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Adoptive Transfer of Infectious Bronchitis Virus Primed $\alpha\beta$ T Cells Bearing CD8 Antigen Protects Chicks from Acute Infection

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Infectious bronchitis virus (IBV) infection and associated illness may be dramatically modified by passive transfer of immune T lymphocytes. Lymphocytes collected 10 days postinfection were transferred to naive chicks before challenge with virus. As determined by respiratory illness and viral load, transfer of syngeneic immune T lymphocytes protected chicks from challenge infection, whereas no protection was observed in the chicks receiving the MHC compatible lymphocytes from uninfected chicks. Protection following administration of T lymphocytes could be observed in chicks with three distinct MHC haplotypes: B^8/B^8, B^{12}/B^{12}, and B^{19}/B^{19}. Nearly complete elimination of viral infection and illness was observed in chicks receiving cells enriched in $\alpha\beta$ lymphocytes. In contrast, removal of $\gamma\delta$ T lymphocytes had only a small effect on their potential to protect chicks. The adoptive transfer of enriched $CD8^+$ or $CD4^+$ T lymphocytes indicated that protection was also a function primarily of CD8-bearing cells. These results indicated that $\alpha\beta$ T lymphocytes bearing $CD8^+$ antigens are critical in protecting chicks from IBV infection.

Key Words: IBV; coronavirus; protection; immune T lymphocytes; adoptive transfer; MHC haplotype.

INTRODUCTION

Infectious bronchitis virus (IBV) is a prototype of the *Coronaviridae* family (Collisson *et al.,* 1992; Siddell *et al.,* 1983). Infection with IBV causes a highly contagious, respiratory disease in chickens with high mortality in young chicks (Alexander *et al.,* 1978; Crinion and Hofstad, 1972; Hofstad, 1984). In addition to the upper and lower respiratory tract, IBV affects the genital and urinary systems and has been isolated from many tissues, including the lung, trachea, cecal tonsils, kidneys, Harderian (paraocular) glands, and spleen (Alexander *et al.,* 1978; Crinion and Hofstad, 1972; Hofstad, 1984). In laying flocks, IBV infection can cause complete cessation of egg production or production of thin-shelled, rough, and misshapen eggs, and in broilers, infection can result in decreases in feed consumption.

Cytotoxic T lymphocytes (CTL) specific for IBV, associated with the control of acute infection in chicks, have been shown *in vitro* to be MHC restricted and mediated primarily by cells with the CD8 phenotype (Seo and Collisson, 1997, 1998). Initial elimination of virus following infection has been associated with this CTL response (Seo and Collisson, 1997). Furthermore, the nucleocapsid polypeptide, known to induce CTL activity, could protect chicks from IBV challenge, when expressed *in vivo* by a eukaryotic expression plasmid (Seo *et al.,* 1997). Protection occurred in the absence of neutralizing antibody, which is induced later during a viral infection by the spike, not the nucleocapsid, protein (Ignjatovic and Galli, 1994).

Adoptive transfer of immune lymphocytes has been used to evaluate their role in protecting mice from severe viral infections (Offit and Dudzik, 1990; Cannon *et al.,* 1987; Klavinskis *et al.,* 1989). For example, suckling mice were protected from murine rotavirus-induced gastroenteritis after adoptive transfer of splenic lymphocytes from immunized animals. Protection against disease occurred in the absence of rotavirus-specific neutralizing antibodies in the sera of suckling mice (Offit and Dudzik, 1990). In a study with respiratory syncytial virus (RSV), primed RSV-specific immune T cells collected between days 5 and 8 of infection cleared the lungs of nude and irradiated mice of RSV within 10 days of transfer (Cannon *et al.,* 1987). When adoptively transferred at the time of infection, MHC Class I restricted CTL protected mice from lymphocytic choriomeningitis virus infection (Klavinskis *et al.,* 1989).

The chicken is an especially appealing model for doing adoptive transfer studies. In addition to the ease of synchronizing hatching and maintaining these animals, a number of stocks are available with well-defined MHC haplotypes (Briles and Briles, 1982, 1987) and recombinants within the MHC chromosomal region (Miller *et al.,* 1988; Aeed *et al.,* 1993; Golemboski *et al.,* 1995). In this

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TABLE 1

Effects of Adoptive Transfer of Syngeneic Lymphocytes on Acute Respiratory Illness 5 Days p.i. with IBV

Transferred lymphocytes	−[a]	+	++	+++
None	0	0	5	0
Nonimmune	0	0	5	0
Immune, not depleted*	4	2	0	0

Note. Data are representative of three experiments.

