Natural killer cell activity of chicken intraepithelial leukocytes against rotavirus-infected target cells

T.J. Myers and K.A. Schat*

Department of Avian and Aquatic Animal Medicinie, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, U.S.A.

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ABSTRACT

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Intraepithelial leukocytes (IEL) and splenocytes collected from uninfected and rotavirus-infected chickens were evaluated for cytotoxic activity against a natural killer (NK) cell-susceptible lymphoblastoid cell line (LSCC–RP9) and against rotavirus-infected chick kidney cells in 4-h chromium-release assays. Both splenocytes and IELs from uninfected and rotavirus-infected chickens were cytotoxic for LSCC-RP9, and the levels of this NK cell activity were not altered by infection of the host with rotavirus. IELs but not splenocytes from uninfected and rotavirus-infected chickens were cytotoxic for rotavirus-infected but not for uninfected chick kidney cell targets. Because this cytotoxic activity was not induced nor altered by rotavirus infection of the host, and was not major histocompatibility complex-restricted, it was considered to be due to NK cell activity. The cytotoxicity of IELs against rotavirus-infected target cells was dose-dependent; however, there was some suppression of cytotoxic activity at high effector to target cell ratios. There were no differences in the cytotoxic activities of IELs collected from the duodenum versus the jejunum. The in vitro cytotoxic activity of IELs against rotavirus-infected target cells suggested that NK cell activity may be an important immune response to rotavirus infections in vivo. The absence of cytotoxic activity by splenocytes against rotavirus-infected target cells indicated that there may be different subpopulations of NK cells in the spleen and intestinal epithelium of chickens.

ABBREVIATIONS

CKC; chick kidney cell; CRA; chromium-release assay; DPI; days post inoculation; E:T; effector to target cell ratio; FBS; fetal bovine serum; IEL; intraepithelial leukocytes; MHC; major histocompatibility complex; NK; natural killer; PBS; phosphate-buffered saline; SPF; specific-pathogen-free; $TCID_{50}$; tissue culture infectious dose–50%.

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*To whom correspondence should be addressed.

INTRODUCTION

The immune response to rotavirus infections has been the subject of much study since the recognition of the etiological significance of rotaviruses in human and domestic animal diarrheas (Kurstak et al., 1981). Most of these studies have examined antibody-mediated responses. In mammals, rotavirus-specific intestinal antibodies clearly mediate passive immunity (Offit and Clark, 1985; Archambault et al., 1988), and are thought to be important in the active immune response to primary (Sheridan et al., 1983; Van Zaane et al., 1986) and secondary (Bishop et al., 1986) rotavirus infections. However, there is evidence to suggest that mechanisms besides rotavirus-specific intestinal antibodies may participate in recovery and resistance. Clinical studies in humans show that the presence of rotavirus-specific fecal IgA does not always correlate with resistance to diarrhea following challenge (Bernstein et al., 1986). Also, studies in experimentally infected calves have shown that calves lacking coproantibodies to a heterologous secondary challenge virus can still be protected against developing diarrhea (Bridger and Oldham, 1987). Furthermore, neonatal nude (athymic) mice are able to recover normally from rotavirus infection despite the inability to mount a T cell or an intestinal antibody response (Eiden et al., 1986). Conversely, neonatal mice with severe combined immunodeficiency, which are also incapable of normal T and B cell responses, exhibit persistent rotavirus infections following inoculation (Riepenhoff-Talty et al., 1987).

A limited number of studies have examined non-antibody responses to rotavirus infections. Offit and Dudzik (1988) have demonstrated MHC-restricted, rotavirus-specific cytotoxic T cell activity in vitro using splenocytes from rotavirus-infected mice at 7 DPI. This cytotoxic activity was directed against target cells infected with rotaviruses either homotypic or heterotopic to the virus inoculum. Kohl et al. (1983) demonstrated that human interferon can induce in vitro NK cell activity of human peripheral blood leukocytes (collected from randomly selected individuals) against rotavirus-infected target cells. In calves, La Bonnardiere et al. (1981) showed that intestinal interferon is produced during rotavirus infection and Van den Broecke et al. (1984) found a correlation between high levels of intestinal interferon and reduced clinical disease. Thus, there is evidence to indicate that cytotoxic T cells, NK cells, and interferon may participate with intestinal antibodies in mediating rotavirus immunity.

