Immunology 1985 **54** 265

Reversibility of lymphokine-induced NK-like activity in virus-specific cytotoxic T-lymphocyte clones

FRANCA RONCHESE, D. COLLAVO, PAOLA ZANOVELLO, V. CERUNDOLO & G. BIASI
Laboratory of Oncology, University of Padova, Italy

Accepted for publication 11 September 1984

Summary. A limiting dilution microculture system, supplemented with a source of interleukin-2 (IL-2), was employed to evaluate the frequency of Moloney-murine leukaemia/sarcoma virus (M-MuLV/M-MSV)-specific cytotoxic T-lymphocyte precursors (CTL-p) which also exhibited NK-like activity. Spleen cells, obtained from M-MuLV/M-MSV regressor mice, were restimulated in bulk secondary mixed leucocyte-tumour cell cultures (MLTC), and subsequently plated in a culture medium supplemented with two different supernatants (SN) produced following PMA-stimulation of the same EL-4 thymoma cell line. SN 20, obtained from the cell line maintained *in vitro*, contained IL-2 and only negligible amounts (< 3 U/ml) of interferon (IFN), while SN 19, obtained after passage of the ascitic form of EL-4 thymoma in syngeneic mice, contained both IL-2 and IFN in high titres. The frequency of CTL-p specific for MBL-2 lymphoma cells was high and comparable in cultures supplemented with both SN (1/2·84 cells and 1/2·40 cells, respectively), while the frequency of CTL-p directed against NK-susceptible YAC-1 target cells was low in SN 20 (1/90 cells) and high in SN 19 (1/5·40 cells). An analysis of individual microcultures established at low cell dose (1 cell/well) indicated that specific and NK-like activity could be ascribed to the same precursor cells. Furthermore, using different long-term CTL clones, we observed that, after passage in SN 20, double-reactive clones gradually lose the capacity to lyse NK-susceptible targets, while most of MBL-2 specific clones acquired NK-like activity following a few passages in SN 19. Therefore, the induction of NK-like activity is reversible and may be modulated by soluble factors present in supernatant in which CTL clones are maintained. Double-reactive clones were unable to lyse NK-resistant allogeneic tumour cells or normal syngeneic blast cells. A few clones cross-reacting with H-2^d alloantigens also exhibited NK-like activity when maintained in SN 19. The different pattern of CTL clone activity was associated with a morphological change in the clones themselves: the acquisition of double activity was accompanied by an increase in cell size and the appearance of numerous cytoplasmic granules. All CTL clones were phenotypically Thy-1^+ and Lyt-2^+ on indirect immunofluorescence and complement-dependent cytotoxicity investigation. However, following preincubation with anti-Lyt-2 monoclonal antibodies in the absence of complement, some clones were inhibited in their M-MuLV/M-MSV specific cytotoxicity, while none were affected when assayed against the YAC-1 target cells. Therefore, it appears that different recognition structures are in-

Abbreviations: CTL, cytotoxic T lymphocytes; CTL-p, cytotoxic T-lymphocyte precursor; IFN, interferon; IL-2, interleukin-2; M-MuLV/M-MSV, Moloney-murine leukaemia/sarcoma virus complex; MLTC, mixed leucocyte-tumour cell culture; PMA, phorbol myristate acetate; SN, supernatant.

Correspondence: F. Ronchese, Cattedra di Oncologia, Ospedale Busonera, via Gattamelata 64, 35128 Padova, Italy.

volved in the specific and the NK-like activities of CTL clones.

INTRODUCTION

Immunological defence mechanisms against altered cell antigens are mainly mediated by cells with killer activity. While antigen-specific killing is characteristic of cytotoxic T lymphocytes (CTL), non-specific killing of a wide spectrum of target cells has been ascribed to another group of cells which still lacks precise identification, and which includes natural killer (NK) and activated lymphocyte killer (ALK) cells. Klein (1983) proposed a relationship between these different cell types; this suggestion has been supported by the observation that numerous CTL clones exhibit NK-like activity, besides their typical target cell specificity (Neefe & Carpenter, 1982; Pawelec *et al.*, 1982; Moretta *et al.*, 1983; Lopez-Botet, Moretta & Moretta, 1983; Binz *et al.*, 1983; Acha-Orbea *et al.*, 1983; Shortman *et al.*, 1983; Brooks *et al.*, 1983a). In addition, Brooks *et al.* (Brooks, 1983; Brooks, Urdal & Henney, 1983b) recently reported that antigen-specific CTL clones may acquire a new lytic activity, which apparently is identical to that of NK cells, when cultured in a medium containing interleukin-2 (IL-2) and interferon (IFN). Preliminary studies indicated that both factors are required for the induction of this activity (Brooks, 1983).

We observed that a subclone of the IL-2-producing EL-4 lymphoma cell line also produces large amounts of IFN when maintained in ascites form by transfer into syngeneic recipient mice. In addition, the supernatant of this subclone, when added to spleen cells from unprimed mice, supports the growth of cells with NK-like activity (Collavo *et al.*, 1984).