[a] Number of B^8/B^8 chicks with observed respiratory illness, scored as: −, no illness; +, sneezing or nasal discharge; ++, plus dyspnea or rales; +++, plus lack of appetite or pale color ataxia. Five or six chicks per group.

*Statistically significant ($P < 0.05$) by analysis of variance.

article, the role of T lymphocytes with CTL activity in protecting chicks against acute IBV infection was confirmed through the adoptive transfer of immune cells to naive syngeneic chicks prior to challenge. The present study also investigated the *in vivo* role of subtypes of T lymphocytes in protecting chicks from IBV infection. The transfer of $\alpha\beta$ T lymphocytes bearing CD8 antigens protected chicks from acute IBV infection.

RESULTS

Adoptive transfer of immune T lymphocytes protected chicks against IBV infection. Previous studies indicated that maximum CTL activity could be observed in lymphocytes prepared from spleens of chicks, 10 days postinfection (p.i.) with the Gray strain of IBV. The elimination of virus during acute infection was found to be closely correlated with CTL activity, and to a lesser extent with the presence of IgM antibody, but not with the presence of IgG antibody. The correlation of CTL with elimination of virus *in vivo* was further investigated through transfer of 4×10^6 immune (group 1) or nonimmune (group 2) lymphocytes to naive 5-day-old B^8/B^8 chicks, 1 day prior to infection with IBV. A third group with age-matched birds received PBS in the absence of lymphocytes. After challenge with the Gray strain, the effects of lymphocyte transfer on associated respiratory illness were examined for 5 days. The transfer of immune cells delayed the onset of clinical illness, reduced the number of chicks infected, and reduced the clinical illness observed (Table 1). On day 3, only two of six chicks displayed any signs of respiratory illness and they experienced only mild signs of occasional sneezing. In contrast, five of five chicks receiving normal lymphocytes or four of five inoculated with only buffer showed mild clinical signs of respiratory illness by day 2. On days 3, 4, and 5, all chicks inoculated with only buffer or with nonimmune lymphocytes were sneezing, in addition to experiencing more severe signs of tracheal and lung congestion as indicated by observed rales.

The effects of immune lymphocytes on viral replication

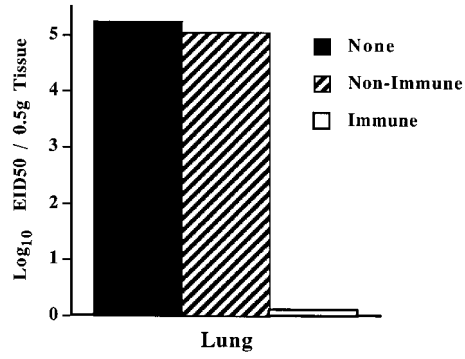

FIG. 1. Effects of transferred immune cells on viral titers in the lungs of chicks 5 days after infection with IBV. Transferred lymphocytes were collected from the spleens of 38-day-old chicks 10 days p.i. or from uninfected age-matched chicks. T lymphocytes were transferred to naive chicks 1 day before challenge with IBV. The mean titer of five animals/group is shown. The standard deviation of the mean for chicks receiving no lymphocytes and that for chicks receiving nonimmune lymphocytes were both 0.17. No virus was detected in the lungs of chicks receiving immune lymphocytes. The results are representative of three experiments.

were determined in lungs collected at 5 days p.i. No virus was detected in the lungs of chicks given immune lymphocytes, whereas the viral load in lungs from the chicks receiving nonimmune lymphocytes or buffer alone prior to infection was $10^{5.06}$ and $10^{5.24}$ EID_{50}, respectively, per 0.5 g of tissue (Fig. 1). Transfer of immune lymphocytes protected chicks against clinical illness and viral replication in the lungs. Because lymphocytes from uninfected chicks had no impact, protection was not the result of providing a more mature population of T lymphocytes.

Varying doses of immune lymphocytes were given to B^{19}/B^{19} chicks 1 day prior to infection with IBV. Protection from transferred immune lymphocytes at 5 days p.i. is shown in Table 2. Protection was negligible or marginal when chicks were given 5×10^4 (data not shown) or 10^5 lymphocytes, respectively.