Recent studies in our laboratory demonstrated that embryonally bursectomized (B cell-deficient) chickens showed a slightly delayed but complete recovery from, and resistance to, avian rotavirus infection (Myers and Schat, 1990), providing further evidence for non-antibody mechanisms of rotavirus immunity. This report characterizes the cytotoxic activity of intestinal IELs from rotavirus-infected chickens using a 4-h CRA.

MATERIALS AND METHODS

Virus

The Ch2 strain (Yason and Schat, 1985) of avian rotavirus was propagated and semipurified as described (Myers et al., 1989).

Birds

P2a and N2a strain SPF chickens homozygous for the MHC alleles B^{19} and B^{21}, respectively (Weinstock and Schat, 1987), were obtained from departmental flocks. Effector cell donor birds were P2a chickens inoculated either with 10^5 $TCID_{50}$ Ch2 rotavirus in 1 ml PBS, pH 7.2, by esophageal cannulation or with a sham inoculation of PBS alone. Infected and uninfected birds were housed in separate isolation units. At specified times following inoculation, serum samples and duodenal, splenic, and thymic tissues were collected. Exposure to infectious rotavirus was confirmed by evaluating serum samples for the presence of rotavirus-specific IgM in an enzyme-linked immunosorbent assay (Myers et al., 1989).

Effector cells

Duodenal and jejunal IELs were isolated by the procedure of Chai and Lillehoj (1988). Briefly, the duodenum or jejunum from each bird was removed, cut into 1 cm pieces, and incubated first with 5 mM dithiothreitol (to remove mucus) and then with 10^{-4} M EDTA (to remove the epithelial cells from the mucosal basement membrane). IELs were separated from this population of cells by passing the cell suspensions twice through a nylon wool column, followed by purification of the IELs on a discontinuous Percoll gradient. Thymocytes and splenocytes were isolated as described by Schat and Calnek (1980). All effector cells were suspended in LM-Hahn basal medium (Calnek et al., 1981) supplemented with 20% heat-inactivated FBS (LM20) prior to their addition to target cell cultures.

Target cells

Lymphoblastoid cell line targets: LSCC-RP9 is an avian leukosis virus-transformed lymphoblastoid cell line (MHC alleles B^2B^{15}) sensitive to in vitro NK cell lysis (Sharma and Okazaki, 1981). RECC-CU60 is a reticuloendotheliosis virus-transformed lymphoblastoid cell line (MHC alleles $B^{19}B^{19}$) refractory to in vitro NK cell lysis (Weinstock et al., 1989). These two cell lines were grown in LM-Hahn basal medium supplemented with 10% heat-inactivated FBS (LM10) at 41°C in a humidified atmosphere of 5% CO_2. Prior to their use in a CRA, these cells were centrifuged over Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and labeled with 300 μCi Na^{51}CrO$_4$ per 10^7 cells as described (Schat and Calnek, 1980). Labeled cells were resuspended in LM20 and dispensed to round-bottomed 96-well tissue culture plates

(Corning Glass Works, Corning, NY) at 5×10^4 cells per well just prior to the addition of effector cells.

