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Natural Antitumour Cytotoxicity of Mouse Lymph Node Cells; Characterization and Specificity Studies

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Abstract—*Effector cells, present in the mesenteric lymph nodes of normal C57Bl mice, were shown to be cytotoxic towards cell targets derived from* in vivo *or* in vitro *cultured tumour lines, but not cells freshly prepared from normal syngeneic mouse tissue. Targets from continuously passaged* in vitro *lines were also susceptible to this form of killing. The observed cytotoxicity was restricted to one effector cell type, which proved to be adherent to glass and nylon wool, sensitive to carbon treatment, and resistant to treatment with anti-Thy* 1:2 *serum and complement, trypsin, ammonium chloride and* 1500 rad *irradiation. These effector cells therefore represent a population distinct from natural killer cells, with properties similar to those of macrophages. Killing of* in vivo *derived EL4 targets was rapid and detected after* 1 hr *of co-culture; this cytotoxicity was maximum after* 3 hr *co-culture. Using unlabelled EL4 tumour cells as competitors for cytotoxicity tests, it was concluded that killing results following cell to cell contact, occurring through membrane associated receptor interaction.*

INTRODUCTION

NATURALLY occuring cytotoxic lymphocytes with selective reactivity towards tumour cells have been demonstrated in both animal and human tumour systems, and have been termed natural killer (NK) cells. They represent a subpopulation distinct from those of T or B cells and possess characteristics dissimilar to those of macrophages [1–11]. In most previous investigations cell susceptibility to lysis has almost entirely been studied using *in vitro* cultured tumour cells, and in particular cells derived from the lymphoblastoid lines. Other naturally cytotoxic antitumour mechanisms have been reported recently [12–14] and *in vitro* and *in vivo* studies have inferred a role for macrophages in immune surveillance against tumours [15–17]. Human blood monocytes have been reported to kill tumour targets *in vitro* [18, 19], and in the mouse natural cytotoxicity has been shown using isolated monocytes and macrophages [20]. The assays used to demonstrate monocyte/macrophage antitumour reactivity however, are long term 48–72 hr tests, whereas NK cell reactivity is manifest in 4–6 hr.

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The present study was prompted by the observation in our laboratory that C57Bl mice possessed relatively low levels of spleenic natural cytotoxicity towards tumour targets (Rees and Hassan, in preparation), whereas effector cells derived from the mesenteric lymph nodes proved highly reactive. Studies by other workers have indicated the potential cytotoxicity of mesenteric lymph node cells [2, 12], but detailed studies establishing the effector cell types have not been performed. We report here that C57Bl lymph node cells prove to be highly cytotoxic towards tumour cell targets in a short-term cytotoxicity assay, and that this reactivity is entirely due to an adherent cell faction possessing the properties of phagocytic macrophages.

MATERIALS AND METHODS

Animals and tumours

C57Bl mice, aged 4–8 weeks, were obtained from our own inbred colony. An ascites line of the T-lymphoma EL4 was kindly supplied by Dr. E. Purves, St. Mary's Hospital Medical School, London, and the T-lymphoma TLX9 was obtained from Dr. R. Evans, The Chester Beatty Research Institute,

Sutton, Surrey. Both lymphoma lines were maintained by weekly transplantation of ascites cells into the peritoneal cavity of the male C57Bl mice.

The Mc2B-sarcoma line was originally derived by inoculation of 500 μg of 3-methylcholanthrene (MCA) subcutaneously (s.c.) into a 4-week-old male C57Bl mouse. The HSV2-333-8-9 hamster tumour, originally derived by transformation of hamster embryo cells *in vitro* by herpesvirus hominis Type 2, was kindly supplied by Dr. F. Rapp. *In vivo* transplant lines established from these cells, give rise to undifferentiated fibrosarcomas. The SV40-induced F-5-1 hamster fibrosarcoma was obtained from Flow Laboratories, Irvine, Scotland. All solid tumour lines have been maintained by s.c. trocar implantation of tissue fragments.