TABLE 2

Effects of Dose of Immune Lymphocytes on the Number of Chicks Displaying Respiratory Illness 5 Days Following IBV Infection[a]

Transferred lymphocytes[b]	Cells given per bird	Clinical illness		
		−	+	++
PBS	0	0	1	4
Immune	10^5	0	4	1
Immune	10^6	4	1	0
Immune	10^7	5	0	0

Note. Significant differences ($P < 0.05$) were determined between chicks given 0 or 10^5 and chicks given 10^6 or 10^7. The results are representative of two experiments.

[a] B^{19}/B^{19} chicks; five chicks/group.

[b] Syngeneic lymphocytes.

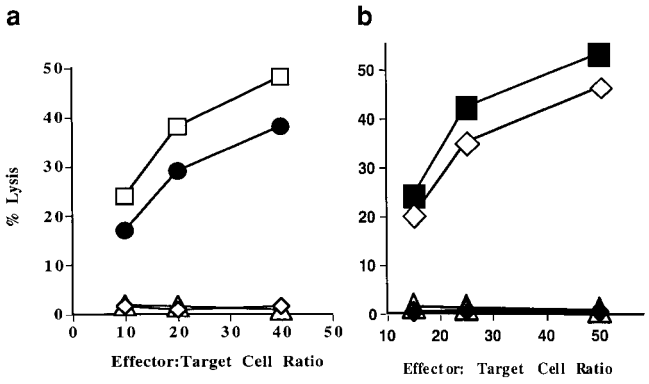

FIG. 2. IBV-specific CTL activity of fractionated immune T lymphocytes following *in vitro* stimulation with IBV-specific APC. T lymphocytes were prepared from the spleens of 38-day-old chicks, 10 days p.i. with IBV, and cultured for 10 days *in vitro* with irradiated, IBV-infected CK cells. (a) Cytolytic activity determined following depletion of CD8 or CD4 lymphocytes. Closed circles represent lysis of target cells by undepleted effector cells; open squares represent lysis of target cells by effector cells depleted of CD4 T cells; open diamonds represent lysis of target cells by effector cells depleted of CD8 T cells, and open triangles represent lysis of uninfected target cells by undepleted T cells. (b) Responses of cells depleted of $\alpha\beta$ or $\gamma\delta$ lymphocytes. Open diamonds represent lysis of target cells by undepleted T cells; closed squares represent lysis of target cells by effector cells depleted of $\gamma\delta$ T cells; closed diamonds represent lysis of target cells by effector cells depleted of $\alpha\beta$ T cells, and open triangles represent lysis of uninfected target cells by undepleted T cells. The results are representative of three experiments.

Cytolytic responses following APC stimulation depended on CD8$^+$ and $\alpha\beta$ T lymphocytes. Before the phenotypes of T lymphocytes responsible for *in vivo* decreases in viral replication were determined, it was necessary to increase the numbers by stimulating immune lymphocytes in the presence of IBV-infected and irradiated APC. Because the available cultured chicken kidney (CK) cells used as stimulators originated from B^{12}/B^{12} Hy-VAC chicks, effector immune T lymphocytes were prepared from B^{12}/B^{12} chicks. Following stimulation, cells were separated by depletion of CD8-, CD4-, TCR1 ($\gamma\delta$)-, or TCR2 ($\alpha\beta$)-bearing cells using mouse monoclonal antibody. The purity of the depleted cells was determined by flow cytometry. The purities of the CD8$^+$ enriched and CD4$^+$ enriched T lymphocytes were 96 and 95%, respectively.

IBV-specific CTL activity of the *in vitro* stimulated and depleted cell preparations was verified. The nondepleted immune lymphocytes or preparations depleted of CD4$^+$ cells lysed target IBV-infected CK cells in a dose-dependent manner, whereas the cytolytic activity of effector cells depleted of CD8$^+$ T lymphocytes was negligible (Fig. 2a). At an effector:target cell ratio of 40:1, about 48% killing was observed with effectors enriched in CD8$^+$ lymphocytes. Lysis from unfractionated effectors was about 10% less at the same effector:target cell ratio. Uninfected target CK cells were not killed with immune unfractionated lymphocytes.

