Immunology 1988 **63** 111–117

Isolation and functional characterization of chicken intestinal intra-epithelial lymphocytes showing natural killer cell activity against tumour target cells

J.-Y. CHAI & H. S. LILLEHOJ *Protozoan Diseases Laboratory, Animal Parasitology Institute, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Maryland, U.S.A.*

Accepted for publication 17 August 1987

SUMMARY

Intestinal intra-epithelial lymphocytes (IEL) of SC or FP chickens were isolated and examined for their natural killer (NK)-cell activity against chicken tumour cell lines, LSCC-RP9 (RP9), LSCC-RP12 (RP12), MDCC-MSB-1 (MSB-1) and MDCC-CU36 (CU36). In general, IEL of satisfactory yield and of good viability were obtained with EDTA treatment of the gut tissues, followed by rapid passages of the resultant cells through nylon-wool columns and centrifugation on two-step Percoll density grandients (45% and 80%). In 4-hr and 16-hr ^{51}Cr-release assays, the NK-cell activity of chicken IEL depended not only upon the type of target cells but also upon the incubation time and the host genetic background. RP9, MSB-1 and CU36 were susceptible to NK lysis by IEL and by spleen cells, while RP12 was resistant to lysis even after a prolonged incubation. In kinetic studies the cytotoxicity was detactable from 2 hr after incubation and progressively increased up to 16 or 18 hr. The IEL of SC chickens revealed significantly higher levels of NK-cell activity against RP9 than FP-strain chickens, whereas their splenic NK-cell activity was not significantly different. Against MSB-1 targets, however, IEL of SC and FP chickens showed similar levels of NK-cell activity while their spleens did not (being higher in FP). When tested in FP chickens, IEL NK-cell activity was inhibited by the addition of unlabelled homologous target cells. In general, NK-cell activity was higher in the jejunum and ileum than in the duodenum and caecum. Efforts to enrich IEL NK-effector cells by discontinuous Percoll gradients were not successful. The results of the present study show that IEL of chicken intestine contain effector cells that can mediate NK-cell activity against chicken tumour cells.

INTRODUCTION

A considerable amount of interest has been paid to the gut mucosal lymphoid populations recently, especially intra-epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) of humans and animals. It is suggested that the IEL and LPL are active in the first line of host defence (Ernst, Befus & Bienenstock, 1985; Elson *et al.*, 1986) because of their close proximity to the gut lumen where a variety of antigenic substances are introduced into the host. Some reports have indicated that IEL are mainly T cells and, to a lesser extent, non-T, non-B cells (Ferguson, 1977; Guy-Grand, Griscelli & Vassalli, 1978), whereas LPL are relatively enriched with immunoglobulin-producing B cells (Arnaud-Battandier, Lawrence & Blaese, 1980; Chiba *et al.*, 1981). Therefore, it is postulated that IEL play an important role in local cell-mediated immunity.

Correspondence: Dr H. S. Lillehoj, Protozoan Diseases Laboratory, Animal Parasitology Institute, U.S. Dept. Agriculture, Agricultural Research Service, Beltsville, MD 20705, U.S.A.

Natural killer (NK) cells, a subpopulation of lymphocytes showing non-specific cytotoxicity against tumour cells and microbially infected cells, are an important group of effector cells mediating a type of cell-mediated immunity (Herberman, 1981). NK-cell activity has been reported to be present in the peripheral blood and spleen, and recently also in the intestinal IEL population, of mice (Tagliabue *et al.*, 1982; Dillon & MacDonald, 1984), rats (Flexman, Shellam & Mayrhofer, 1983; Nauss *et al.*, 1984), pigs (Wilson, Stokes & Bourne, 1986) and guinea-pigs (Arnaud-Battandier *et al.*, 1978). In chickens, NK-cell activity has been demonstrated in the spleen (Sharma & Coulson, 1979; Lam & Linna, 1980; Sharma & Okazaki, 1981) and peripheral blood (Leibold, Janotte & Peter, 1980; Fleischer, 1980; Mandi *et al.*, 1985), but the presence of NK cells in their intestine has not yet been described. In the present study we have successfully isolated the intestinal IEL from two inbred strains of chickens, SC and FP, and examined their NK-cell activity against chicken tumour cell lines. The results of the present study demonstrate for the first time that chicken intestinal IEL contain a population of lymphocytes that mediate NK-cell cytotoxicity against chicken tumour cells.

MATERIALS AND METHODS

Chickens

The embryonated eggs of two strains of chickens, SC (B^2B^2) and FP ($B^{15}B^{21}$), were purchased from Hy-line International Production Center, Dallas Center, IA, and hatched in this institute. They were housed in clean wire-floored cages and provided with food and water *ad libitum*. Special care was taken not to allow any chickens to be exposed to specific pathogens such as Marek's disease virus, herpes virus, mycoplasma or chicken coccidia. Unless otherwise stated, healthy chickens of both sexes were used as donors of effector cells (IEL and spleen cells) at the age of 4–8 weeks.

Media

Two types of media, designated 'complete' or 'washing' medium, were used throughout the study. The complete medium was RPMI-1640 supplemented with 10% fetal calf serum (FCS), 5% tryptose phosphate broth, 10 mM HEPES buffer (pH 7·5), 2 mM glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 0·1 mM non-essential amino acids, 100 U/ml penicillin and 100 ug/ml streptomycin (all from Flow Laboratories Inc., McLean, VA). Washing medium used during the cell isolation was calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS) supplemented with 5% FCS, 10 mM HEPES and antibiotics.