CKC targets. The development of CKC targets was modeled after a procedure described by Offit and Dudzik (1988). Primary CKC cultures were established from 2- to 3-week-old SPF P2a and N2a chicks as described by Calnek and Madin (1969). The growth medium (M25) used for CKC cultures has been described (Myers and Schat, 1989). These cultures were trypsinized at 48 h in culture and frozen in liquid nitrogen for use as secondary CKCs. Twenty-four hours prior to their use in a CRA, an ampule of CKCs was thawed, and cells were counted, resuspended at 0.5×10^6 cells per ml in M25 medium, dispensed into a 96-well flat-bottomed tissue culture plate (Corning) at 200 μl per well, and allowed to grow at 38°C in a humidified atmosphere of 5% CO_2. The next day, cultures in a row of 12 wells were trypsinized and these cells were collected and counted to determine the number of target cells per well (counts averaged about 5×10^4 cells per well). The remaining wells were then washed twice in PBS, and inoculated with 7.5×10^5 $TCID_{50}$ Ch2 in 25 μl M20 (M25 without FCS) medium (or with 25 μl M20 medium alone) resulting in an average multiplicity of infection of 15:1. Prior to inoculation of cultures, the purified Ch2 virus had been diluted 1:3 in M20 supplemented with 5 μg/ml trypsin and incubated in a 37°C waterbath for 1 h. The cultures were incubated at 38°C for 1 h, the inoculum was aspirated, and, after washing once with PBS, the wells received 200 μl of M20. Each well then received 2.5 μCi $Na^{51}CrO_4$ in 10 μl M20 and the cultures were incubated at 38°C for 3 h (Schat and Heller, 1985). Wells were washed three times in M20 with 30 min between washes. Each well then received 100 μl LM20 just prior to the addition of effector cells. CKC cultures grown on glass coverslips in 24-well tissue culture plates (Corning) in parallel with the 96-well cultures and evaluated with a direct fluorescent antibody assay (Yason and Schat, 1985) demonstrated from 75 to 90% rotavirus-positive cells 5 h post inoculation when the CKC cultures were ready to receive the effector cell suspensions.

Chromium release assays

Effector cells were added to target cells at an effector to target cell (E:T) ratio of 50:1 unless otherwise noted. Effector cells added to CKC targets were centrifuged onto the CKC monolayers at a low speed for 1 min while effector cells added to suspensions of lymphoblastoid cell targets were not. Cultures were set up in duplicate or in triplicate and were incubated at 38°C for 4 h. Culture supernatant fluids and pellets were harvested separately and counted in a gamma counter as described (Weinstock et al., 1989). Percent release was calculated for each well as follows: 100 × (cpm of supernatant fluid)/ (cpm of supernatant fluid + cpm of pellet). Mean percent spontaneous release was determined to be the mean percent release of 12 wells containing

target cells without the addition of effector cells. The average spontaneous release values observed were for LSCC-RP9: 26.81%; uninfected P2a cells: 14.78%; rotavirus-infected P2a cells: 20.88%; uninfected N2a cells: 14.53%; rotavirus-infected N2a cells: 21.23%. Percent specific release was determined for duplicate or triplicate experimental wells as follows: mean percent release experimental – mean percent spontaneous release. Mean percent specific release values were compared using Student's t test (Bishop,1966).

Experimental design

Cytotoxic activity against lymphoblastoid cells. Duodenal IELs (experiment 1) and splenocytes (experiment 2) from four or five uninfected and four or five rotavirus-infected chickens were evaluated approximately every other day for 10 DPI in a CRA using LSCC-RP9 target cells. Thymocytes from uninfected birds, which have been shown to exhibit little or no NK cell activity against LSCC-RP9 (Sharma and Coulson, 1979) were used as negative controls in both experiments. Two additional control trials, each consisting of IELs from four uninfected and four rotavirus-infected (7 DPI) birds incubated either with LSCC-RP9 or RECC-CU60 target cells, were also performed.