CTL responses were observed with immune lymphocytes depleted of $\gamma\delta$ T cells and with undepleted immune effector cells. Responses were dose-responsive with approximately 50% lysis at an effector:target ratio of 50:1. In contrast, lysis of IBV-infected target cells by effector cells depleted of $\alpha\beta$ T cells or lysis of uninfected target cells by effector cells from unfractionated lymphocyte preparations was undetectable (Fig. 2b).

Adoptive transfer of immune CD8 T cells protected chicks from acute IBV infection. Stimulated T cells depleted of CD8$^+$ or CD4$^+$ cells (1×10^7) were transferred to naive chicks before challenge with IBV. Clinical signs of respiratory illness were minimal in chicks receiving the immune cells depleted of CD4$^+$ T lymphocytes. In contrast, chicks receiving immune lymphocytes depleted of CD8$^+$ cells presented clinical signs that were similar to those of control infected chicks that did not receive immune lymphocytes (Table 3). Virus was not detected in either lungs or kidneys of chicks receiving immune T lymphocytes depleted of CD4$^+$ cells. In contrast, viral titers in both lungs and kidneys of chicks receiving immune T cells depleted of CD8$^+$ T cells were not significantly reduced ($P > 0.05$) compared to infected control chicks (Fig. 3). CD8$^+$ T lymphocytes are critical in protecting chicks from IBV infection.

Adoptive transfer of immune $\alpha\beta$ T cells, not $\gamma\delta$ T cells, protected chicks from acute IBV infection. Similarly, 10^7 immune lymphocytes depleted of cells bearing TCR1 ($\gamma\delta$) or TCR2 ($\alpha\beta$) antigens were inoculated in chicks (five per group) prior to challenge with virus. Following challenge, chicks receiving immune lymphocytes depleted of $\gamma\delta$ T cells suffered only minor respiratory illness, whereas

TABLE 3

Phenotypes of Transferred T Lymphocytes Reducing Acute Respiratory Illness 5 Days p.i. with IBV

Transferred lymphocytes	−[a]	+	++	+++
None	0	0	2	3
Immune CD4 depleted[b]	4	1	0	0
Immune CD8 depleted	0	0	3	2
None	0	0	2	3
Immune $\gamma\delta$ depleted[c]	3	2	0	0
Immune $\alpha\beta$ depleted	0	1	2	3

Note. The results are representative of three experiments.

[a] Number of B^{12}/B^{12} chicks with observed respiratory illness, scored as: −, no illness; +, sneezing or nasal discharge; ++, plus dyspnea or rales; +++, plus lack of appetite or pale color ataxia. Five or six chicks per group.

[b] Statistically significant difference ($P < 0.05$) from chicks not receiving transferred lymphocytes and from those receiving CD8-depleted lymphocytes.

[c] Statistically significant difference ($P < 0.05$) from chicks not receiving transferred lymphocytes and from those receiving $\alpha\beta$-depleted lymphocytes.

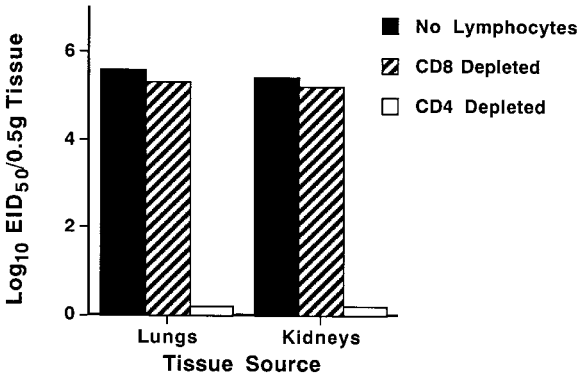

FIG. 3. Viral titers in lungs and kidneys of chicks receiving the immune T cells depleted of $CD4^+$ or $CD8^+$ T cells before challenge with IBV. Viral replication was determined in kidneys and lungs collected and pooled from each group of five chicks. The viral load of chicks that did not receive lymphocytes is shown by the black bar, that of chicks that received lymphocytes depleted of $CD8^+$ lymphocytes is shown by the hatched bar, and that of chicks receiving lymphocytes depleted of $CD4^+$ cells is shown by the white bar. Standard deviations were less than 0.5. The results are representative of three experiments.