Target cells

Chicken lymphoblastoid tumour cell lines were tested for their susceptibility to NK cytolysis. LSCC-RP9 (RP9) and LSCC-RP12 (RP12) are cell lines derived from the avian lymphoid leukosis tumour transplant, LSCT-RP6 (Sharma & Okazaki, 1981). MDCC-MSB-1 (MSB-1) and MDCC-CU36 (CU36) are cell lines derived from Marek's disease lymphomas (Sharma & Okazaki, 1981; Schat & Shek, 1984). All these cell lines were maintained *in vitro* as suspension cultures and subcultured every other day in complete medium, except CU36 cells that were grown in McCoy–Leibovitz-L-15 (ML-15) medium supplemented with 10% FCS and antibiotics instead.

Isolation of IEL

The preparation of IEL was carried out applying previously described techniques (Dillon & MacDonald, 1984; Wilson *et al.*, 1986; Davis & Parrott, 1981) with some modifications. In most experiments only the duodenal c-loops, 12–15 cm long, were removed from two or three individuals of SC or FP chickens, pooled, and used as the source of intestinal IEL. The duodenal segments were cut open longitudinally and then cut into 1–2 cm fragments. The gut pieces were swirled in more than three changes of cold washing medium until free of detritus. Then the gut pieces were put in a bottle containing 1–10 mM dithiothreitol (DTT) (Sigma, St Louis, MO) in 30 ml washing medium for 5–10 min at room temperature, to remove the intestinal mucus. The supernatants containing mucus and tissue debris were discarded. The gut pieces were then gently stirred at 41° in 10^{-4}M EDTA for 15–20 min followed by several rinses with fresh washing medium. The cell-containing supernatants were pooled, washed twice, and resuspended in complete medium. In some experiments the jejunum (down to the yolk sac), ileum (remaining small intestine) and two caeca were additionally removed and their comparative cytotoxicities tested.

The cells at this time consisted of large, viable or dead epithelial cells and their clumps, as well as small, round and mostly viable IEL. In order to get rid of the dead cells, cell clumps and epithelial cells, passages through a nylon-wool column (NWC) and Percoll density gradient separation were performed. Briefly, the washed cell suspensions were rapidly passed two or three times through a 30-ml syringe which had been loosely packed with 2 g of nylon-wool. NWC-passed cells were adjusted to 10–20 ($\times 10^6$)/ml and resuspended in 45% Percoll (density 1·058 g/ml). Three to four millilitres of this cell suspension were carefully overlayed on 3–4 ml of 80% Percoll (density 1·098 g/ml) in a 15-ml conical centrifuge tube. After spinning at 600 ***g*** for 20 min at room temperature, the cell band formed at the interface of two-step Percoll gradients was pipetted carefully and transferred to a 50-ml tube, washed twice and resuspended in complete medium. Where necessary, further fractionation of IEL was performed by multiple-step (25, 35, 40, 45, 50, 70 and 80%) Percoll density-gradient separation. The cell viability was examined by a trypan blue dye exclusion test.

Preparation of spleen cells

Spleen cells of SC and FP chickens were prepared by the mechanical separation technique. Two or three spleens of SC or FP chickens were teased gently through a stainless steel mesh into a petri-dish containing washing medium. After 2–3 min settling, the single-cell containing supernatant was collected, washed twice, and resuspended in complete medium. Where necessary, further purification of spleen lymphocytes was performed by layering them on Ficoll–Hypaque separation medium (density 1·077 g/ml).

Cytotoxicity assay

The standard ^{51}Cr-release assay (Brunner *et al.*, 1968) was adopted with some modifications. The tumour target cells were mixed with 100–150 μCi/10^7 cells of $Na_2{}^{51}CrO_4$ (specific activity 200–400 mCi/mg; New England Nuclear Co., Boston, MA) in 0·3–0·5 ml complete medium. The mixture was incubated for 45–60 min in a gently stirring water bath at 41°. After the incubation, the target cell suspension was washed with cold washing medium, kept on ice for 30 min, and washed again. Then the cell suspension was preincubated at 41° for 30–45 min in a CO_2 incubator to reduce spontaneous release during the assay. The target cells were washed again immediately before use, and adjusted to 2×10^5/ml. One hundred microlitres of the target cell suspension were placed in each well of U-bottomed 96-well microtitre plates. Various effector cell concentrations were prepared so that the effector to target (E:T) ratio was 200:1 through 3:1; 100 μl of any concentration were put into three wells. Wells containing only target cells and medium served for controls (spontaneous release), while wells containing target cells and Nonidet-P 40 detergent (added directly before harvest) were used for the measurement of total releasable radioactivity. The plates were spun at 50 ***g*** for 5 min at room temperature and then incubated for 4 or 16 hr (in some experiments 2–18 hr) at 41° in a humidified atmosphere of 5% CO_2 and 95% air. The plates were spun at 600 ***g*** at 4° for 5 min and the supernatants harvested using the Skatron supernatant collection system (Skatron Inc., Sterling, VA). The radioactivity released in each supernatant was counted using a Beckman 4000 counter (Beckman Instruments, Fullerton, CA). Spontaneous release never exceeded 2–3%/hr of the total releasable (or

Table 1. Purity and viability of intestinal cells after each step of the isolation procedure

Treatment*	IEL No. ($\times 10^6$)	IEL Viability (%)	Epithelial cells No. ($\times 10^6$)	Epithelial cells Viability (%)	IEL (% of total cells) Mean	IEL (% of total cells) Range
EDTA	62±30	75±15	61±40	70±22	50	35–70
Nylon-wool	45±16	85±10	21±22	66±19	68	59–86
Percoll	16+12	99±1	4±3	56±20	80	70–93

Values are expressed as mean ± SD per chicken from seven experiments, each group consists of two to three chickens (FP or SC strain).