Using a limiting dilution microculture system, which allows CTL generation under clonal conditions (Brunner, MacDonald & Cerottini, 1980; Collavo *et al.*, 1982), we have now found that a high percentage of CTL precursors (CTL-p) specific for antigens induced by Moloney-murine leukaemia/sarcoma virus complex (M-MuLV/M-MSV) acquires NK-like activity when cultured in medium supplemented with a supernatant containing high IL-2 and IFN titres. This ability to lyse NK-susceptible target cells by double-reactive clones appears reversible and could depend on the activity of different lymphokines present in the supernatants added to the culture medium.

MATERIALS AND METHODS

Mice

Inbred C57BL/6 mice, originally purchased from the Jackson Laboratory (Bar Harbor, ME), and maintained in our colony for several generations by sister × brother matings, were used throughout this study.

Virus

M-MuLV/M-MSV extract 0·05 ml, which had an *in vitro* titre of 3×10^6 focus-forming units per ml on 3T3/FL cells, was injected intramuscularly in the thigh region of 6–8-week-old mice. Mice were killed 2–3 weeks later and used as spleen cell donors.

Tumour cell lines

The NK-resistant MBL-2 (M-MuLV-induced lymphoma in C57BL/6 mice), YAC (M-MuLV induced lymphoma in A/SN mice) and YC8 (M-MuLV-induced lymphoma in BALB/c mice) cell lines were all maintained by weekly intraperitoneal passages of 2×10^6 cells in syngeneic hosts.

The NK-sensitive YAC-1 (a subclone of YAC selected for NK sensitivity) and Jurkat (a human T leukaemia) cell lines were maintained by continuous *in vitro* culture in complete medium. Complete medium consisted of Dulbecco's modified minimal essential medium (MEM, GIBCO, Glasgow, Scotland) supplemented with L-glutamine, HEPES, 2-mercaptoethanol, antibiotics and 10% heat-inactivated fetal calf serum (FCS, GIBCO).

Cultures

Secondary mixed leucocyte-tumour cell cultures (MLTC) were carried out as previously described (Collavo *et al.*, 1982). Briefly, 2×10^7 responder spleen cells from M-MuLV/M-MSV injected mice and 5×10^6 mitomycin *c* (m)-treated (40 μg/5×10^6 cells/ml) MBL-2 stimulator cells were cultured for 7 days in a 50 ml flask (Sterilin, Teddington, Middlesex, U.K.) in a total volume of 15 ml complete medium. Micro-MLTC were established by plating limiting numbers of responder cells in round-bottomed microplates (Sterilin) in the presence of 3×10^4 m-treated MBL-2, and 5×10^5 m-treated syngeneic accessory spleen cells (Collavo *et al.*, 1982). Cells were cultured for 7 days in a final volume of 0·2 ml complete medium supplemented with 5% v/v EL-4 supernatant as a source of growth factors. In some experiments, on day 7, cultures were split into different microplates and

cultured further for 4 days before testing for cytotoxicity against different targets, or for further expansion.

Derivation and maintenance of CTL clones

All clones, except clone C11-D2 which was derived by single cell micromanipulation, were expanded and recloned by limiting dilution at a density of 0·3–0·1 cells per well. Selected clones were maintained by seeding, at 4–6 days intervals, 5×10^4 clone cells in 16 mm multiwell plates (Costar 3596, Cambridge, U.K.) containing 3×10^5 m-treated MBL-2 cells and 5×10^6 accessory cells in 1·5 ml complete medium supplemented with 5% v/v EL-4 supernatant. Cell aliquots were periodically frozen at −80° in culture medium containing 10% dimethyl sulphoxide and 70% FCS.

Supernatants (SN)

SN 20 was obtained from an EL-4 lymphoma cell line subclone (Farrar *et al.*, 1980) producing high titres of IL-2 following *in vitro* stimulation for 48 hr with phorbol myristate acetate (PMA, 30 μg/10^6 cells/ml; Sigma, St Louis, MO). SN 19 was obtained from the same subclone, which was maintained by one or two weekly intraperitoneal passages of the ascites form in syngeneic recipient mice before *in vitro* PMA stimulation.

Lymphokine assays

IL-2 activity was measured as [^{3}H]thymidine (TdR) incorporation by an IL-2-dependent CTL clone as described by Gillis *et al.* (1978). IL-2 activity was expressed as the reciprocal of the SN dilution producing 50% maximal [^{3}H]TdR incorporation with reference to a standardized IL-2 preparation. IFN activity of supernatant fluids was titrated with respect to their ability to inhibit the cytopathic effects of vesicular stomatitis virus on a monolayer of L929 cells as described by Campbell *et al.* (1975). IFN units were expressed as the reciprocal of the dilution inhibiting cytopathic effects by 50%.

Cytolytic assays

Cytolytic activities of micro-MLTC were assessed by removing 100 μl of supernatant from each well and adding 2×10^3 ^{51}Cr-labelled target cells; the plates were incubated for 4 hr at 37°, then centrifuged and the supernatants removed for counting. Spontaneous release values were determined in control cultures prepared in the same manner as the experimental groups, but without responder cells. For the calculation of CTL-p frequencies, microcultures were scored as positive or negative, with positive cultures defined as those with ^{51}Cr-release values exceeding the mean spontaneous release values by more than three standard deviations (SD). Minimal estimates of CTL-p frequencies were then calculated from the single hit Poisson model equation according to Taswell, MacDonald & Cerottini (1979). Cytolytic activities of CTL clones were assayed by incubating in round-bottomed microtitre plates (Sterilin) three-fold dilutions of effector cells with 2×10^3 ^{51}Cr-labelled target cells in a final volume of 0·2 ml. After 4 hr, plates were centrifuged and processed as detailed above.