Cytotoxic activity against CKC targets

In experiment 3, duodenal IELs from four uninfected and four rotavirus-infected birds were assessed approximately every other day for 8 DPI in a CRA against uninfected and rotavirus-infected P2a and N2a CKC targets. In experiment 4, duodenal IELs and splenocytes from five uninfected and five rotavirus-infected birds were evaluated at 5, 7, and 9 DPI in a CRA against uninfected and rotavirus-infected P2a CKC targets. Rotavirus-infected birds were re-inoculated with the virus at 14 DPI, and IELs and splenocytes from five uninfected and five rotavirus-infected birds were then evaluated at 16 and 19 DPI. In experiment 5, duodenal IELs collected from uninfected birds (two birds in trial 1 and four birds in trial 2) were tested at various E:T ratios against uninfected and rotavirus-infected P2a target cells. Experiment 6 compared the cytotoxic activities of IELs collected from the duodenum versus the jejunum of uninfected birds (four birds in each trial) in a CRA against uninfected and rotavirus-infected P2a CKC targets.

RESULTS

Cytotoxic activity against lymphoblastoid target cells

The ability of IELs and splenocytes from rotavirus-infected chickens to lyse a known avian NK cell target (LSCC-RP9) was assessed in experiments 1 and 2 (see Fig. 1). The cytotoxic activity of IELs and splenocytes from both uninfected and rotavirus-infected birds was significantly ($P < 0.05$) greater than that seen with thymocytes for all assay days. With the exception of IELs

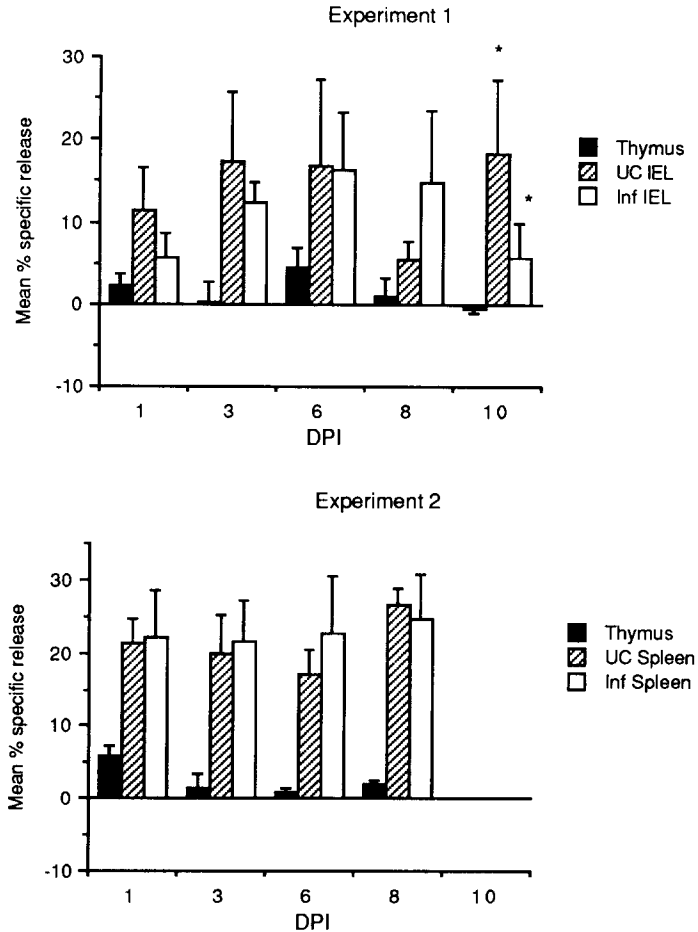## Experiment 1

Experiment 2

Fig. 1. Cytotoxic activity of thymocytes, IELs, and splenocytes against LSCC-RP9 target cells at an E:T ratio of 50:1. Each bar represents the mean percent specific release (± standard deviation) for five birds in experiment 1 and four birds in experiment 2. IELs and splenocytes were collected from uninfected control (UC) and rotavirus-infected (Inf) chickens in separate experiments, while thymocytes were collected from UC chickens in both experiments. *Significantly different at $P < 0.05$.