* Treatments of cell preparations were done as described in the Materials and Methods.

incorporated) radioactivity. Percentage specific release was calculated by the following formula:

$$100 \times \frac{\text{test release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}.$$

Cold-target inhibition assay

Various concentrations of unlabelled (cold) inhibitors, homologous or heterologous to each target cell, were added to the wells containing effector cells and labelled target cells at E:T ratios of 100 :1 and 50:1. The total volume of each well was maintained to be 200 μl and unlabelled chicken red blood cells were added to make the total cell number constant, so as to provide negative controls. The labelled target/unlabelled inhibitor ratios were set at 1:1, 1:5 and/or 1:10.

Morphological observation of gut tissue sections and isolated IEL

In order to verify that the treatment of gut tissues with DTT and EDTA, as described, isolated only the basement membrane of the epithelial layer, and thus extracted a pure population of IEL (with epithelial cells) devoid of LPL and other cells, some of the treated gut pieces were processed to histological sections followed by routine haematoxylin and eosin stain. In addition, the final IEL preparations obtained were fixed with methanol, stained with Giemsa or Wright solution, and their morphological features observed.

RESULTS

Purity and viability of IEL

Extraction of IEL using DTT and EDTA with subsequent steps of NWC passages and Percoll-gradient separation produced high yields and a relatively pure population of IEL with good viability (Table 1). Contamination with LPL or other cells of lamina propria was not recognizable, as shown by the histological sections of the gut pieces treated with DTT and EDTA. The stained preparations of IEL almost exclusively revealed the characteristic morphology of lymphocytes, together with a few contaminating epithelial cells. No difference in IEL yield was observed between the two strains of chickens. The cell viability was largely dependent upon the duration of time taken from the killing of the chickens to the processing of the intestines. Poor viability of IEL (and/or epithelial cells) resulted if the time was delayed longer than 30 min, even though the resected gut had been preserved on ice. Only the gut samples processed within 5–20 min were used for the test of cell viability as well as for the examination of NK-cell cytotoxicity (Tables 1 to 5, Figs 1 and 2). Optimum separation of IEL by Percoll gradients was achieved when the input cell concentration was dilated to 10–20 ($\times 10^6$)/ml in 45% Percoll. The number of IEL finally obtained per chicken was 16±12 ($\times 10^6$) and their viability 99% (Table 1).

Table 2. NK-cell activity of intestinal IEL

Exp. no.	Target cell	% cytotoxicity 4-hr 100:1*	4-hr 50:1	16-hr 100:1	16-hr 50:1
I	RP9	1·2	1·4	14·2	19·6
	MSB-1	ND	ND	46·0	42·0
	RP12	1·1	0·8	5·0	−1·2
	CU36	−0·4	1·0	12·6	29·3
II	RP9	3·0	1·2	18·5	7·5
	MSB-1	12·5	2·8	68·2	63·2
	RP12	2·0	1·7	ND	8·8
	CU36	−4·1	−2·9	46·5	28·0
III	RP9	4·2	1·4	38·7	23·1
	MSB-1	5·8	2·2	87·5	72·7
	RP12	−0·3	−1·3	11·3	2·6
	CU36	1·8	1·5	64·2	43·1

Values are expressed as mean of triplicate wells. The standard deviation never exceeded 20–30% of the mean value. Each experiment was performed with IEL from three FP chickens.

ND, not determined.

* E:T ratio.

Table 3. NK-cell activity of Percoll-fractionated IEL

Target cell	E:T	Percoll-fractionated IEL (% cytotoxity) Input	25–35%	35–40%	40–50%	50–70%
RP9	50:1	12·0	19·8	16·1	14·6	8·6
	25:1	9·1	12·5	5·5	6·9	12·3
MSB-1	50:1	46·1	44·6	53·1	54·1	53·9
	25:1	51·9	54·7	50·5	56·2	48·8
RP12	50:1	7·3	9·8	3·6	6·4	10·1
	25:1	2·6	5·3	1·4	2·1	4·1
CU36	50:1	60·6	39·3	ND	19·5	21·1
	25:1	44·2	18·5	ND	20·5	18·5

Values are mean of triplicate wells in a 16-hr ^{51}Cr-release assay. Standard deviations were usually less than 20% of the mean value. Effector cells were obtained from pooled IEL of the duodenum of SC chickens, that had been harvested from the interface of layers on discontinuous Percoll gradients.

ND, not determined.