chicks receiving immune cells depleted of $\alpha\beta$ T cells displayed clinical signs that were similar to those in IBV-infected chicks not given immune lymphocytes (Table 3). The viral titers in lungs and kidneys of chicks receiving immune cells depleted of either $\alpha\beta$ or $\gamma\delta$ T lymphocytes were also examined. Viral replication in both lungs and kidneys from infected chicks having received immune cells depleted of $\alpha\beta$ T cells was not significantly different ($P > 0.05$) from that in control infected chicks receiving only buffer, while no virus was detected in chicks given lymphocytes depleted of $\gamma\delta$ cells (Fig. 4). Therefore, $\alpha\beta$ T lymphocytes are capable of controlling IBV infection and associated disease.

DISCUSSION

The capacity for transferred syngeneic immune T lymphocytes to protect birds from challenge infection further indicated that T lymphocytes play a critical role in resolving acute IBV infection. Consistent with observations that the kinetics of MHC restricted CTL response can be associated with decreases in viral load, $CD8^+$ lymphocytes were mostly responsible for the observed protection. In addition, cells that play a critical role in resolving early IBV infection in chicks were TCR2-bearing ($\alpha\beta$) lymphocytes. Thus, the transfer of immune cells depleted of $CD4^+$ T cells or $\gamma\delta$ T cells protected chicks from acute IBV infection.

Antibody responses do not appear to participate in the initial elimination of viral replication following infection, although their control of chronic infection is probably critical (Ignjatovic and Galli, 1994; Seo and Collisson, 1997). Cook *et al.* (1991) also showed that *in ovo* bursectomy did not appear to increase the level of mortality or early control of IBV replication in leghorn chickens although bursectomized chicks experienced longer lasting infection and illness than intact chicks. The cellular immune responses were not evaluated. However, we have found that bursectomy does not decrease, but may actually increase, the IBV-specific CTL response (Seo and Collisson, unpublished data).

In evaluating the role of T lymphocytes in murine hepatitis virus (MHV) infection in the central nervous system, the adoptive transfer of both a $CD8^+$ T cell clone and a $CD4^+$ T cell clone was shown to suppress viral growth and viral antigen-positive cells in the brain (Yamaguchi *et al.,* 1988). In addition, the transfer of MHV nucleoprotein-specific CTL protected mice from a subsequent lethal MHV challenge by reducing virus replication within the central nervous system (Stohlman *et al.,* 1995). Transfer of these CTL directly into the central nervous system was at least 10-fold more effective than peripheral transfer. Histological analysis demonstrated that CTL reduced virus replication in ependymal cells, astrocytes, and microglia. However, $CD4^+$ T lymphocytes do not appear to play a critical role in resolving the viral load after infection with IBV in chicks. Therefore, the impact of various cellular immune responses to distinct coronaviruses differs, depending on the host and virus.

The distribution of chicken $\alpha\beta$ and $\gamma\delta$ T lymphocytes can be differentiated in developing chicks and chick embryos (Chen *et al.,* 1988, 1990). The $\gamma\delta$ T lymphocytes appear earlier and migrate more quickly from thymic cortex to medulla and to peripheral lymphoid tissues and differ in their composition of CD4- and CD8-bearing cells compared to mammalian $\gamma\delta$ T cells (Bucy *et al.,* 1988, 1990; George and Cooper, 1990). Greater numbers of T lymphocytes in chickens are $\gamma\delta$ cells than in mammals. Thirty percent of the splenic T lymphocytes in the

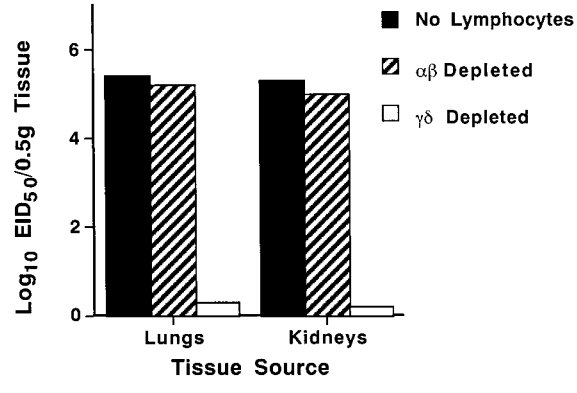

FIG. 4. Viral titers of IBV target organs from chicks receiving immune cells depleted of $\gamma\delta$ or $\alpha\beta$ T cells before challenge with IBV. Viral replication was determined in kidneys and lungs collected and pooled from each group of five chicks. The viral load of chicks that did not receive lymphocytes is shown by the black bar, that of chicks that received lymphocytes depleted of $\alpha\beta^+$ lymphocytes is shown by the hatched bar, and that of chicks receiving lymphocytes depleted of $\gamma\delta^+$ cells is shown by the white bar. Standard deviations of viral titers were less than 0.5. The results are representative of three experiments.

