na-SFV (b), Nb-SFV (c), Nc-SFV (d), Nd-SFV (e), and Ne-SFV (f) before collection for analysis. Control cells were stained with normal mouse serum and then with fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody. (B) Detection of viral protein expression in CK cells transfected with pTargeT recombinants, using flow cytometry. CK cells transfected with DNA recombinants Nd-pTargeT (a′) and Ne-pTargeT (b′) by electroporation were collected for analysis.

pressed in target cells infected with Na-SFV and Nb-SFV (Table 1). Splenic T cells collected from IBV-infected chickens were stimulated in vitro with irradiated IBV-infected CK cells for 8 days before use as CTL effector cells. Effector cells lysed target cells infected with both N-SFV and Nb-SFV with a maximum of 60% at an effector/target ratio of 40:1. Lysis of target cells infected with Na-SFV was minimal. Therefore, the IBV-specific CTL epitope(s) in the nucleocapsid protein resides within carboxyl 238 amino acids (Fig. 4A).

To further map CTL determinants in the nucleocapsid, SFV vectors encoding the overlapping fragments Nc, Nd, and Ne were used to generate CK target cells. Splenic T cells from IBV-infected chicks lysed syngeneic CK target cells infected with IBV, N-SFV, and Nd-SFV in a dose-dependent manner but not target cells expressing Nc-SFV or Ne-SFV or the uninfected target cells (Fig. 4B). These results indicate that at least one IBV-specific CTL epitope exists in Nd, the carboxyl-terminal 120 amino acids of nucleocapsid.

IBV nucleocapsid-specific CTL were generated in vivo and were cross-reactive. The nucleocapsid protein and polypep-