Table 4. Cold-target inhibition of NK-cell activity of IEL and spleen cells

Exp. no.	Labelled target cell (L)	Unlabelled inhibitor (U)	L:U	% cytotoxicity IEL 100:1*	IEL 50:1	Spleen cells 100:1	Spleen cells 50:1
I	RP9	—	—	36·4	33·5	42·4	31·4
	RP9	RP9	1:1	11·5	17·6	13·5	9·7
	RP9	RP9	1:5	2·5	1·8	10·2	14·8
	RP9	MSB-1	1:10	2·4	0·5	18·5	20·2
II	MSB-1	—	—	34·5	34·2	36·7	30·6
	MSB-1	MSB-1	1:5	4·5	5·1	8·6	11·4
	MSB-1	MSB-1	1:10	−0·1	−9·9	7·6	15·1

Values are mean of triplicate wells in a 16-hr ^{51}Cr-release assay. Standard deviations are less than 20% of the mean value. The effector cells were obtained from FP chickens.

* E:T ratio.

Table 5. Comparative NK-cell activity of IEL from the duodenum, jejunum, ileum and caecum with that of spleen cells

Target cell	E:T*	% cytotoxicity Duodenum	Jejunum	Ileum	Caecum	Spleen
RP9	100:1	12·0	17·3	30·2	−3·7	39·6
	50:1	7·3	16·0	12·1	0·5	17·3
MSB-1	100:1	9·7	26·3	28·0	9·8	9·7
	50:1	14·8	27·6	33·0	13·9	14·8
RP12	100:1	2·6	ND	ND	0·1	1·3
	50:1	−2·3	−6·2	ND	ND	−2·3
CU36	100:1	11·4	35·1	39·4	26·8	11·4
	50:1	17·8	34·7	42·9	23·5	17·8

Values are mean of triplicate wells in a 12-hr ^{51}Cr-release assay and standard deviations were usually less than 20% of the mean.

ND, not determied

* Effector cells were obtained from SC chickens.

NK-cell activity of IEL and spleen cells of FP chickens

A standard 4-hr ^{51}Cr-release assay was performed to see whether IEL from FP chickens exhibited any cytotoxicity against chicken tumour cell targets. Although the results were somewhat variable, FP IEL did not show high levels of cytotoxicity against any of the four kinds of targets at E:T ratios from 200:1 to 3:1 (data not shown). As a next step, 4-hr and 16-hr assays were performed simultaneously. High levels of cytotoxicity were obtained by both IEL and spleen cells against all tumour targets except RP12 when the assay time was extended to 16 hr (Table 2). RP12 appeared not to be a susceptible target to NK cytolysis by IEL (Table 2) and by spleen cells (Table 5). The cytotoxicity by both kinds of effector cells was usually highest at the E:T ratio of 50:1 or 100:1 (data not shown).

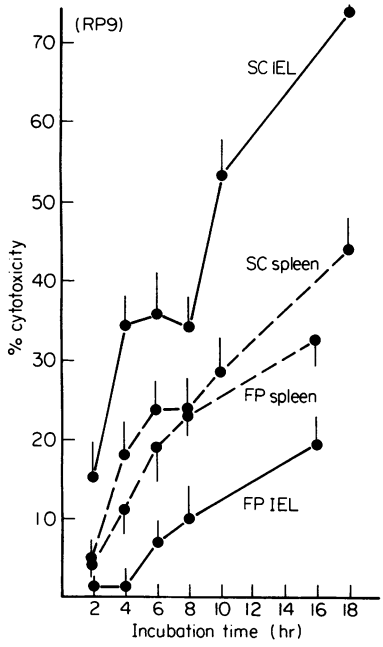

Figure 1. Kinetics of NK-cell activity of IEL and spleen cells obtained from SC and FP chickens. Target cells are RP9. Values represent the mean cytotoxicity with standard deviation of triplicate wells at E:T ratio of 50:1.

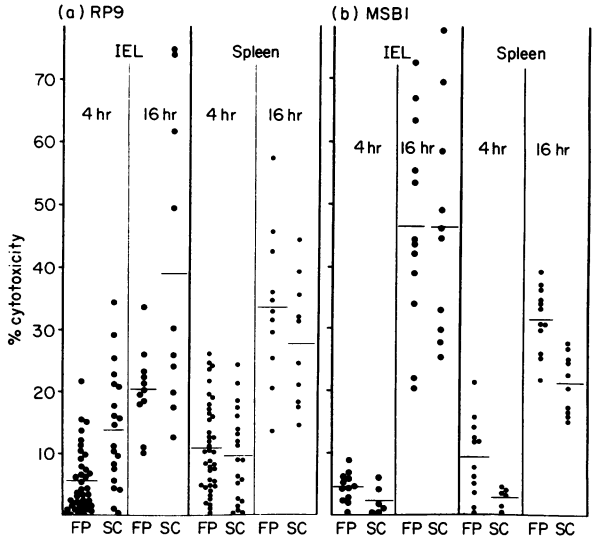

Figure 2. NK-cell activity of IEL and spleen cells of FP and SC chickens in 4-hr and 16-hr ^{51}Cr release assays, at E:T ratio of 50:1. (a) Against RP9 target; (b) against MSB-1 target. Bar (-) represents the mean value.

Kinetics of NK-cell activity

The kinetics of NK-cell cytotoxicity were studied in FP and SC chickens by setting the assay time at 2, 4, 6, 8, 10, 16 and/or 18 hr and using RP9 cells as the target. Figure 1 shows the results of one of three experiments that gave similar results. the cytotoxicity was detected as early as after 2 hr of incubation in all groups, although low by FP IEL, and progressively increased as the incubation time was prolonged. SC IEL showed higher cytotoxicity against RP9 than FP IEL throughout the assay time, and spleen cells of FP and SC revealed similar levels and kinetics of NK-cell activity.