Percent specific ^{51}Cr-release was calculated according to the formula:

$$100 \times \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}$$

with spontaneous release and maximum release determined by incubating 2×10^3 labelled cells with the medium alone or with a 5% solution of Triton-X.

Inhibition of cytolytic activity by anti-Lyt-2 monoclonal antibodies

CTL clone cells, 30×10^3, were preincubated with either medium or four-fold dilutions of anti-Lyt-2 monoclonal antibodies in round-bottomed microplates for 30 min at 20°. At the end of incubation, 2×10^3 ^{51}Cr-labelled target cells were added to give a final volume of 0·2 ml. After 4-hr incubation at 37°, cytolytic activities were assayed as reported above. The supernatant of the hybridoma cell line 3.239.2, kindly provided by Dr F. Fitch (University of Chicago), was used as a source of monoclonal antibodies against the polymorphic Lyt-2.2 determinant.

RESULTS

Frequency of M-MuLV/M-MSV-specific CTL-p with NK-like activity

Farrar *et al.* (1980) reported that PMA stimulates an EL-4 lymphoma subline to produce high IL-2 titres. We observed that this cell line, after passage in syngeneic recipient mice, also acquired the ability to produce IFN. As shown in Table 1, SN 20 obtained from the EL-4 cells maintained *in vitro* contained IL-2, but little, if any, IFN. However, SN 19, which was obtained from cultures of these cells harvested from the ascites tumour, contained both IL-2 and IFN in high titres. Since Brooks *et al.* (Brooks, 1983; Brooks *et al.*, 1983b) recently suggested that both IL-2 and

Table 1. IL-2 and IFN titres of supernatants obtained from PMA-stimulated EL-4 tumour cells maintained *in vivo* or *in vitro*

	IL-2*	IFN†
SN 20‡	19 U/ml	<3 U/ml
SN 19§	117 U/ml	729 U/ml

* IL-2 titre is expressed as the reciprocal of the dilution giving 50% of the maximal proliferation of an IL-2 dependent CTL clone.

† IFN titre is expressed as the reciprocal of the dilution giving 50% protection of a monolayer of L929 cells from the cytopathic effects of vesicular stomatitis virus.

‡ SN 20 was obtained following PMA-stimulation of the EL-4 lymphoma subline maintained *in vitro*.

§ SN 19 was obtained following PMA-stimulation of the same EL-4 subline which was maintained *in vivo* by passaging the ascitic form in syngeneic mice.

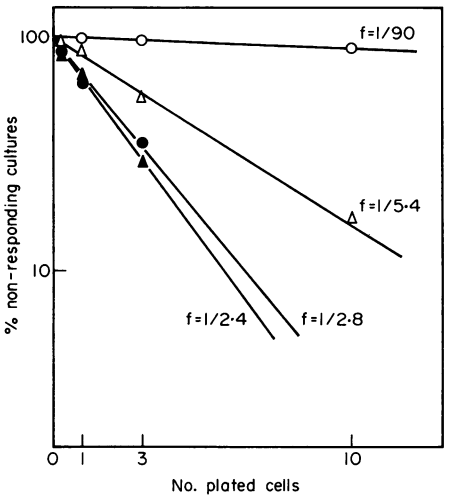

Figure 1. Frequencies of CTL-p having M-MuLV/M-MSV-specific and NK-like activity. Limiting numbers of MLTC cells were cultured with m-treated MBL-2 stimulator and syngeneic accessory cells in the presence of 5% v/v SN 19 (▲,△) or SN 20 (●,○). After 7 days, each microculture was split into two aliquots, cultured further for 4 days, and assayed against the relevant MBL-2 (▲,●) and the NK-sensitive YAC-1 (△,○) target cells in a 4-hr ^{51}Cr-release assay.

IFN are involved in the expression of NK-like activity by antigen-specific CTL clones, we considered it interesting to study whether M-MuLV/M-MSV-specific CTL-p could acquire NK-like cytotoxicity when cultured in medium supplemented with SN 19. Therefore, cells recovered from bulk secondary MLTC were cultured under limiting dilution conditions (10–0·3 cells/well) with m-treated syngeneic accessory and MBL-2 stimulator cells, in the presence of SN 19 or SN 20, which served as source of growth factors. After 7 days, each individual culture was split and assayed for lytic activity against the relevant target cells (i.e. MBL-2 lymphoma cells) and against NK-susceptible target cells (i.e. the YAC-1 cell line). As shown in Fig. 1, similar MBL-2-specific CTL-p frequencies were obtained using both supernatants (1/2·40 with SN 19 and 1/2·84 with SN 20), while the precursor frequency for YAC-1 target cells was 1/5·40 and 1/90 in a medium supplemented with SN 19 and SN 20, respectively. An analysis of the lytic activity in individual microcultures established at low cell dose (1 cell/well) disclosed that 12 of 26 cultures with specific activity in the presence of SN 19 were also lytic for YAC-1 cells, while in the presence of SN 20, the majority of cultures exhibited specific activity only (Fig. 2). The high percentage of double-reactive cultures, in view of the low number of cells plated, and the observation that in no culture were only YAC-1 cells lysed, strongly indicate that most of the cytotoxic cells contained in the microwells were derived from the same precursor cells.