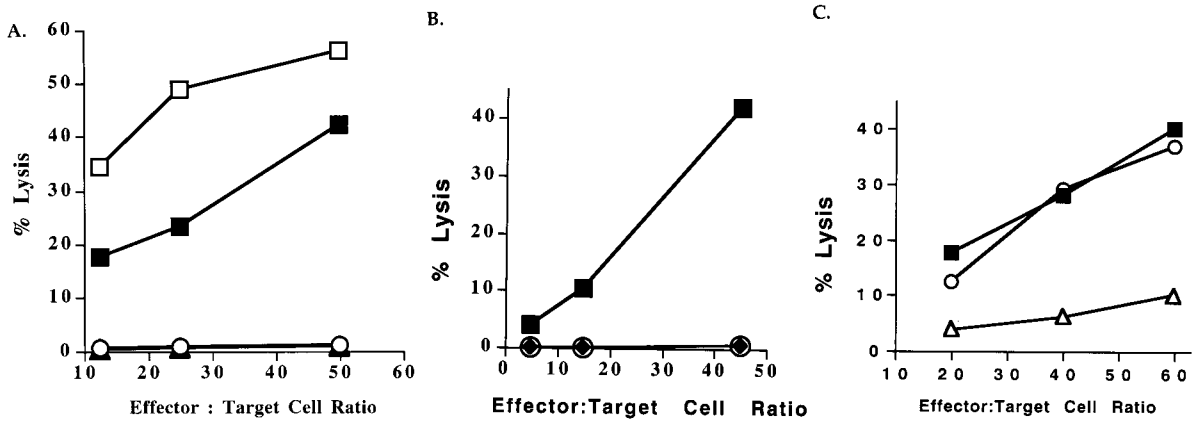

FIG. 3. (A) Lysis of target cells infected with SFV encoding whole nucleocapsid by IBV-specific CTL. Splenic T cells collected from chickens infected with the Gray strain of IBV were stimulated in vitro with irradiated IBV-infected syngeneic CK cells before use as effector cells. Lysis of target cells infected with IBV is represented by open squares, lysis of target cells infected with an SFV recombinant encoding whole nucleocapsid is represented by closed squares, lysis of target cells infected with SFV alone is represented by closed triangles, and lysis of target cells infected with IBV by stimulated effector cells from uninfected chickens is represented by open circles. (B) MHC-restricted lysis of target cells expressing nucleocapsid protein. Effector cells collected from IBV-infected chickens were stimulated in vitro before CTL assay. Lysis of syngeneic CK cells infected with an SFV recombinant encoding nucleocapsid is represented by closed squares, lysis of heterologous CK cells infected with an SFV recombinant encoding nucleocapsid is represented by closed diamonds, and lysis of uninfected syngeneic CK cells is represented by open circles. (C) $CD8^+$ T-cell-mediated lysis of target cells infected with an SFV recombinant encoding nucleocapsid. In vitro-stimulated splenic T cells were depleted of $CD4^+$ or $CD8^+$ T cells before CTL assay. Lysis of target cells infected with an SFV recombinant encoding nucleocapsid protein by undepleted effector cells is represented by closed squares, lysis of target cells infected with an SFV recombinant encoding nucleocapsid protein by effector cells depleted of $CD4^+$ T cells is represented by open circles, and lysis of target cells infected with an SFV recombinant encoding nucleocapsid protein by effector cells depleted of $CD8^+$ T cells is represented by open triangles.

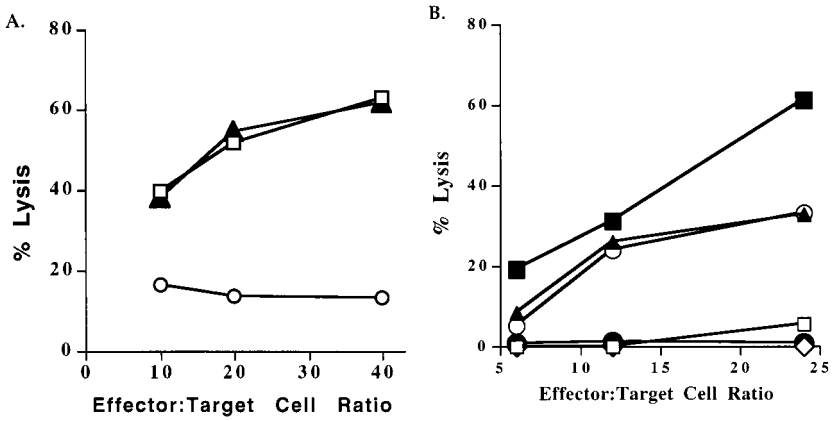

FIG. 4. (A) Mapping of the CTL epitope to nucleocapsid protein. CK cells were infected with Na-SFV or Nb-SFV before use as target cells for effector cells stimulated with irradiated IBV-infected syngeneic CK cells. Lysis of target cells infected with Nb-SFV is represented by closed triangles, lysis of target cells infected with N-SFV is represented by open squares, and lysis of target cells infected with Na-SFV is represented by open circles. (B) Further mapping of CTL epitopes to nucleocapsid protein. CK cells were infected with IBV, N-SFV, Nc-SFV, Nd-SFV, or Ne-SFV before use as target cells for effector cells stimulated with irradiated IBV-infected syngeneic CK cells. Lysis of target cells infected with IBV is represented by closed squares, lysis of target cells infected with N-SFV is represented by closed triangles, lysis of target cells infected with Nd-SFV is represented by open circles, lysis of target cells infected with Nc-SFV is represented by closed circles, lysis of target cells infected with Ne-SFV is represented by open squares, and lysis of uninfected target cells is represented by open diamonds.

tides expressed by the Nb and Nd sequences in target cells were recognized by CTL from virus-infected chickens. In vivo induction of CTL with endogenously expressed nucleocapsid in the absence of other viral polypeptides was examined. pTargeT DNA plasmids encoding Nd or Ne of the Gray strain of IBV were constructed for inoculation of chicks. Transfection and expression potential of pTargeT plasmids encoding Nd or Ne were first examined in vitro in CK cells. More than 85% of these cells were shown to express viral polypeptide (Fig. 2B).