Comparative NK-cell activity between SC and FP chickens

Since RP9 and MSB-1 were susceptible targets, these cell lines were used to determine further the differences in NK-cell activity of IEL and spleen cells of two strain chickens. In 4-hr and 16-hr assays, their IEL and spleen cells showed wide ranges of cytotoxicity, as shown in Fig. 2a,b.

In Fig. 2a, approximately 40% of SC chickens had elevated IEL NK-cell levels against RP9 targets. On the other hand, the cytoxicity of SC and FP spleen cells was not significantly different. In SC chickens the cytotoxicity of IEL was of a similar level to that of their spleen cells, whereas in FP chickens the cytotoxicity of IEL was lower than that of their spleen cells ($P < 0{\cdot}05$). These results suggest that there is a strain difference in the NK-cell activity of chicken intestinal IEL against RP9 target.

Interestingly, when MSB-1 was used as the target the effector cells of SC and FP chickens showed slightly different patterns of cytotoxicity (Fig. 2b). The NK-cell activity of IEL populations of SC and FP chickens did not differ significantly, whereas the spleen cells of SC chickens revealed lower cytotoxicity than those of FP chickens ($P < 0{\cdot}01$). In the SC strain, the cytotoxicity of their IEL and spleen cells was not different in a 4-hr assay, but later, at 16 hr, their IEL showed higher cytotoxicity than their spleen cells ($P < 0{\cdot}01$). In FP chickens, IEL revealed lower cytotoxicity than spleen cells in a 4-hr assay ($P < 0{\cdot}05$), but the situation reversed when the assay was continued for 16 hr ($P < 0{\cdot}01$).

NK-cell activity of different Percoll fractions of IEL

Efforts to enrich for the IEL NK-effector cells by discontinuous Percoll density gradients were not successful (Table 3). The 25–35% interface fractions of SC IEL contained as many as 40% epithelial cells of mixed viability, and the 35–40% interface fractions were contaminated with over 30% epithelial cells. Even the 40–50% and 50–70% Percoll interfaces contained a significant proportion of epithelial cells. Moreover, as can be seen in Table 3, no specific enrichment of cytotoxicity was accomplished against any of the target cells tested in a 16-hr assay. In general the cytotoxicity appeared slightly higher in the lower density fractions.

Cold-target inhibition

Cold unlabelled targets were added to observe their inhibitory effect on the cytotoxicity by IEL and spleen cells of FP chickens. Experiments were repeated four times with similar results, and the data from two representative experiments are presented in Table 4. When RP9 was used as the target cell the cytotoxicity of IEL at E:T ratios of 100:1 and 50:1 was 36·4% and 33·8%, respectively, whereas the cytotoxicity of spleen cells was 42·4% and 31·4%, respectively. The addition of unlabelled homologous cells resulted in an inhibition of the cytotoxicity. The extent of inhibition was stronger in IEL than in spleen cells. Similar results were obtained when MSB-1 was used as the labelled/unlabelled targets. When unlabelled heterologous cells, MSB-1, were added to RP9 target cells at a labelled : unlabelled cell ratio of 1:10, complete inhibition was found with IEL while the inhibition was less remarkable with spleen cells.

NK-cell activity of different segments of intestine

The NK-cell activity of IEL populations in the duodenum, jejunum, ileum and caecum was compared with each other and with that of spleen cells in SC chickens (Table 5). The NK-cell activity of IEL from each segment of intestine and spleen cells varied from experiment to experiment depending on the kinds of target cells used. In three separate experiments, the cytotoxicity in a 12-hr assay against RP9, MSB-1 and CU36 was generally higher in the jejunum and ileum than in the duodenum and caecum. RP12 was a poor target for all IEL groups as well as for spleen cells. IEL from the caecum exhibited their highest cytotoxicity against CU36 cells.

DISCUSSION

The present study demonstrated that chicken intestinal IEL, of high yield and good viability, can be obtained by sequential steps of EDTA treatment, passages through NWC and centrifugation on Percoll density gradients. The separation of IEL (with epithelial cells) but not LPL from the gut mucosa was vertified by histological observation of the gut pieces treated with EDTA. The IEL isolated from SC and FP chickens showed NK-cell acivity against chicken tumour cells in a 4–16-hr ^{51}Cr-release assay. The degree of cytotoxicity depended upon the host genetic background, the target cell, the area of the intestine where the cells were harvested, as well as upon the incubation time. The specificity of NK-cell activity to tumour targets was verified by the inhibition of cytotoxic activity after the addition of cold unlabelled target cells.