chicken have the γδ, CD3 phenotypes (Bucy *et al.,* 1988; Sowder *et al.,* 1988). Avian γδ T cells are found predominantly in sinusoid areas of the spleen and intestinal epithelium, whereas αβ T cells reside primarily in the splenic periarterial sheath (Vainio *et al.,* 1990). The mammalian γδ lymphocytes, displaying less diversity than αβ T cells, are present in smaller numbers in the peripheral lymphoid tissues, but are relatively abundant in the epithelia of the intestines, skin, and reproductive organs, such as the uterus and vagina (Durum *et al.,* 1998; Guehler *et al.,* 1999; Livak *et al.,* 1999; Salerno and Dieli, 1998; Zorbas and Scollay, 1995).

The mammalian γδ T cells appear to play a protective role in early infection with intracellular bacteria, such as *Listeria monocytogenes* (Hasegawa *et al.,* 1992), *Mycobacterium bovis* (Inoue *et al.,* 1991), and *Salmonella cholerasuis* (Emoto *et al.,* 1992). Murine γδ T lymphocytes were prevalent within inflammatory lesions late, rather than early, in the course of pneumonia caused by influenza virus A (Carding *et al.,* 1990) or Sendai virus (Hou *et al.,* 1992) and might, therefore, be important for the resolution of the inflammatory process. In mice infected with Sendai virus (Ogasawara *et al.,* 1994), the time course of virus propagation was immediately and coincidentally followed by the developmental course of γδ T cells. In our study, γδ T lymphocytes do not appear to be important in resolving IBV infection in chicks, although we cannot rule out the possibility that they produce cytokines that influence αβ T cell function. Human γδ T lymphocytes from peripheral blood lymphocytes of healthy donors secreted IL-2 following exposure to mycobacteria (Munk *et al.,* 1990). Consistent with IBV infection in chickens, the CTL response to reticuloendotheliosis virus infection in chickens was mediated by αβ, but not by γδ, T cells (Merkle *et al.,* 1992). Because avian γδ cells represent a large proportion of T lymphocytes in peripheral organs in the chicken, the actual role of γδ T cells in chicks infected with avian viruses should be further examined.

The chicken is proving to be a rewarding model in examining the role of CTL in the pathogenesis and control of viral infection. Ongoing studies making use of αβ, $CD8^+$ T lymphocytes from chicks exposed to IBV are critical in determining factors that protect chickens against IBV infection. Future experiments will utilize available MHC recombinants. Because cells with the MHC restricted capacity to protect against challenge virus correlated with the CTL response, it may be suggested that the major mechanism for control of acute infection is due to $CD8^+$, MHC restricted αβ T lymphocytes. Because IBV-specific CTL tend to recognize epitopes conserved among serologically distinct strains (Seo *et al.,* 1997), mechanisms that induce CTL should be exploited with the development of effective vaccines.

MATERIALS AND METHODS

Experimental animals. Chicks used in these studies were Hy-VAC B^{12}/B^{12} (Hy-VAC, Adel, IA) as well as B^{8}/B^{8} B^{19}/B^{19} from the Department of Biological Sciences, Northern Illinois University (DeKalb, IL) (Briles and Briles, 1982). Chicks were hatched in our laboratory and housed in a specific, pathogen-free environment at the Laboratory Animal and Resources Facility (Texas A & M University, College Station, TX).

Viral inoculations. The nephrotrophic Gray strain of IBV, used as the source for viral inoculation, was propagated by inoculating the allantoic sac of 11-day-old chicken embryos and harvesting the allantoic fluid 36 h p.i. (Sneed *et al.,* 1989).