Splenic T cells collected from chickens inoculated with pTargeT plasmids were stimulated in vitro with irradiated syngeneic CK cells infected with Nd-SFV or Ne-SFV for 8 days before use as CTL effector cells. Nd-specific CTL were detected in assays using target cells infected with the Gray strain of IBV or Nd-SFV in a dose-responsive manner resulting in maximum lysis of 45% at an effector/target ratio of 70:1 (Fig. 5A). In contrast, splenic T cells from uninoculated chickens or chickens inoculated with Ne-pTargeT did not lyse target cells infected with the Gray strain of IBV.

The cross-reactivity of nucleocapsid-specific CTL was examined with two serologically and genetically distinct strains of IBV. Splenic T cells from chickens inoculated with Nd-pTargeT lysed target cells infected with the heterologous Arkansas and Mass41 strains of IBV in a dose-dependent manner. Therefore, IBV-specific CTL epitopes are conserved in the nucleocapsid proteins of various strains of IBV (Fig. 5B).

Nd-specific CTL protected chicks from acute infection. Chickens inoculated with Nd-pTargeT or Ne-pTargeT were challenged with the Gray strain of IBV. Chickens immunized with Nd-pTargeT were protected from severe illness (two of six chickens experienced mild respiratory signs of sneezing or nasal discharge). In contrast, all of six chicks immunized with

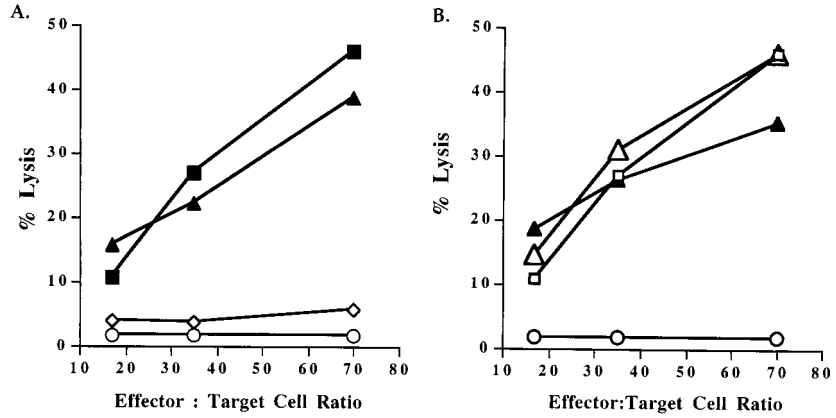

FIG. 5. (A) In vivo induction of nucleocapsid-specific CTL by DNA inoculation. Splenic T cells collected from chickens inoculated with DNA recombinants were stimulated in vitro with irradiated syngeneic CK cells infected with N-SFV before use as effector cells. Lysis of target cells infected with Nd-SFV by effector cells from chickens inoculated with Nd-pTargeT is represented by closed triangles, lysis of target cells infected with the Gray strain of IBV by effector cells from chickens inoculated with Nd-pTargeT is represented by closed squares, lysis of target cells infected with the Gray strain of IBV by effector cells from chickens inoculated with Ne-pTargeT is represented by open diamonds, and lysis of target cells infected with the Gray strain of IBV by effector cells from DNA uninoculated chickens is represented by open circles. (B) Cross-reactive CTL activity by splenic T cells from chickens inoculated with Nd-pTargeT. CK cells were infected with different strains of IBV before use as target cells for Nd-specific effector cells stimulated in vitro with irradiated syngeneic CK cells infected with N-SFV. Lysis of target cells infected with the Gray strain of IBV is represented by closed triangles, lysis of target cells infected with Arkansas strain of IBV is represented by open triangles, lysis of target cells infected with the Mass41 strain of IBV is represented by open squares, and lysis of uninfected target cells is represented by open circles.