Techniques used to isolate intestinal lymphocytes, IEL and/or LPL, are known to affect their functional capabilities. Chicken gut mucosal lymphocytes isolated by a mechanical separation technique (Arnaud–Battandier *et al.*, 1980) did not show any proliferative functional response to mitogenic stimulation. In a human study (Bland *et al.*, 1979), the large bowel mucosal lymphocytes isolated by a mechanical method failed to show cellular cytotoxicity, whereas cytotoxic activity was retained in the lymphocytes isolated by an enzymatic method. The loss of cellular cytotoxicity after a mechanical separation was attributable to a release of prostaglandin E_2 which suppresses cytotoxic activity (Bland *et al.*, 1979). On the other hand, the disadvantage of using EDTA is also known. High concentrations of EDTA used to isolate IEL are reported to have caused substantial loss of cellular cytotoxicity by human colonic IEL (Chiba *et al.*, 1981). In the present study we used a low concentration of EDTA to eliminate such a deleterious effect. In our experience, the cytotoxic potential of chicken IEL was dependent mainly on the freshness of the gut samples and on the duration of DTT or EDTA treatment. Incubation of the gut

pieces with DTT for longer than 30 min and/or incubation with EDTA for longer than 1 hr tended to result in both poor viability of IEL and depressed levels of cytotoxicity, even in a 16-hr assay (data not presented).

The NK-cell activity of intestinal IEL has been studied in a variety of mammalian species (Tagliabue *et al.*, 1982; Flexman *et al.*, 1983; Wilson, *et al.*, 1986; Arnaud-Battandier *et al.*, 1978; Mowat *et al.*, 1983). According to these reports, in general, IEL demonstrated low levels of NK-cell activity compared to spleen cells in a short-term assay (4 hr) but showed comparable levels of cytotoxicity when the assays were continued up to 16–24 hr. The present study agrees with the above findings. As for the reason for the delayed expression of cytotoxicity, it was suggested that a 'NK receptor' on IEL was modified *in vivo* or during the isolation procedure and overnight culture was required for the regeneration of this structure (Mowat *et al.*, 1983). A different suggestion is that, although IEL and spleen cells have an equal frequency of NK cells that would form conjugates with target cells, IEL are less efficient than spleen cells in their lytic interactions with target cells or in their ability to recycle to new targets after lytic interactions (Flexman *et al.*, 1983). However, all these suggestions may not apply to all of the subpopulations of IEL NK cells that show different and distinct target specificity. In fact, very rapid cytotoxic activities such as NK and antibody-dependent cellular cytotoxicity (ADCC) were demonstrated against a bacterium, *Salmonella typhimurium*, after only 2 hr of incubation by IEL and Peyer's patch lymphocytes (Nencioni *et al.*, 1983). Very high natural anti-viral activity was also reported by IEL of specific pathogen-free mice within 6 hr (Carman *et al.*, 1986). The relatively rapid induction of killing of tumour targets in the present study, from as early as 2 hr by SC chicken IEL against RP9 (Fig. 1), appears to agree with the above observations. On the other hand, the delayed and less efficient killing of RP9 by FP IEL contrasts with them. Both FP IEL and SC IEL showed a delayed but efficient killing against MSB-1, a different susceptible target. These results strongly suggest that the efficiency of NK-cell killing by chicken IEL is influenced by the host genetic background as well as by the type and nature of the target cells.

In humans and rodents, the effector cells responsible for natural, non-MHC restricted cytotoxicity are complex and heterogeneous. Other than classical NK cells, macrophages and granulocytes can express natural cytotoxicity (Herberman & Ortaldo, 1981) and, among the lymphocyte subpopulations, non-MHC-restricted cytotoxic T lymphocytes (CTL) (Brooks, 1983; Lanier *et al.*, 1986), and even lymphocytes of B-cell phenotype (Welsh *et al.*, 1986), can lyse NK-sensitive tumour targets or virally infected murine cells. Recently, another type of T cell that can kill tumour targets when stimulated by IL-2 was termed lymphokine-activated killer (LAK) cells (Rosenberg, 1985; Malkovsky *et al.*, 1987). Also, in avian species several studies have characterized the properties of effector cells mediating non-MHC-restricted cellular cytotoxicity. Chicken splenic effector cells responsible for natural resistance to Marek's disease were non-T, non-B and non-phagocytic in nature (Lam & Linna, 1980), which can be regarded as NK cells. Phagocytic and adherent cells (Fleischer, 1980) as well as granulocytes (Mandi *et al.*, 1985) were also reported to be the effector populations that can kill tumour cells of allogeneic or xenogeneic origin. Several NK-like clones of chicken spleen cells have been developed, using a medium containing IL-2 (Schat, Calnek & Weinstock, 1986), that could represent LAK cells since they are dependent upon the lymphokine in the culture medium.

In the present study, efforts to enrich for the IEL NK-effector cells by Percoll gradients were not successful. It is well known that in humans peripheral blood NK-effector cells can be easily enriched by Percoll-density gradients (Timonen, Ortaldo & Herberman, 1981). For chicken spleen cells, the NK-effector cells have been reported to become enriched at the density range of 1·060 g/ml (47·5% Percoll) to 1·070 g/ml (55% Percoll) (Schat & Shek, 1984); however, the extent of enrichment was not so remarkable when compared with human peripheral blood NK cells.

It is strongly suggestive that the IEL effector cells that have shown natural cytotoxicity in the present study are NK cells. However, the participation of other cell types such as macrophages in IEL NK-cell killing is possible from the two observations made in this study. First SC IEL demonstrated a biphasic pattern of lysis of RP9 targets (Fig. 1). Secondly the kinetics of target cell lysis by IEL is considerably slower than that of splenic NK-cell killing. B cells are not good candidates since it is known that chicken intestinal IEL contain few B cells (Arnaud-Battandier *et al.*, 1980). The possibility for T cells, especially non-MHC-restricted CTL, is still retained. At present, however, no appropriate surface markers or monoclonal antibodies than can efficiently discriminate NK cells from CTL of chickens are available. Further studies are needed to elucidate the nature and properties of IEL NK cells of chickens.