A small percentage (7%) of M-MuLV-specific CTL clones have been reported cytotoxic also for allogeneic Moloney leukaemia cells (Brunner *et al.*, 1980). Since the YAC-1 cell line derives from a M-MuLV-lymphoma originally induced in A/Sn mice, we considered the possibility that the high frequency of double reactive CTL-p, observed when SN 19 was used, could be due to the increase in such cross-reactive precursors. Thus, micro-MLTC, obtained by plating 1 cell/well in a medium containing SN 19, were also tested against the NK-resistant YAC cell line, and against the NK-susceptible Jurkat cell line of human origin. As shown in Fig. 3, only a few cultures lysed the YAC cells, while the majority of cultures were positive for both YAC-1 and Jurkat NK-susceptible target cells.

Effect of different supernatants on M-MuLV/M-MSV-specific CTL clone cytotoxicity

The clonality of cells obtained by limiting dilution was further confirmed in subcloning experiments. Cells

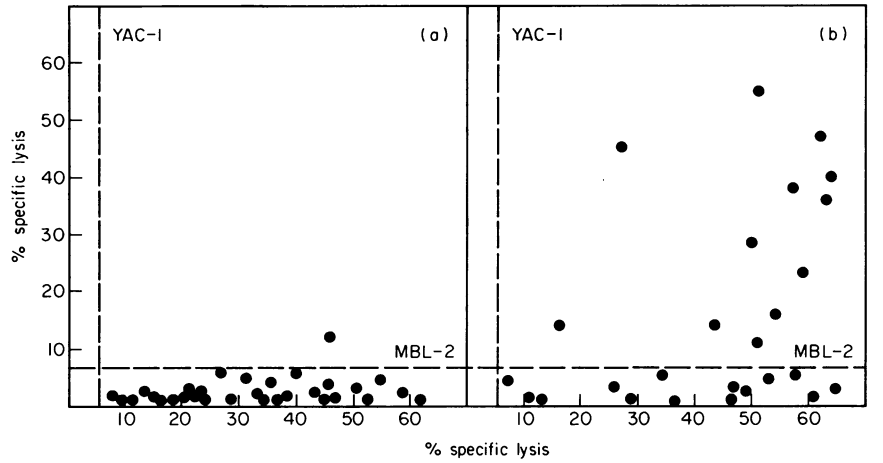

Figure 2. Comparison of the specific and NK-like cytotoxicity of individual micro-MLTC obtained in a culture medium supplemented with (a) SN 20 or (b) SN 19. After 7 days, microcultures established with 1 MLTC cell/well, were split into two aliquots, cultured further for 4 days, and assayed against MBL-2 and YAC-1 target cells. The dashed lines represent the mean + three SD ^{51}Cr-release of control cultures. Only results for positive microcultures are shown.

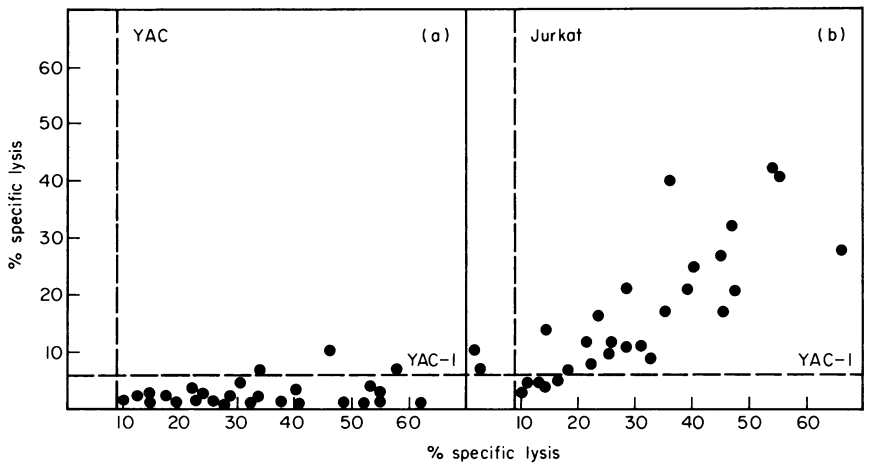

Figure 3. Pattern of cytotoxicity of individual micro-MLTC obtained in a culture medium supplemented with SN 19. Individual microcultures, established as reported in Fig. 2, were assayed against (a) the NK-sensitive YAC-1 and NK-resistant YAC target cells, or (b) against the NK-sensitive YAC-1 and Jurkat target cells. The dashed lines represent the mean + three SD ^{51}Cr-release of control cultures. Only results for positive microcultures are shown.

from different micro MLTC were transferred into 24-well plates containing stimulator and accessory cells; responding cells were subsequently recloned by plating 0·3–0·1 cells/well in the same supernatant in which they were originally obtained. In the majority of cases, the cytolytic pattern of recloned cells was similar to that of the parental population, thus confirming the clonality of the original isolates (data not shown).