TABLE 2. Clinical signs in DNA-inoculated chickens[a]

Inoculation	Clinical illness (no. ill/6)
Nd-pTargeT	+ (2), − (4)
Ne-pTargeT	++ (3), +++ (3)
No DNA	++ (2), +++ (4)

[a] Each group of six chickens was inoculated two times with 150 μg of pTargeT recombinant before being challenged with IBV. The clinical illness was recorded 5 days p.i. according to the following criteria: −, no clinical signs of illness; +, sneezing or nasal discharge; ++, plus dyspnea or rales; +++, plus lack of appetite, pale color, or ataxia; ++++, plus abnormal stools, inability to move, or death. Six uninfected chickens were used for the negative control group.

Ne-pTargeT showed signs of severe clinical illness, such as dyspnea, rales, lack of appetite, or ataxia, similar to those in unimmunized chicks (Table 2).

Viral replication in lungs and kidneys from chickens inoculated with pTargeT plasmids encoding Nd or Ne and challenged with the Gray strain of IBV was quantitated. The viral burden in both lungs and kidneys of chickens immunized with Ne-pTargeT was similar to that in the unimmunized control chickens (Fig. 6). In contrast, compared with unvaccinated chickens, the viral replication in chickens immunized with Nd-pTargeT was reduced by 7 logs in lungs and to undetectable levels in kidneys.

DISCUSSION

This is the first description of CTL activity specific for the nucleocapsid of IBV. The vigorous CTL response from IBV-infected chicks was directed primarily toward the carboxyl-terminal 120 residue (Nd). Similarly, a CTL epitope for murine hepatitis virus was mapped to the 178-residue carboxyl terminus (30). SFV vectors were used to generate target cells expressing individual polypeptides. This system was preferred because IBV polypeptides were efficiently expressed in the cytoplasm in the absence of structural proteins from the vector. The presence of an IBV epitope or epitopes in the Nd region was further substantiated through the in vivo inoculation of DNA plasmids expressing the fragment. The second Ne fragment expressing residues in the amino-terminal 75% of nucleocapsid, which was not recognized by effectors from IBV-infected chickens, did not induce CTL upon in vivo expression using the cDNA expression plasmids.

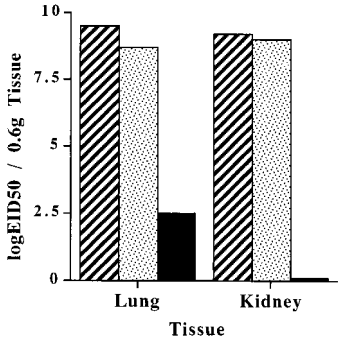

FIG. 6. Viral titers of IBV target organs from chickens inoculated with DNA recombinants before challenge with homologous IBV. Lungs and kidneys from six chickens unimmunized or immunized with DNA recombinants before challenge with the Gray strain of IBV were collected and pooled. Viral titers of lungs and kidneys from unimmunized chickens are represented by hatched squares, viral titers of lungs and kidneys from chickens immunized with Ne-pTargeT are represented by dotted squares, and viral titers of lungs and kidneys from chickens immunized with Nd-pTargeT are represented by shaded squares.

The inoculation of DNA plasmids encoding N polypeptides proved to be an efficient and effective means of inducing CTL. This procedure is technically simple and results in the endogenous expression of antigen necessary for optimal CTL activity. As might be predicted of the highly conserved N, immunity induced through in vivo expression of Nd was cross-reactive with two serologically distinct strains of IBV. Immunization with naked DNA vectors has proven to be valuable in inducing protective immunity to other viral proteins, such as the influenza virus nucleocapsid (11, 20, 22). In the present study, acute IBV infection was reduced by 7 logs in the lungs and eliminated in the kidneys of chicks inoculated with Nd DNA but not reduced in either organ of chickens receiving DNA encoding Ne. More complete protection of the lungs might be achieved with direct inoculation of Nd within the respiratory tract.

In our previous study, the IBV-specific CTL response following infection correlated with a decrease in detectable virus in both the lungs and kidneys. Specific IgG antibodies were not detectable until viral loads and CTL activity declined. However, because N does not induce viral neutralizing antibody and the Ne fragment did not protect chickens, it seems likely that protection against acute infection was the result of the specific CTL response. In addition, preliminary studies in which $CD8^+$ T cells stimulated with antigen-presenting cells endogenously expressing IBV were adoptively transferred to chickens suggest that IBV-specific CTL can eliminate virus in acute infection (26a).