Interestingly the present study shows that the NK-cell activity against certain tumour cell lines is slightly stronger in the middle and lower parts of the small intestine than in the duodenum or caecum. Whether or not this difference is due to any abundance of gut-associated lymphoid tissues (GALT) at these sites remains unclear. If this is the case, it would be of value to compare the NK-cell activity between IEL and LPL, IEL and Peyer's patches, and IEL and mesenteric lymph nodes, since this would provide information on the origin(s) of chicken intestinal IEL. In chickens, IEL are suggested to have a different origin from that of LPL, since IEL contain a smaller number of immunoglobulin-bearing B cells compared to LPL (Arnaud-Battandier *et al.*, 1980). In mammals, contradictory results have been published concerning the ontogeny of IEL and LPL (Wilson *et al.*, 1986; Mayrhofer & Whately, 1983) and this subject needs further investigation in many species.

The NK-cell activity in IEL of FP and SC chickens is intriguing for gut immunity implicated in avian coccidiosis. It was demonstrated that the sporozoites of *Eimeria tenella* first penetrate enterocytes and then enter IEL that leave the epithelium, pass through LPL and enter the crypt where the parasites are liberated (Lawn & Rose, 1982). Whether the sporozoites can enter NK cells among the IEL population or not is as yet uncertain. However, the potential invasion of coccidia into chicken intestinal IEL is of considerable importance for the study of mucosal immunity against this protozoan infection. The genetic susceptibility of different strains of chickens to coccidiosis (Lillehoj & Ruff, 1987) might be determined at this level within the gut mucosa. Further characterization of host–parasite interaction in the gut should shed light on the mechanisms of pathogenesis in avian coccidiosis.

ACKNOWLEDGMENTS

The authors wish to thank Ms Marjorie Nichols and Ms Kimberly Lee for their excellent assistance in the preparation of effector cells used in this study. Special thanks are also given to Dr K. Nazarian. at the Regional Poultry Research Laboratory, East Lansing, MI and to Dr K. A. Schat at the Department of Avian and Aquatic Animal Medicine. Cornell University, Ithaca, NY for providing the tumour cell lines.

This work was supported by the U.S. Department of Agriculture, Agricultural Research Service, under Agreement No. 86-CRCR-1-2099. Dr J.-Y. Chai was on Sabbatical leave from Department of Parasitology, College of Medicine, Seoul National University, Seoul 110, Korea.

REFERENCES

ARNAUD-BATTANDIER F., BUNDY B.M., O'NEILL M., BIENENSTOCK J. & NELSON D.L. (1978) Cytotoxic acivities of gut mucosal lymphoid cells in guinea pigs. *J. Immunol.* **121,** 1059.

ARNAUD-BATTANDIER, F., LAWRENCE E. C. & BLAESE R.M. (1980) Lymphoid populations of gut mucosa in chickens. *Diges. Dis. Sci.* **25,** 252.

BLAND P.W., RICHENS E.R., BRITTON D.C. & LLOYD J.V. (1979) Isolation and purification of human large bowel mucosal lymphoid cells: effect of separation technique on functional characteristics. *Gut,* **20,** 1037.

BROOKS C.G. (1983) Reversible induction of natural killer cell activity in cloned murine cytotoxic T lymphocytes. *Nature (Lond.),* **305,** 155.

BRUNNER K.T., MAUEL J., CEROTTINI J. C. & CHAPUIS B. (1968) Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells *in vitro;* inhibition by isoantibody and by drugs. *Immunology,* **14,** 181.

CARMAN P.S., ERNST P.B., ROSENTHAL K.L., CLARK D.A., BEFUS A.D. & BIENENSTOCK J. (1986) Intraepithelial leukocytes contain a unique subpopulation of NK-like cytotoxic cells active in the defence of gut epithelium to enteric murine coronavirus. *J. Immunol.* **136,** 1548.

CHIBA M., BARTNIK W., REMINE S.G., THAYER W.R. & SHORTER R.G. (1981) Human colonic intraepithelial and lamina proprial lymphocyte: cytotoxicity *in vitro* and the potential effects of the isolation method on their functional properties. *Gut,* **22,** 177.

DAVIS M.D.J. & PARROTT D.M.V. (1981) Cytotoxic T cells in small intestine epithelial, lamina propria and lung lymphocytes. *Immunology,* **44,** 367.

DILLON S.B. & MACDONALD T.T. (1984) Functional properties of lymphocytes isolated from murine small intestinal epithelium. *Immunology,* **52,** 501.

ELSON C.O., KAGNOFF M.F., FIOCCHI C., BEFUS A.D. & TARGAN S. (1986) Intestinal immunity and inflammation: recent progress. *Gastroenterology,* **91,** 746.

ERNST P.B., BEFUS A.D. & BIENENSTOCK J. (1985) Leukocytes in the intestinal epithelium: an unusual immunological compartment. *Immunol. Today,* **6,** 50.

FERGUSON A. (1977) Intraepithelial lymphocytes of the small intestine. *Gut,* **18,** 921.