The erythro-leukaemic cell line K562 was obtained as a gift from Dr. M. Moore, Paterson Laboratories, Manchester and the Raji Burkitts lymphoma cell line from Dr. R. Walker, The Department of Biological Studies, University of Warwick. Both lines have been maintained *in vitro* as suspension cultures in RPMI-1640 medium supplemented with 10% fetal calf serum.

Preparation of target cells

EL4 and TLX9 T-lymphoma cell targets were harvested from the peritoneal cavity of C57Bl mice by injection of 8 ml of medium 199 with added heparin (10 U/ml), and the ascitic fluid aspirated using a multi-perforated needle. The cells were washed three times in medium 199, and adjusted to the required concentration for use in experiments.

K562 and Raji cells from *in vitro* cultures were washed three times in medium 199, adjusted to the required concentration and used in experiments. Single cell suspensions were prepared from solid tumour tissue by Trypsin digestion (0.2% Difco 1:250). The cells were prevented from clumping by the addition of DNase (0.02% w/v), washed three times in medium 199, adjusted to the required concentration and used in experiments. The viability of target cell suspensions was determined by Trypan Blue exclusion, and only cell preparations with greater than 90% viability were used in tests.

Preparation of effector cells

Effector cells were derived from the lymph nodes, thymus and spleens of healthy normal C57Bl mice aged 4–8 weeks. Lymphoid organs were removed, and passed through a 120 gauge stainless steel mesh, and the cells harvested in medium 199. Contaminating RBC were removed from the spleen cell suspensions by treatment with ammonium chloride (0.33% w/v); all effector cell preparations were washed threee times in medium 199 prior to use in experiments.

Preparation and use of anti-Thy 1:2 *antisera*

Anti-Thy 1:2 serum was prepared by immunization of AKR mice with 3 weekly injections of 1×10^7 CBA thymocytes, and the antiserum was shown to be selectively cytotoxic in the presence of complement, for thymocytes prepared from CBA and C57Bl mice, but not AKR mice. Anti-Thy 1:2 treatment of effector cells was performed as follows: mesenteric LNC (2×10^7 cells) were treated with the appropriate dilution of AKR anti-Thy 1:2 antiserum for 45 min at 4°C, centrifuged, and the supernatant removed; the cells were incubated with guinea pig complement for a further 30 min at 37°C, and the remaining effector cells washed three times in medium 199 prior to use in experiments.

Nylon wool column fractionation

This method is described elsewhere [21, 22]. Briefly, 0.5 g of nylon fibre was packed in 5 ml plastic syringes and saturated with medium 199 containing 5% FCS. Up to 1×10^8 cells were loaded onto each column and incubated at 37°C in 5% CO_2 in air for 45 min. Both column eluted and column retained cell fractions were collected, and the cells used as effectors.

Carbonyl iron treatment

Effector cells were adjusted to $2–3 \times 10^7$ cells/ml and 4 mg of carbonyl iron powder added to 3 ml of cells, and the mixture incubated at 37°C for 30 min. Carbonyl iron was sedimented on a magnet for 10 min at 4°C, the supernatant removed and the sedimentation repeated. The recovered cells were washed and adjusted to 5×10^7/ml and used in experiments.

Glass adherence

Effector cells (1×10^7) were incubated in serum free medium on glass Petri dishes for 2 hr in an atmosphere of 5% CO_2/95% air at 37°C. Non-adherent cells were recovered by vigorous washing of the Petri dish surface with medium 199.

Treatment with trypsin and ammonium chloride

Effector cells (1×10^7) were incubated with either 10 ml of 0.2 Trypsin or 0.8% NH_4Cl_2 for 10, 20 and 30 min, after which the cells were sedimented and washed three times in medium 199 prior to use in experiments.