Nucleocapsid protein closely associated with genomic RNA plays diverse roles in the structure and replication of IBV (29). This phosphorylated and unglycosylated protein is produced in large quantities during replication, and its predicted amino acid sequence is highly conserved among various IBV strains (27, 33). Although serotyping is generally based on epitopes in the spike protein, we now know that the nucleocapsid is highly immunogenic at both the antibody and CTL levels (28). Therefore, considering the continuously evolving antigenic diversity of S1 among IBV strains, effective subunit vaccine strategies should incorporate the IBV nucleocapsid. Future studies should identify the peptide sequence(s) of the CTL epitope(s) and define the apparent central role of CTL to nucleocapsid in clearing acute IBV infection.

ACKNOWLEDGMENTS

This research was supported by Southeast Poultry & Egg Association no. 142, USDA Animal Health (section 1433) no. TEXO-6824, and USDA Cooperative State Research Service no. 93-37207-9296.

We thank Betty Rosenbaum for help with flow cytometry.

REFERENCES

1. **Bangham, C. R. M., P. J. M. Openshaw, L. A. Ball, A. M. Q. King, G. W. Wertz, and B. A. Askonas.** 1986. Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleocapsid (N), but not the major glycoprotein (G), expressed by vaccinia virus recombinants. J. Immunol. **137:**3973–3977.
2. **Bevan, M. J.** 1989. Stimulating killer cells. Nature **342:**478–479.
3. **Bingham, R. W.** 1975. The polypeptide composition of avian infectious bronchitis virus. Arch. Virol. **49:**207–216.
4. **Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley.** 1987. Structure of the human class I histocompatibility antigen, HLA-A2. Nature **329:**506–512.
5. **Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley.** 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature **329:**512–518.
6. **Boots, A. M. H., J. G. Kusters, J. M. Van Noort, K. A. Zwaagstra, E. Rijke, B. A. M. Van Der Zeijst, and E. J. Hensen.** 1991. Localization of a T-cell epitope within the nucleocapsid protein of avian coronavirus. Immunology **74:**8–13.