By using this procedure, we obtained CTL clones with specific cytotoxicity in SN 19 or in SN 20, as well as clones also showing NK-like activity in both supernatants. Following three passages in the original

supernatant, selected clones were then transferred into both supernatants in the presence of stimulator and accessory cells, and tested repeatedly for lytic activity. As shown in Table 2, most of the clones originally obtained in SN 20 either lysed YAC-1 cells (clone E7-G2) or showed increased NK-like activity (clone C11-D2) after passage in SN 19; only a few (clone B4-C11) remained specific following repeated passages in SN 19. Clones originally obtained in SN 19 (Table 3) and showing double reactivity became exclusively M-MuLV/M-MSV-specific after three to four passages in SN 20 (clone D7-D6). Clones with specific activity in SN 19 acquired NK-like activity when repeatedly passaged in SN 19, but reverted to specific activity when cultured in SN 20 (clone C11-C4). It is worth mentioning that the specific or double activity acquired by all CTL clones became stable starting from three to four passages in the same SN.

When analysed by light microscopy, all clones maintained in SN 19 consisted of round, large cells with numerous prominent cytoplasmic granules, while in SN 20 cells were smaller and without apparent granules. As Brooks *et al.* (1983b) previously reported, it was possible to predict whether CTL clones would present specific or double activity from their morphological feature.

In order to define the activity pattern more clearly, several clones were assayed against different target cells. As shown in Table 4, clones lysing MBL-2 and YAC-1 cells in SN 19 were ineffective against the NK-resistant YAC target cells, as well as allogeneic (H-2^d) YC8 tumour and normal BALB/c blast cells. Clone E5-C2 in SN 20 cross-reacted with allogeneic H-2^d target cells (YC8 and BALB/c cells), and in SN 19 acquired the capability to lyse YAC-1 cells as well. Finally, clone B4-C11 in both supernatants was cytotoxic only against MBL-2 cells. None of these clones were cytotoxic for syngeneic Con A-induced blast cells.

Effect of anti-Lyt-2 monoclonal antibody treatment on CTL clone activity

All CTL clones obtained in SN 19 or SN 20 expressed Thy-1 and Lyt-2 antigens, as evaluated by complement-dependent lysis or indirect immunofluorescence. We found that pre-treatment of clones with anti-Lyt-2 monoclonal antibodies and complement abolished cytotoxic activity against both relevant and NK-susceptible target cells (data not shown).

MacDonald, Glasebrook & Cerottini (1982) reported that M-MuLV-specific CTL clones are heterogeneous in terms of their susceptibility to inhibition of lytic activity by anti-Lyt-2 monoclonal antibodies. In addition, when cross-reacting clones are incubated with these antibodies, lysis of allogeneic tumour cells is abolished, while lysis of syngeneic M-MuLV-infected cells is only weakly inhibited, if at all (MacDonald

Table 2. Modulation of cytotoxic activity of CTL clones originally obtained in SN 20

CTL clones	Target cells	Cytotoxic activity of clones from:* Original culture	Subculture in SN 20	Subculture in SN 19†
E7-G2	MBL-2	22	31	50
	YAC-1	1	3	26
C11-D2	MBL-2	37	30	42
	YAC-1	13	17	54
B4-C11	MBL-2	31	36	46
	YAC-1	0	1	2

* Percent specific lysis at an effector to target cell ratio of 20:1. Each value refers to the activity of the original culture or is representative of the activity of subcultures assayed at different passages (three to ten).

† Clones transferred in SN 19 after three to four passages in SN 20.

Table 3. Modulation of cytotoxic activity of CTL clones originally obtained in SN 19

CTL clones	Target cells	Cytotoxic activity of clones from:* Original culture	Subculture in SN 19	Subculture in SN 20†
D7-D6	MBL-2	46	61	36
	YAC-1	64	41	4
C11-C4	MBL-2	31	56	38
	YAC-1	6	39	3

* Percent specific lysis at an effector to target cell ratio of 20:1. Each value refers to the activity of the original culture or is representative of the activity of subcultures assayed at different passages (three to ten).

† Clones were transferred in SN 20 after three to four passages in SN 19.

Table 4. Reactivity pattern of M-MuLV/M-MSV-specific CTL clones

CTL clone*	SN	Target cells					
		MBL-2	YAC-1	YAC	YC-8	BALB/c†	C57BL/6†
E7-G5	20	++	–	–	–	–	–
	19	+++	++	–	–	–	–
D7-D6	20	++	–	ND‡	ND	–	–
	19	+++	++	ND	ND	–	–
C11-D2	20	+++	+	ND	ND	–	–
	19	+++	+++	ND	ND	–	–
E7-G2	20	++	–	–	–	–	–
	19	+++	++	–	–	–	–
C11-C4	20	++	–	–	–	–	–
	19	+++	+++	–	–	–	–
E5-C2	20	++	–	–	++	++	–
	19	++++	++++	–	+++	+++	–
B4-C11	20	++	–	–	–	–	–
	19	++++	–	–	–	–	–

* CTL clones were maintained in SN 19 or in SN 20 and, after three to five passages, assayed against different target cells in a 4 hr ^{51}Cr-release assay. Percent specific lysis at an effector-to-target cell ratio of 20:1: (++++) 55–70%; (+++) 40–54%; (++) 25–39%; (+) 10–24%; (–) 10%.