evaluated at 10 DPI, there was no significant difference between the mean % specific release values from uninfected versus infected birds at any DPI for IELs in experiment 1 or splenocytes in experiment 2. Cytotoxic activity was not detectable for IELs from uninfected or rotavirus-infected birds incubated with RECC-CU60 target cells (data not shown). Serum samples collected from birds at 10 and 8 DPI in experiments 1 and 2, respectively, were tested for rotavirus-specific IgM. These assays demonstrated rotavirus-specific IgM re-
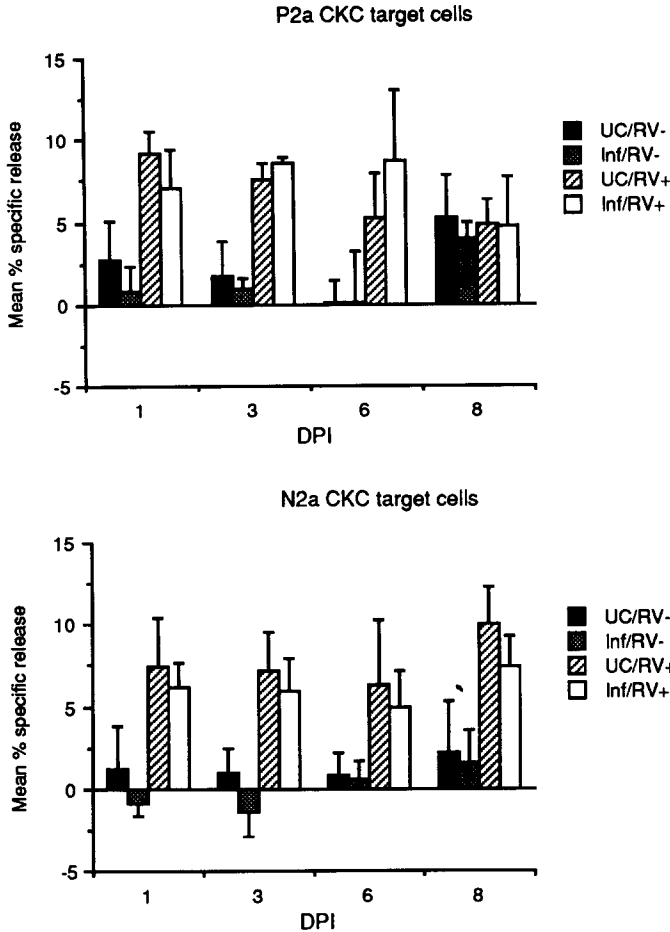
P2a CKC target cells

N2a CKC target cells

Fig 2. Cytotoxic activity of IEL from uninfected control (UC) and rotavirus-infected (Inf) P2a chickens against uninfected (RV−) and rotavirus-infected (RV+) P2a and N2a chick kidney cell (CKC) targets at an E:T ratio of 50:1. Each bar represents the mean percent specific release (±standard deviation) for four birds.

sponses for the rotavirus-inoculated birds but not for the uninoculated control birds.