Chromium-51 release assay

In the present study we have used a 4-hr ^{51}Cr-release test to measure natural cytotoxicity. Target cells at 1×10^7 cells/ml were labelled with $Na_2{}^{51}CrO_4$ (100 μ Ci) (Radiochemical Centre, Amersham, Bucks) for 1 hr at 37°C, after which time the cells were washed three times with medium 199, resuspended in medium 199 and incubated for a further hour at 37°C. The cells were then washed three times and re-suspended at a concentration of 10^6 cells/ml in medium 199. Effector cells (0.1 ml in medium 199) at various ratios with target cells, were added to 0.1 ml of ^{51}Cr-labelled target cells (in triplicate) in Nunc-U-bottomed microtest plates (Gibco Biocult, Paisley, Scotland), and incubated at 37°C in a 5% CO_2 atmosphere for 4 hr. The cells were sedimented by centrifuging at 200 *g* for 5 min and 0.1 ml of supernatant removed into separate wells. The plates were dried, sealed with parafilm and the individual wells counted in a gamma spectrophotometer. The ^{51}Cr-release was determined using the following formula:

$$\text{percentage of } {}^{51}\text{Cr-release} = \frac{(1/2 \text{ supernatant}) \times 2}{(1/2 \text{ supernatant}) + (\text{cells} + 1/2 \text{ supernatant})} \times 100$$

The results presented here represent the percentage of ^{51}Cr-release in the presence of test lymphoid cells minus the spontaneous release, which was usually in the range of 5–10%. Statistical analysis was performed using Students t-test and the significance indicated by, $*P<0.001$; $\dagger P<0.01$; $\ddagger P<0.05$. The results presented in Figs. 1–3 are given as the mean percentage cytotoxicity ± S.E.

RESULTS

Cytotoxicity towards tumour cell targets

The distribution of cytotoxic effector cells from the spleen, lymph nodes and thymus, reactive towards EL4 lymphoma targets was assessed, and results, typical of those routinely obtained, are shown in Fig. 1. Effector cells derived from the mesenteric lymph nodes, used in E:T ratios of 10:1 to 100:1, were cytotoxic for EL4 targets; the level of reactivity being distinctly higher than that observed with effector cells derived from the spleen, thymus or other lymph nodes (inguinal and axillary lymph nodes). Low but significant mesenteric LNC cytotoxicity was observed after only 1 hr, and cytotoxicity reached a maximum, and was complete after 3 hr (50:1, E:T ratio) (Fig. 2).

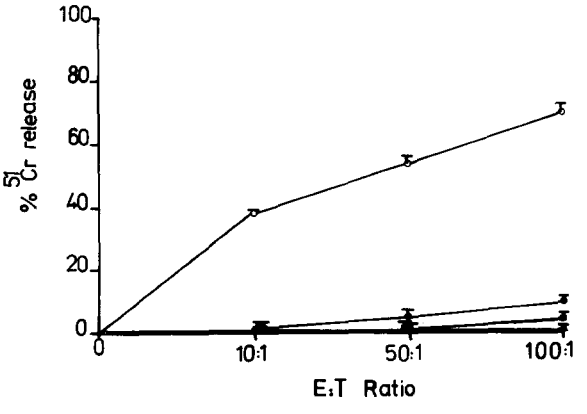

Fig. 1. Cytotoxicity of C57Bl mouse lymphoid cells prepared from various lymphoid organs towards EL4 lymphoma target cells. Effector cell prepared from spleen (●—●), thymus (△—△), axillary lymph nodes (■—■), inguinal lymph nodes (▲—▲) and mesenteric lymph nodes (○—○).

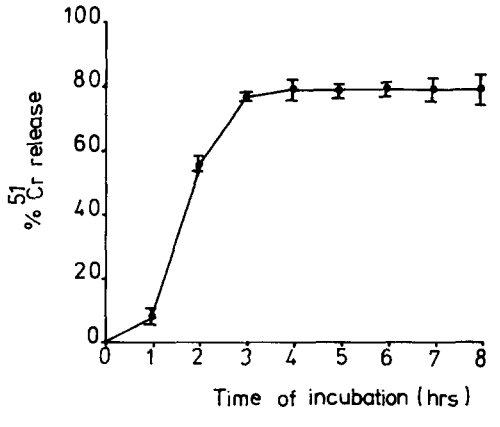

Fig. 2. Natural cytotoxicity of C57Bl mouse mesenteric lymph node cells against EL4 lymphoma target cells; time course of reactivity.