Preparation of donor lymphocytes. Chicks at the age of 28 days were infected with 10^7 EID_{50} of the Gray strain of IBV. Splenocytes were collected from IBV-infected chicks at 10 days p.i. Control donor cells were prepared from age-matched uninfected chicks. Lymphocytes were separated with a Ficoll-Hypaque density gradient (Histopaque 1.077; Sigma, St. Louis, MO), adherent cells were removed by incubation on plastic petri dishes, and B lymphocytes were depleted using nylon wool columns (Seo and Collisson, 1997). T-cell-enriched preparations were transferred directly to naive chicks or were first stimulated *in vitro* for 10 days with antigen-presenting cells (APC) in RPMI with 10% chicken serum, 50 μg/ml gentamycin, 5×10^{-5} M 2-mercaptoethanol, and 2 mM L-glutamine from Gibco BRL (Grand Island, NY). IBV-infected CK cells (Seo and Collisson, 1997), irradiated with 10,000 rad, were used as APC.

Inoculation of donor lymphocytes and challenge virus. Each group consisted of at least five chicks. Immune or nonimmune donor lymphocytes were given at 5 days of age through a wing vein. One day after receiving donor lymphocytes, chicks were challenged with 2 to 4×10^6 EID_{50} of the Gray strain of IBV by the nasal–ocular route. Birds were observed twice each day for clinical signs of illness, such as coughing, sneezing, rales, or dyspnea. Statistical differences in illness were determined by analysis of variance (Ott, 1984). Viral load was evaluated in lungs and kidneys collected 5 days p.i. (Seo and Collisson, 1997).

Depletion of T lymphocyte subtypes. Stimulated T cell subtypes were enriched, after B cells and adherent cells were removed, with antisera specific for CD4, CD8, TCR1, or TCR2 (Seo and Collisson, 1997). Briefly, tissue culture flasks were coated with goat anti-mouse IgG. The immune T cells were labeled with mouse anti-chicken CD4, CD8, TCR1, or TCR2 monoclonal antibody (Southern Biotechnology Associates, Birmingham, AL) before being added to IgG-coated plates and being incubated at room temperature for 30 min. The suspensions were swirled gently before being incubated for an additional 30 min at room temperature. Nonadherent cells were decanted

from the plates following gentle agitation, centrifuged for 10 min at 600 *g,* and resuspended in PBS before being transferred to chicks.

CTL assay. In vitro stimulated T lymphocytes depleted of T cell subtypes were tested for CTL activity using target CK cells infected with the Gray strain of IBV as described previously (Seo and Collisson, 1997; Seo *et al.,* 1997). Target cells were labeled with ^{51}Cr for 90 min before being washed and mixed with effector lymphocytes (Seo and Collisson, 1997). Varying amounts of effector cells in 100 μl of RPMI with 10% chicken sera were added to each well in 96-well round-bottom microtiter plates, and 100 μl of 2×10^4 of ^{51}Cr-labeled target cells that were infected with IBV or uninfected was added to each well. Microtiter plates were centrifuged at 100 *g* for 5 min before being incubated for 4 h at 40°C. After being centrifuged at 600 *g* for 10 min, 100 μl of the supernatants in triplicate samples was collected for determining ^{51}Cr release using a gamma radiation counter (Cobra Autogamma, Canberra Packard). The percentage of specific lysis was calculated as $(E\text{-}S/M\text{-}S) \times 100$, 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 ^{51}Cr release of target cells was less than 15% of the maximum release.

Determination of viral load in chicken lungs or kidneys. Lungs and kidneys were collected and pooled from syngeneic chicks in each group (Seo and Collisson, 1997). Tissues from the chicks in each group were pooled before being weighed and homogenized. A 10% w/v tissue suspension was prepared in Eagle's MEM containing 200 U/ml of penicillin and 200 μg/ml streptomycin. After standing for 90 min at 4°C, cells were pelleted by centrifuging for 20 min at 2000 *g* and serial 10-fold dilutions of supernatants were prepared in Eagle's MEM. One-tenth of a milliliter of each virus dilution was injected into the allantoic sac of six 11-day-old embryonated chicken eggs (Cook, 1983). The eggs were sealed with collodion before being incubated at 38°C for 5 days and then the embryos were examined for dwarfing by weighing. EID_{50} was calculated by the Reed and Muench method (Burleson *et al.,* 1992).

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