Cytotoxic activity against CKC targets

The cytotoxic activity of IELs and splenocytes for rotavirus-infected CKC targets was evaluated in experiments 3 through 6. Experiment 3 (Fig. 2) demonstrated that IELs from both uninfected and rotavirus-infected P2a chickens were cytotoxic for both P2a and N2a rotavirus-infected target cells, but not for uninfected target cells. The mean percent specific release values for rotavirus-infected target cells was significantly greater ($P<0.05$) than for un-
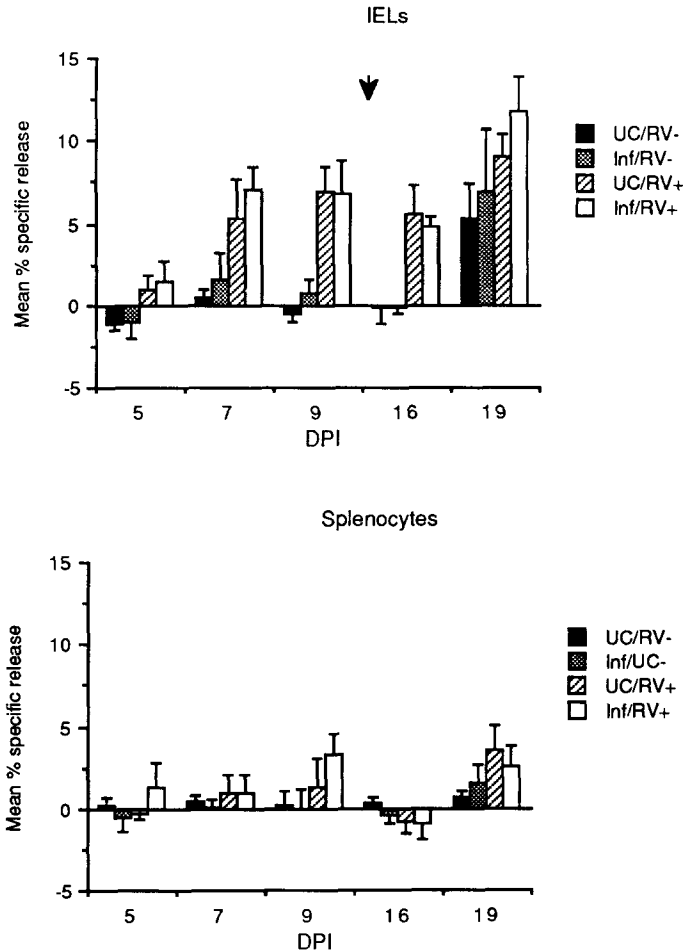
IELs

Splenocytes

Fig. 3. Cytotoxic activity of IELs and splenocytes from uninfected control (UC) and rotavirus-infected (Inf) P2a chickens against uninfected (RV−) and rotavirus-infected (RV+) P2a chick kidney cell targets at an E:T ratio of 50:1. Each bar represents the mean percent specific release (±standard deviation) for four birds. Arrowhead indicates rotavirus-infected birds were reinoculated at 14 DPI.

infected target cells in each assay except for P2a target cells at 8 DPI. The levels of cytotoxic activity induced by IELs from uninfected versus rotavirus-infected birds were not significantly different at any DPI for either P2a or N2a rotavirus-infected target cells. Serum samples collected at 8 DPI showed that the rotavirus-inoculated birds were positive for rotavirus-specific IgM while the uninoculated control birds were not. Experiment 4 (Fig. 3) showed that while IELs from uninfected and rotavirus-infected P2a chickens were cytotoxic for rotavirus-infected P2a CKCs, splenocytes from uninfected and rotavirus-infected chickens were not. The mean percent specific release val-
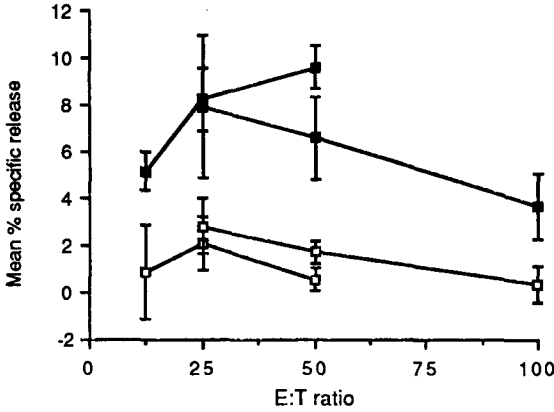
Fig. 4. Cytotoxic activity of IEL from uninfected P2a chickens against uninfected control (□) and rotavirus-infected (■) P2a chick kidney cell targets at various effector to target cell (E:T) ratios. Each point represents the mean percent specific release (± standard deviation) for two birds in trial 1 (E:T ratios of 12.5:1 to 50:1) and four birds in trial 2 (E:T ratios of 25:1 to 100:1).
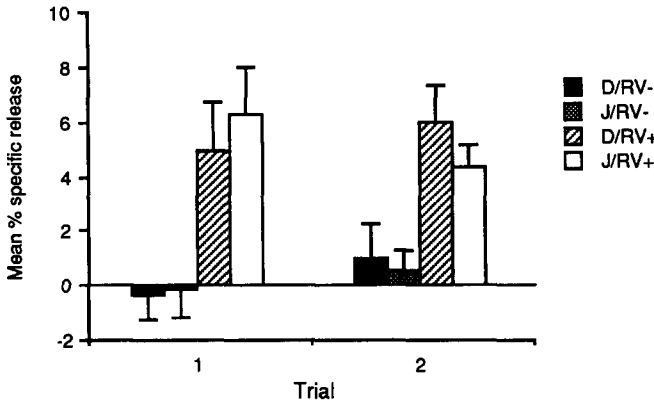