The specificity of this rapid cytotoxicity was assessed against several *in vitro* cultured, and *in vivo* derived, tumour and normal cell targets (Table 1). *In vivo* derived EL4 and TLX9 lymphoma cells and Mc2B sarcoma cells of C57Bl origin, were susceptible to killing by

Table 1. *Natural cytotoxicity against tumour and normal cell targets*

Target cells	Origin of targets	Percentage cytotoxicity Exp.1 (E:T ratio) 10:1	50:1	100:1	Exp.2 (E:T ratio) 10:1	50:1	100:1
Hep-2	*In vitro* cell line—human	33*	59*	54*	38*	52*	59*
K562	*In vitro* cell line—human	50*	70*	64*	72*	68*	68*
RAJ1	*In vitro* cell line—human	77*	76*	74*	74*	72*	71*
BHK-21	*In vitro* cell line—hamster	39*	60*	72*	74*	79*	78*
HSV2-333-8-9	*In vivo* cell line—hamster	7⁻	59*	63*	—	—	—
SV40-F-5-1	*In vivo* cell line—hamster	29*	62*	77*	—	—	—
Mc2B	*In vivo* line—C57Bl mouse	−2	6	64*	−5	1	47*
TLX9	*In vivo* line—C57Bl mouse	48*	73*	76*	33*	62*	65*
EL4	*In vivo* line—C57Bl mouse	−5	23*	47*	75*	51*	88*
14d embryo fibroblasts	C57Bl mice	1	+1	2	−1	−1	1
Lymph node cells	C57Bl mice	−1	8	11	NT	NT	NT
Spleen cells	C57Bl mice	−7	9	4	NT	NT	NT
Kidney cells	C57Bl mice	3	−1	−1	8	2	2
Thymocytes	C57Bl mice	−15	−10	9	19	20†	20‡

C57Bl mesenteric LNC, and target cells prepared from *in vivo* hamster transplant tumour lines HSV2-333-8-9- and SV40-F-5-1 were also highly susceptible to killing by mesenteric LNC. The human lymphoblastoid NK-sensitive line K562 and the NK-resistant line Raji ([3] and Rees, unpublished observations) were both shown to be susceptible to mesenteric effector cell killing. Cells derived from normal C57Bl mouse tissue (embryo and kidney fibroblasts, spleen, LNC and thymus cells) were resistant to killing, but mesenteric LNC cytotoxicity could be demonstrated towards cells from long-term tissue culture lines of HEP-2 or BHK-21. These results infer a preferential killing by mesenteric cytotoxic cells of tumour targets, or long-term tissue culture cells, and also that this form of killing is not species restricted. Unlike previous studies relating to NK activity, mouse mesenteric LNC cytotoxicity was not age dependent (Table 2).

Table 2. *Natural mesenteric lymph node cell cytotoxicity: lack of age restriction in C57Bl mice*

Age (weeks)	Percentage cytotoxicity§ Exp. 1	Exp. 2
1	84*	85*
4	75*	41*
8	80*	43*
16	82*	87*
45	80*	77*

§E:T ratio of 100:1 was used in all experiments.

Characterization of effector cells

Using as target cells *in vivo* transplant lines of EL-lymphoma and Mc2B-sarcoma, experiments were performed to establish the characteristics of the cytotoxic effector cell present in the mesenteric lymph nodes of C57Bl mice. The results using EL4 and Mc2B target cells (Table 3), clearly show the mesenteric effector cells to be adherent and possess characteristics similar to those of macrophages. More precisely, cytotoxicity was removed following carbonyl iron treatment, adherence to glass, and nylon wool fractionation; effector cells could not be recovered from the nylon wool column-retained fraction. Treatment of the effector cells with anti-Thy 1:2 serum and complement failed to significantly decrease the cytotoxic potential of mesenteric LNC, which also proved resistant to treatment with trypsin, ammonium chloride or 1500 rad X-irradiation. In Table 4, results are presented further substantiating adherent cells as the main cytotoxic type; reactivity towards HEP-2, BHK-21 and K562 was completely abolished by removal of glass adherent cells. Experiments were performed using cells adherent to the surface of micro-test plate (flat-bottomed) wells. In these studies C57Bl mesenteric LNC were added to wells for 60 min at 37°C at cell concentrations equivalent to those normally used in 4-hr assays, after which the non-adherent cells were removed by successive washings with medium 199. ^{51}Cr-labelled EL4 or Mc2B targets (10^5 cells in 0.2 ml), were then added to each well, and the percentage ^{51}Cr-release estimated after a 4-hr incubation period. The results of these

experiments are shown in Table 5, and prove conclusively that an adherent cell population is responsible for cytotoxicity.