† Con A-induced splenic blast cells.

‡ ND, not done.

et al., 1982). In this regard, we investigated the effect of anti-Lyt-2 antibodies on clones with NK-like activity, and observed that the cytotoxic effect against MBL-2 cells of clones C11-D2, D7-D6 and D8-F8 was reduced in a dose-dependent fashion by anti-Lyt-2 antibodies. In addition, the activity of clones E7-G2, C11-C4 and E5-C2 was not affected by this treatment, and no clone was inhibited when tested against allogeneic NK-susceptible YAC-1 cells (Fig. 4). These data indicate that different recognition structures are involved in specific

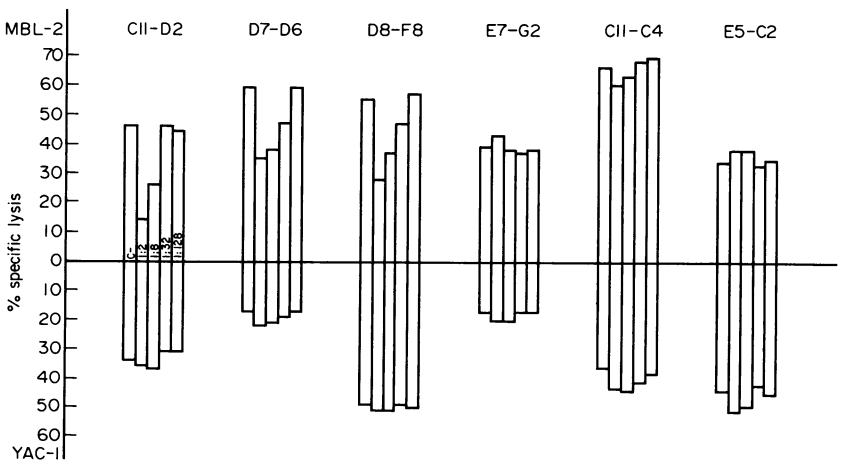

Figure 4. Effect of anti-Lyt-2 monoclonal antibodies on the specific and NK-like activities of different M-MuLV/M-MSV-specific CTL clones. Cloned cells, 30×10^3, were preincubated for 30 min at 20° with four-fold dilutions of anti-Lyt-2 monoclonal antibodies and assayed for lytic activity against 2×10^3 ^{51}Cr-labelled MBL-2 or YAC-1 target cells.

and NK-like activity by CTL clones, and confirm that the CTL clones are not directed against cross-reactive antigens present on the YAC-1 cell line.

DISCUSSION

CTL antigen specificity has been better evaluated recently by means of limiting dilution analysis of precursors and by the derivation of cloned cell lines (Nabholz *et al.*, 1978; Ryser & MacDonald, 1979; Baker, Gillis & Smith, 1979; Engers *et al.*, 1980); both methods have confirmed the exquisite specificity of cloned cells, even though non-specific lysis of a broad range of target cells has also been observed (Engers *et al.*, 1980; Nabel *et al.*, 1981; Russel & Dobos, 1983; Teh & Yu, 1983). Recent studies in mice and humans have indicated that CTL clones can also kill NK-susceptible target cells (Neefe & Carpenter, 1982; Pawelec *et al.*, 1982; Moretta *et al.*, 1983; Lopez-Botet *et al.*, 1983; Binz *et al.*, 1983; Acha-Orbea *et al.*, 1983; Shortman *et al.*, 1983; Brooks *et al.*, 1983a). The present study shows that NK-like cytotoxicity may be induced in M-MuLV/M-MSV specific CTL-p by the addition of supernatants that, like SN 19, were obtained from the *in vivo* passage of the EL-4 lymphoma cell line and which contain IL-2 and IFN; supernatants obtained from *in vitro* passage did not show this effect. This difference in the expression of NK-like function is apparently not due to a dissimilar IL-2 titre in the two supernatants. In fact, by varying the SN dilutions so that the IL-2 concentration in both was similar, we observed that most CTL-p were specific in a medium containing 15% of SN 20, while a high frequency of precursors showed double reactivity in medium containing 2·5% of SN 19 (data not shown). In addition, most of the CTL-p maintained specificity even when they were derived in medium supplemented with higher (40%) SN 20 concentrations. In view of the observation that IFN induces an increase in NK cytotoxicity by normal spleen cells (Djeu *et al.*, 1979), Brooks (1983) proposed that NK-like activity expression by CTL clones might be ascribed to a synergistic effect of IFN and IL-2. It is, however, more difficult to clarify the role of IFN, since we observed that specific and double-reactive clones derived in either SN, when washed and restimulated for two days in SN-free medium, produce low but detectable IFN titres (data not shown). This finding suggests that IFN apparently plays a minor role in NK-like activity acquisition by CTL clones. On the other hand, we cannot discard the possibility that other unidentified factors are present in SN 19. In this regard, Olabuenaga *et al.* (1983) have recently shown that, besides IL-2 and IFN, other additional factor(s) are required for long-term growth of cloned NK-like cytotoxic cells.