Fig. 5. Cytotoxic activity of intraepithelial leukocytes from the duodenum (D) and jejunum (J) of uninfected P2a chickens against uninfected (RV−) and rotavirus-infected (RV+) P2a chick kidney cell targets at an E:T ratio of 50:1. Each bar represents the mean percent specific release (± standard deviation) for four birds.

ues for rotavirus-infected target cells was significantly greater ($P < 0.05$) than for uninfected target cells in assays using IELs but not those using splenocytes. The levels of cytotoxic activity induced by IELs from uninfected versus rotavirus-infected birds were not significantly different at any DPI for rotavirus-infected target cells, despite reinoculation of the birds at 14 DPI. Serum samples collected from rotavirus-inoculated birds at 19 DPI were positive for rotavirus-specific IgM while sera from the uninoculated control birds were negative. Experiment 5 (Fig 4) demonstrated that the cytotoxic activity of

IELs from uninfected P2a chickens for rotavirus-infected P2a cells was dose dependent. Cytotoxic activity peaked at E:T ratios of 25:1 and 50:1, and had decreased at 100:1. The mean percent specific release values for rotavirus-negative versus rotavirus-infected target cells were significantly different at all E:T ratios in both trials except at 12.5:1 ($P < 0.05$). Experiment 6 (Fig. 5) compared the cytotoxic activity of IELs collected from the duodenum and jejunum. While the mean percent specific release values for uninfected versus rotavirus-infected target cells were significantly different ($P < 0.01$) in both trials, the mean percent specific release values for duodenum versus jejunum were not.

DISCUSSION

This study demonstrated that NK cell-like activity was expressed by chicken IELs against rotavirus-infected target cells. Furthermore, we confirmed the NK cell activity of chicken IELs against the lymphoblastoid target cell LSCC-RP9 demonstrated by Chai and Lillehoj (1988).

The results of experiments 1 and 2 showed that both IELs and splenocytes expressed cytotoxic activity against the accepted chicken NK target cell, LSCC-RP9. The absence of significant chromium release from RECC-CU60 target cells incubated with IELs demonstrated that the IEL preparations did not exhibit non-specific toxicity for lymphoblastoid cells. These experiments confirmed the reports by Chai and Lillehoj (1988) and Lillehoj and Chai (1988) which first demonstrated NK cell activity for chicken IELs against LSCC-RP9 and other lymphoblastoid cell lines. We also demonstrated that the NK activity of splenocytes and IELs measured against LSCC-RP9 cells was not affected by primary rotavirus infection of the host. This was in contrast to work performed by Lillehoj (personal communication) who showed that splenic and IEL NK activity was transiently depressed during the early phase of *Eimeria* primary infections. The subclinical nature of Ch2 rotavirus infections in chickens (Yason and Schat, 1986) is one possible explanation for the difference seen between these two systems.