Table 3. Characterization of natural cytotoxic effector cells from C57Bl mouse mesenteric lymph nodes

Effector cells—Treatment	Exp. No.	Percentage cytotoxicity§ 1 EL4	2 EL4	3 Mc2B
Carbonyl iron		−1	0	−2
Untreated		95*	58*	50*
Glass adherence		0	1	−1
Untreated		95*	58*	50*
Nylon wool column eluted fraction		0	1	−1
Nylon wool column retained fraction		9	8	−1
Unfractionated		95	76*	50*
Anti-Thy 1:2		62*	51*	18*
Anti-Thy 1:2 + complement		70*	52*	17*
Complement		73*	56*	26*
Untreated		74*	62*	26*
Trypsin				
2 min		74*	73*	25*
10 min		82*	86*	25*
20 min		85*	70*	25*
Untreated		80*	86*	36*
Ammonium chloride				
10 min		65*	72*	26*
20 min		64*	78*	25*
Untreated		80*	86*	36*
X-irradiation (rad)				
500		62*	72*	22*
1000		64*	71*	26*
1500		56*	70*	24*
Untreated		60*	86*	36*

§E:T ratio of 50:1 was used in all experiments.

Table 4. Cytotoxicity by adherent mesenteric lymph node cells from C57Bl mice

Exp. No.	Target cells	Treatment	Percentage Cytotoxicity (E:T ratio) 10:1	50:1	100:1
1	HEP-2	Glass adherence§	3	5	0
		Untreated	2	8	45*
2	BHK-21	Glass adherence	0	0	1
		Untreated	24‡	28†	24†
3	K562	Glass adherence	−1	−2	−2
		Untreated	−3	14‡	71*

§Glass adherent cells removed, and non-adherent fraction assayed for cytotoxicity.

Table 5. Natural cytotoxicity by adherent effector cells from C57Bl mesenteric lymph nodes

Exp. No.	Target cells	Percentage cytotoxicity§ (E:T ratio‖) 10:1	50:1	100:1
1	EL4	64*	72*	80*
2	EL4	50*	51*	55*
3	EL4	23*	68*	74*
4	Mc2B	21*	26*	26*
5	Mc2B	23*	32*	40*

§Cytotoxicity test performed using plastic adherent effector cells.
‖E:T ratio before removal of non-adherent effector cells.

Competition experiments

Experiments, using cold competitor cells to interfere with the cytotoxic reaction between isotope-labelled targets and effector cells, have been performed by several groups of workers in attempts to determine the specificity of NK receptor sites on target cells. We have used this approach to investigate whether or not target–effector cell binding occurs in our system, and is a prerequisite of the cytotoxic process. Results typical of those obtained in several experiments are shown in Fig. 3.

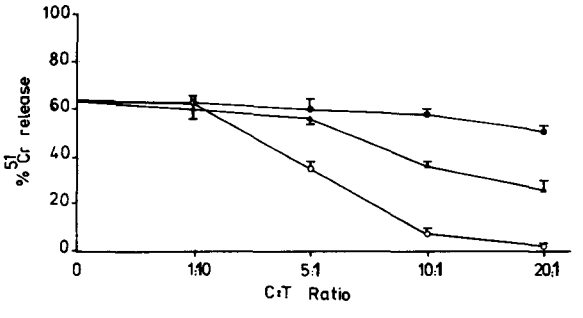

Fig. 3. Competitive inhibition of natural cytotoxicity by unlabelled EL 4 lymphoma cells. Mesenteric LNC: ^{51}Cr-labelled EL4 targets 50:1. EL4 competitors treated with 10% formol saline (●—●), or 25% glutaraldehyde (▲—▲), or untreated (○—○).