7. **Cavanagh, D.** 1981. Structural polypeptides of coronavirus IBV. J. Gen. Virol. **53:**93–103.
8. **Cavanagh, D., J. H. Darbyshire, P. Davis, and R. W. Peters.** 1984. Induction of humoral neutralizing and haemagglutination-inhibiting antibody by the spike protein of avian infectious bronchitis virus. Avian Pathol. **13:**573–583.
9. **Collins, M. S., and D. J. Alexander.** 1980. Avian infectious bronchitis virus structural polypeptides: effect of different conditions of disruption and comparison of different strains and isolates. Arch. Virol. **63:**239–251.
10. **Davies, H. A., R. R. Dourmashkin, and M. R. MacNaughton.** 1981. Ribonucleoprotein of avian infectious bronchitis virus. J. Gen. Virol. **53:**67–74.
11. **Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson.** 1993. DNA vaccines: protective immunization by parenteral mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. USA **90:**11478–11482.
12. **Gotch, F. M., A. J. McMichael, G. L. Smith, and B. Moss.** 1986. HLA B37 determines an influenza A virus nucleoprotein epitope recognized by cytotoxic T lymphocytes. J. Exp. Med. **165:**1397–1406.
13. **King, D. J., and D. Cavanagh.** 1991. Infectious bronchitis, p. 471–484. *In* B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder (ed.), Iowa State University Press, Ames, Iowa.
14. **Kusters, J. G., E. J. Jager, J. A. Lenstra, G. Koch, W. P. A. Posthumus, R. H. Meloen, and B. A. M. Van Der Zeijst.** 1990. Sequence evidence of RNA recombination in field isolates of avian coronavirus infectious bronchitis virus. Vaccine **8:**605–608.
15. **Lambrechts, C., M. Pensaert, and R. Ducatelle.** 1993. Challenge experiments to evaluate cross-protection induced at the trachea and kidney level by vaccine strains and Belgian nephropathogenic isolates of avian infectious bronchitis virus. Avian Pathol. **22:**577–590.
16. **Lenstra, J. A., J. G. Kusters, G. Koch, and B. A. M. Van Der Zeijst.** 1989. Antigenicity of the peplomer protein of infectious bronchitis virus. Mol. Immunol. **26:**7–15.
17. **Lominiczi, B.** 1977. Biological properties of avian coronavirus RNA. J. Gen. Virol. **36:**531–533.
18. **Luckacher, A. E., V. L. Braciale, and T. J. Braciale.** 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. J. Exp. Med. **160:**814–826.
19. **MacNaughton, M. R., and J. H. Madge.** 1977. The polypeptide composition of avian infectious bronchitis virus particles. Arch. Virol. **55:**47–54.
20. **Manickan, E., R. J. D. Rouse, Z. Yu, W. Wire, and B. T. Rouse.** 1995. Genetic immunization against herpes simplex virus. Protection is mediated by CD4 T lymphocytes. J. Immunol. **155:**259–265.
21. **Offit, P. A., and K. I. Dudzik.** 1988. Rotavirus-specific cytotoxic T lymphocytes cross-react with target cells infected with different rotavirus serotypes. J. Virol. **62:**127–131.
22. **Pardoll, D. M., and A. M. Beckerleg.** 1995. Exposing the immunology of naked DNA vaccines. Immunity **3:**165–169.
23. **Puddington, L., M. J. Bevan, J. K. Rose, and L. Lefrancois.** 1986. N protein is the predominant antigen recognized by vesicular somatitis virus-specific cytotoxic T cells. J. Virol. **60:**708–717.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Schochetman, G., R. H. Stevens, and R. W. Simpson.** 1977. Presence of infectious polyadenylated RNA in the coronavirus avian bronchitis virus. Virology **77:**772–782.
26. **Seo, S. H., and E. W. Collisson.** 1997. Specific cytotoxic T lymphocytes are involved in in vivo clearance of infectious bronchitis virus. J. Virol. **71:**5173–5177.

26a. **Seo, S. H., and E. W. Collisson.** Unpublished data.

27. **Siddell, S. G., H. Wege, and V. Ter Meulen**. 1983. The biology of coronaviruses. J. Gen. Virol. **64:**761–776.
28. **Sneed, L., G. Butcher, L. Wang, R. Parr, and E. W. Collisson.** 1989. Comparisons of the structural proteins of avian bronchitis virus as determined by western blot analyses. Viral Immunol. **2:**221–227.
29. **Stern, D. F., and B. M. Sefton.** Coronavirus proteins: biogenesis of avian coronavirus bronchitis virus. 1982. J. Virol. **44:**794–803.
30. **Stohlman, S. A., C. C. Bergmann, D. Cua, R. van der Veen, and D. R. Hinton.** 1995. Mouse hepatitis virus nucleocapsid-specific cytotoxic T cells protect from lethal infection without eliminating virus from oligodendroglia. J. Virol. **69:**684–694.
31. **Wang, B., J. D. Boyer, V. Srikantan, L. Coney, R. Carrano, C. Phan, M. Merva, K. Dang, M. Agadjanyan, L. Gilbert, K. Ugen, V. W. Williams, and D. B. Weiner.** 1993. DNA inoculation induces neutralizing immune responses against human immunodeficiency virus type I in mice and nonhuman primates. DNA Cell Biol. **12:**799–805.
32. **Wang, L., D. Junker, and E. W. Collisson.** 1993. Evidence of natural recombination within the S1 gene of infectious bronchitis virus. Virology **192:**710–716.
33. **Williams, A. K., L. Wang, L. W. Sneed, and E. W. Collisson.** 1992. Comparative analyses of the nucleocapsid genes of several strains of infectious bronchitis virus and other coronaviruses. Virus Res. **25:**213–222.