FLEISCHER B. (1980) Effector cells in avian spontaneous and antibody-dependent cell-mediated cytotoxicity. *J. Immunol.* **125,** 1161.

FLEXMAN J.P., SHELLAM G.R. & MAYRHOFER G. (1983) Natural cytotoxicity, responsiveness to interferon and morphology of intraepithelial lymphocytes from the small intestine of the rat. *Immunology,* **48,** 733.

GUY-GRAND D., GRISCELLI C. & VASSALLI P. (1978) The mouse gut T lymphocyte, a novel type of T cell. Nature, origin and traffic in mice in normal and graft-versus-host conditions. *J. exp. Med.* **148,** 1661.

HERBERMAN R.B. (1981) Natural killer (NK) cells and their possible roles in resistance against disease. *Clin. Immunol. Rev.* **1,** 1.

HERBERMAN R.B. & ORTALDO J.R. (1981) Natural killer cells: their role in defenses against disease. *Science,* **214,** 24.

LAM K.M. & LINNA T.J. (1980) Transfer of natural resistance to Marek's disease (JMV) with nonimmune spleen cells. II. Further characterization of protecting cell population. *J. Immunol.* **125,** 715.

LANIER L.L., PHILLIPS J.H., HACKETT J., TUTT M. & KUMAR V. (1986) Natural killer cells: Definition of a cell type rather than a function. *J. Immunol.* **137,** 2735.

LAWN A.M. & ROSE M.E. (1982) Mucosal transport of *Eimeria tenella* in the cecum of the chicken. *J. Parasitol.* **68,** 1117.

LEIBOLD W., JANOTTE G. & PETER H.H. (1980) Spontaneous cell mediated cytotoxicity (SCMC) in various mammalian species and chickens: selective reaction pattern and different mechanisms. *Scand. J. Immunol.* **11,** 203.

LILLEHOJ H.S. & RUFF M.D. (1987) Comparison of disease susceptibility and subclass-specific antibody response in SC and FP chickens experimentally inoculated with *Eimeria tenella, E., acervulina,* or *E. maxima. Avian Dis.* **31,** 112.

MALKOVSKY M., JIRA M., MADAR J., MALKOVSKA V., LOVELAND B. & ASHERSON G.L. (1987) Generation of lymphokine-activated killer cells does not require DNA synthesis. *Immunology,* **60,** 471.

MANDI Y., SEPRENYI G., PUSZTAI R. & BELADI I. (1985) Are granulocytes the main effector cells of natural cytotoxicity in chickens? *Immunobiol.* **170,** 284.

MAYRHOFER G. & WHATELY R.J. (1983) Granular intraepithelial lymphocytes of the rat small intestine. I. Isolation, presence in T lymphocyte-deficient rats and bone marrow origin. *Intern. Archs. Allergy appl. Immunol.* **71,** 317.

MOWAT A.M., TAIT R.C., MACKENZIE S., DAVIES M.D.J. & PARROTT D.M.V. (1983) Analysis of natural killer effector and suppressor activity by intraepithelial lymphocytes from mouse small intestine. *Clin. exp. Immunol.* **52,** 191.

NAUSS K.M., PAVLINA T.M., KUMAR V. & NEWBERNE P.M. (1984) Functional characteristics of lymphocytes isolated from the rat large intestine. *Gastroenterology,* **86,** 468.

NENCIONI L., VILLA L., BORASCHI D., BERTI B. & TAGLIABUE A. (1983) Natural and antibody-dependent cell-mediated activity against *Salmonella typhimurium* by peripheral and intestinal lymphoid cells in mice. *J. Immunol.* **130,** 903.

ROSENBERG S. (1985) Lymphokine-activated killer cells: a new approach to immunotherapy of cancer. *J. natl. Cancer Instit.* **75,** 595.

SCHAT K.A., CALNEK B.W. & WEINSTOCK D. (1986) Cultivation and characterization of avian lymphocytes with natural killer cell activity. *Avian Pathol.* **15,** 539.

SCHAT K.A. & SHEK W.R. (1984) Separation of cytotoxic lymphocytes against Marek's disease lymphoblastoid cell lines on Percoll gradient. *Avian Pathol.* **13,** 469.

SHARMA J.M. & COULSON B.D. (1979) Presence of natural killer cells in specific-pathogen-free chickens. *J. natl. Cancer Instit.* **63,** 527.

SHARMA J.M. & OKAZAKI W. (1981) Natural killer cell activity in chickens: target cell analysis and effect of antithymocyte serum on effector cells. *Infect. Immun.* **31,** 1078.

TAGLIABUE A., BEFUS A.D., CLARK D.A. & BIENENSTOCK J. (1982) Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. *J. exp. Med.* **155,** 1785.

TIMONEN T., ORTALDO J.R. & HERBERMAN R.B. (1981) Characterization of human large granula lymphocytes and relationship to natural killer and K cells. *J. exp. Med.* **153,** 569.

WELSH R.M., HASPEL M.V., PARKER D.C. & HOLMES K.V. (1986) Natural cytotoxicity against mouse hepatitis virus-infected cells. II. A cytotoxic effector cell with a B lymphocyte phenotype. *J. Immunol.* **136,** 1454.

WILSON A.D., STOKES C.R. & BOURNE F.J. (1986) Morphology and functional characteristics of isolated porcine intraepithelial lymphocytes. *Immunology,* **59,** 109.