Using unlabelled EL4 cells, competition for mesenteric LNC effector cells was observed, and a rapid decrease in cytotoxicity was apparent with increasing concentrations of competitor cells. Competition by unlabelled EL4 cells was not due to mechanical interference, since a reduction in the competitive ability of EL4 cells was observed following fixation in 10% formalin saline or 25% gluteraldehyde solution. Thus, live intact cells provide the

best source of competitor cells, and this observation, together with results showing that supernatant material from cultured mesenteric LNC cultures does not contain lymphotoxic substances (Hassan and Rees, unpublished results), supports the thesis that the rapid killing by adherent mesenteric LNC observed here requires intimate contact between effector and target cells through a receptor site present on the intact cell membrane of the target cell.

DISCUSSION

The present study has shown that unstimulated, adherent effector cells, present in C57Bl mouse mesenteric lymph nodes are highly cytotoxic towards certain types of target cells in short-term (4 hr) ^{51}Cr-release tests (CRT). They appear distinct from NK cells by virtue of their adherent properties to glass and nylon wool. In addition, NK cells have been reported to be radiation sensitive [4], whereas the cells expressing cytoxicity here are radiation resistant. We have also observed that mesenteric LNC killing is not age restricted.

Published reports on macrophage–monocyte killing of tumour cells have shown cytotoxicity to occur following co-culture of target cells with effector cells from 18 hr up to 72 hr [15, 20, 23]. For example, Puccetti and Holden [15] have used an 18 hr cytotoxicity assay to assess peritoneal macrophage killing, and Tagliabue *et al.* [20] have shown mouse monocyte killing to occur between 48 and 72 hr of co-culture. Stutman *et al.* [13, 14] have demonstrated natural cytotoxicity in mice using a 24-hr proline pre-labelling assay, establishing the effector cells to be unlike NK cells, possessing adherent characteristics, as well as lacking the age dependence normally associated with NK cells. Datta *et al.* [12] have recently shown carrageenan sensitive NC reactivity in hamster spleen cell preparations using adherent targets and a 16–18 hr incubation period, while studies in this laboratory have detected rapid killing of hamster and mouse tumour targets by adherent, nylon wool column retained effector cells present in Syrian hamster spleens [24]; in this instance cytotoxicity was detected using a 4-hr assay.

The present paper extends further the investigations into natural cytotoxicity [25], and presents evidence that the innate reactivity of mesenteric lymph node cells is entirely dependent on adherent effector cells. Thus, killing was completely abolished by treatment of the effector cells with carbonyl iron, and by adherence to glass. In addition, using the non-adherent cell fraction, cytotoxicity could be shown towards the NK sensitive target K562; killing of K562 cells by mouse NK effectors has previously been reported [3]. Natural cytotoxicity by mesenteric LNC appeared to be selective for *in vivo* or *in vitro* cultured tumour targets, including mouse and hamster sarcoma cells (Mc2B, SV40-F-5-1 and HSV2-333-8-9 lines) and ascites T-lymphoma targets (EL4 and TLX9 lines). Freshly prepared mouse lymphoid cells or fibroblast targets were insensitive to this cytotoxic activity, but long term *in vitro* cultured lines (HEP2 and BHK-21 cells) acted readily as targets. In contrast to reports pertaining to macrophage killing, mouse mesenteric LNC elaborated rapid cytotoxicity, which appeared to require interaction with target receptor sites. This was concluded following the observed decrease in ^{51}Cr-release in competition experiments using EL4 cells as competitors. In this instance, competition was unlikely to be due to mechanical interference by unlabelled competitors, since formalin or gluteraldehyde fixed EL4 cells failed to abrogate the cytotoxicity, compared with observed inhibition using equivalent numbers of unfixed EL4 cells.

It can be concluded from the present study that there exists a population of naturally cytotoxic cells resident in mesenteric lymph nodes of mice, which are highly reactive towards a wide range of target cells. The observed killing, which was due to adherent phagocytic effector cells, was indeed more rapid than has been observed with adherent peritoneal or intra-tumour macrophages [16, 23, 25, 26].

An understanding of the relationship between the different limbs of the "Natural Immune" response is a prerequisite to determining their relative role as antitumour effector mechanisms, and in particular their relevance in immune surveillance. In this context a careful appraisal of natural cytotoxic and natural killer mechanisms is required.

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