On the basis of limiting dilution analysis, we evaluated the frequency of CTL-p having NK-like activity, and observed that a high number (about 40%) of CTL-p express NK function when cultured in SN 19. In addition, following a few passages in this supernatant, even virus-specific clones acquire the ability to lyse NK-susceptible target cells. Very few clones, such as clone B4-C11, remain virus-specific when maintained in SN 19. On the contrary, following a few passages in SN 20, most double-reactive clones gradually lose their capacity to lyse YAC-1 cells. Therefore, NK-like activity expression is apparently reversible and modulated by soluble factors contained in the culture medium.

The appearance of double reactivity is associated with a rapid morphological modification of CTL clones which, on light microscopy, present a remarkable increase in size and numerous cytoplasmic granules. Electron microscopy analysis disclosed that double-reactive clones and large granular cells have similar ultrastructural features. In fact, the cells contain numerous electron-dense granules and multivesicular bodies located along the cytoplasmic membrane (D. Collavo, manuscript in preparation). As previously observed (Engers *et al.*, 1980), several small granules were also present in the cytoplasm of CTL clones with only specific activity. As suggested by Grossi *et al.* (1983), the presence of granules seems a common feature in all cytotoxic cells, regardless of the mechanisms mediating target cell lysis.

We found that M-MuLV/MSV-specific clones assayed against different target cells are unable to lyse NK-resistant allogeneic tumour cells or normal blast cells, while other workers report that numerous CTL clones may lyse different tumour cell lines, as well as normal syngeneic or allogeneic blast cells (Engers *et al.*, 1980; Nabel *et al.*, 1981; Russel & Dobos, 1983; Teh & Yu, 1983). The discrepancy in these findings may be ascribed to different culture conditions. In fact, multispecific clones are usually obtained when cells are originally isolated or maintained in the absence of antigen stimulation, while clones propagated under continuous stimulation by antigen usually do not acquire this broad cytotoxic activity. A few clones, such as clone E5-C2, lyse allogeneic (H-2^d) YC8

tumour and normal BALB/c blast cells; interestingly enough, these cross-reacting clones also acquired NK-like activity when maintained in SN 19.

It has been demonstrated that CTL, but not NK, activity can be specifically blocked by anti-Lyt-2 monoclonal antibodies (Seaman *et al.*, 1981), and that CTL clones are heterogeneous in their susceptibility to inhibition by these antibodies (MacDonald *et al.*, 1982). We observed that the addition of anti-Lyt-2 monoclonal antibodies affects the activity of only some virus-specific CTL clones; in these cases, however, lysis of NK-susceptible YAC-1 cells is not inhibited. This observation confirms that specific lysis and NK-like activity by double-reactive CTL clones is mediated by two independent recognition structures (Binz *et al.*, 1983).

In conclusion, our results favour Klein's hypothesis (Klein, 1983) that CTL may exhibit NK-killing at a certain stage of their differentiation. The acquisition of such an activity is reversible and may be modulated by lymphokines present in the culture medium. However, it is still unclear whether these factors promote differentiation of individual cells or, alternatively, favour the expansion of variant cells possessing double reactivity within the cloned cell population.

ACKNOWLEDGMENTS

We gratefully acknowledge the competent technical assistance of Mr S. Mezzalira and Mrs G. Miazzo. We also thank Mrs P. Segato for expert help in the preparation of the manuscript, and Mrs W. Tognon for secretarial assistance.

This work was supported by grants from Consiglio Nazionale delle Ricerche, Progetto Finalizzato Controllo della Crescita Neoplastica, nos 83.00779 and 83.00773, Roma, and Associazione Italiana per la Ricerca sul Cancro.

REFERENCES

ACHA-ORBEA H., GROSCURTH P., LANG R., STITZ L. & HENGARTNER H. (1983) Characterization of cloned cytotoxic lymphocytes with NK-like activity. *J. Immunol.* **130,** 2952.

BAKER P.E., GILLIS S. & SMITH K.A. (1979) Monoclonal cytolytic T cell lines. *J. exp. Med.* **149,** 273.

BINZ H., FENNER M., FREI D. & WIGZELL H. (1983) Two independent receptors allow selective target lysis by T cell clones. *J. exp. Med.* **157,** 1252.

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

BROOKS C.G., BURTON R.C., POLLACK S.B. & HENNEY C.S. (1983a) The presence of NK alloantigens on cloned cytotoxic T lymphocytes. *J. Immunol.* **131,** 1391.

BROOKS C.G., URDAL D.L. & HENNEY C.S. (1983b) Lymphokine-driven 'differentiation' of cytotoxic T-cell clones into cells with NK-like specificity: correlations with display of membrane macromolecules. *Immunol. Rev.* **72,** 43.

BRUNNER K.T., MACDONALD H.R. & CEROTTINI J.C. (1980) Antigenic specificity of the cytolytic T lymphocyte (CTL) response to murine sarcoma virus-induced tumors. II. Analysis of the clonal progeny of CTL precursors stimulated *in vitro* with syngeneic tumor cells. *J. Immunol.* **124,** 1627.