Experiments 3 through 6 demonstrated that IELs exhibited cytotoxic activity against rotavirus-infected target cells. Because this activity was not induced by rotavirus infection of the host and was not MHC-restricted, it was considered by definition to be NK cell rather than cytotoxic T cell activity. Furthermore, it is unlikely that the cytotoxic activity observed in these experiments was due to macrophages or antibody-dependent cellular cytotoxicity. IELs isolated by this procedure exhibit the characteristic morphology of lymphocytes but not macrophages (Chai and Lillehoj, 1988). Also, cytotoxic activity in our study was demonstrated for IEL preparations from uninoculated control chickens which were negative for antibodies to rotaviruses. While Kohl

et al. (1983) reported NK activity for human peripheral blood leukocytes against rotavirus-infected target cells, our demonstration of IEL cytotoxicity for rotavirus-infected cells extend these findings to suggest that NK cell activity may indeed be an important intestinal response to rotavirus infection. In similar studies, in vitro spontaneous cytotoxic activity has been demonstrated for murine (Carman et al., 1986) and porcine (Cepica and Derbyshire, 1983) IELs against enteric coronavirus-infected target cells. Our results were in contrast, however, to the findings of Offit and Dudzik (1988), who demonstrated both primary and secondary (after in vitro stimulation) cytotoxic T cell activity for splenocytes against rotavirus-infected target cells. We were unable to demonstrate rotavirus-specific cytotoxic T cell activity for IELs or splenocytes following primary or secondary infection of the host. This does not necessarily indicate that a cytotoxic T cell response was absent from infected birds. First, the response could be masked by a higher level of NK activity in the splenocyte or IEL preparations. Furthermore, MHC-restricted cytotoxic T cell activity has only been documented for chicken splenocytes against reticuloendotheliosis virus-infected cells (MacCubbin and Schierman, 1986; Weinstock et al., 1989) and has yet to be demonstrated for chicken IELs.

While splenocytes were able to lyse LSCC-RP9 cells in an NK fashion, they were unable to lyse rotavirus-infected target cells in this study. This would suggest that there are different NK subpopulations in the spleen and intestine of chickens. This finding is supported by experiments in mice demonstrating that while both IEL and spleen cells exhibit NK activity against YAC-1 tumor cells, there are distinct differences in surface markers between these two populations (Tagliabue et al., 1982; Klein, 1986). Studies are currently planned in our laboratory to examine the surface phenotype of IELs with NK activity described in this report.

The reduced cytotoxic activity of IELs for rotavirus-infected CKC targets at high E:T ratios suggested there may be a subpopulation of IELs or some factor in the IEL preparations which suppressed NK cytolysis. Lillehoj and Chai (1988) did not observe reduced NK activity of IELs at high E:T ratios against LSCC-RP9 target cells in 4-h or 16-h assays. However, Mowat et al. (1983) demonstrated that murine IELs could suppress the NK activity of spleen cells, and that this suppression was not mediated by T lymphocytes or macrophages. Further experimentation will be needed to explore and confirm our observation.

Chai and Lillehoj (1988) demonstrated that the NK activity of IELs for lymphoblastoid target cells was greater in the jejunum than in the duodenum for the SC strain chickens. We did not observe a difference in NK activity between these two sites using the rotavirus-infected target cell assay. It is unclear if this discrepancy is due to differences between the IELs from the two strains of chickens or to differences between the assay systems.

Chai and Lillehoj (1988) commented that the cytotoxic activity of their

IEL preparations was critically dependent upon the expeditious handling of intestinal tissues and cell preparations. We observed a similar decrease in cytotoxic activity when tissues or cell suspensions were not processed quickly. Furthermore, in our assays using rotavirus-infected CKCs, we observed poor cytotoxic activity by IELs if the number of rotavirus-positive CKCs (as demonstrated by the direct fluorescent antibody assay) was less than 50% or if the spontaneous release of chromium was greater than 30% (data not shown).

This study did not attempt to determine the importance of NK cell activity to rotavirus immunity in vivo. This question will be difficult to address experimentally in the chicken system. However, the beige mouse, which exhibits a selective impairment of NK function (Roder, 1979), may prove to be a useful model for examining this question.

ACKNOWLEDGEMENTS

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