CAMPBELL J.B., GRUBERGER T., KOCHMAN M.A. & WHITE S.L. (1975) A microplaque reduction assay for human and mouse interferon. *Can. J. Microbiol.* **21,** 1247.

COLLAVO D., RONCHESE F., ZANOVELLO P., BIASI G. & CHIECO-BIANCHI L. (1982) T cell tolerance in Moloney-murine leukemia virus (M-MuLV) carrier mice: low cytotoxic T lymphocyte precursor frequency and absence of suppressor T cells in carrier mice with Moloney-murine sarcoma (M-MSV)-induced tumors. *J. Immunol.* **128,** 774.

COLLAVO D., RONCHESE F., ZANOVELLO P., CERUNDOLO V. & BIASI G. (1984) Reduction in precursors of cytotoxic T lymphocytes and of cells with natural-killer like activity in spleens of cyclophosphamide-treated mice. *Int. J. Immunopharmacol.* **6,** 529.

DJEU J.Y., HEINBAUGH J.A., HOLDEN H.T. & HERBERMAN R.B. (1979) Augmentation of mouse natural killer cell activity by interferon and interferon inducers. *J. Immunol.* **122,** 175.

ENGERS H., COLLAVO D., NORTH M., VON BOEHMER H., HAAS W., HENGARTNER H. & NABHOLZ M. (1980) Characterization of cloned murine cytolytic T cell lines. *J. Immunol.* **125,** 1481.

FARRAR J.J., FULLER-FARRAR J., SIMON P.L., HILFIKER M.L., STADLER B.M. & FARRAR W.L. (1980) Thymoma production of T cell growth factor (Interleukin 2). *J. Immunol.* **125,** 2555.

GILLIS S., FERM M.M., OU W. & SMITH K.A. (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027.

GROSSI C.E., ZICCA A., CADONI A., MINGARI M.C., MORETTA A. & MORETTA L. (1983) Ultrastructural characteristics of human T cell clones with various cytolytic activities. *Eur. J. Immunol.* **13,** 670.

KLEIN E. (1983) Killing often spoken of as natural. *Immunology Today*, **4,** 97.

LOPEZ-BOTET M., MORETTA A. & MORETTA L. (1983) Natural killer-like cytotoxicity of human T-cell clones against various target cells. *Scand. J. Immunol.* **17,** 95.

MACDONALD H.R., GLASEBROOK A.L. & CEROTTINI J.C. (1982) Clonal heterogeneity in the functional requirement for Lyt-2/3 molecules on cytolytic T lymphocytes: analysis by antibody blocking and selective trypsinization. *J. exp. Med.* **156,** 1711.

MORETTA A., PANTALEO G., MORETTA L., CEROTTINI J.C. & MINGARI M.C. (1983) Direct demonstration of the clono-

genic potential of every human peripheral blood T cell. Clonal analysis of HLA-DR expression and cytolytic activity. *J. exp. Med.* **157,** 743.

NABEL G.L., BUCALO R.L., ALLARD J., WIGZELL H. & CANTOR H. (1981) Multiple activities of a cloned cell line mediating natural killer cell function. *J. exp. Med.* **153,** 1582.

NABHOLZ M., ENGERS H., COLLAVO D. & NORTH M. (1978) Cloned T-cell lines with specific cytolytic activity. In: *Current Topics in Microbiology and Immunology* (eds F. Melcher, M. Potter and N. Warner), p. 176. Spinger, New York.

NEEFE J.R. & CARPENTER R. (1982) Natural killer activity in cloned IL-2 dependent allospecific lymphoid populations. In: *NK Cells and Other Natural Effector Cells* (ed. R.B. Herberman), p. 903. Academic Press, New York, London.

OLABUENAGA S.E., BROOKS C.G., GILLIS S. & HENNEY C.S. (1983) Interleukin 2 is not sufficient for the continuous growth of cloned NK-like cytotoxic cell lines. *J. Immunol.* **131,** 2386.

PAWELEC G., HADAM M., MÜLLER C. & WERNET P. (1982) Cloned human T-cell lines with allospecific or natural killer-like cytotoxicity. *Immunology Letters*, **4,** 237.

RUSSEL J.H. & DOBOS C.B. (1983) Characterization of a 'heteroclitic' cytotoxic lymphocyte clone: heterogeneity of receptors or signals? *J. Immunol.* **130,** 538.

RYSER J.E. & MACDONALD H.R. (1979) Limiting dilution analysis of alloantigen-reactive T lymphocytes. I. Comparison of precursor frequencies for proliferative and cytolytic responses. *J. Immunol.* **122,** 1191.

SEAMAN W.E., TALAL N., HERZENBERG L.A., HERZENBERG L.A. & LEDBETTER J.A. (1981) Surface antigens on mouse natural killer cells: use of monoclonal antibodies to inhibit or to enrich cytotoxic activity. *J. Immunol.* **127,** 982.

SHORTMAN K., WILSON A., SCOLLAY R. & CHEN W.F. (1983) Development of large granular lymphocytes with anomalous, nonspecific cytotoxicity in clones derived from Ly-2^+ T cells. *Proc. natn. Acad. Sci. U.S.A.* **80,** 2728.

TASWELL C., MACDONALD H.R. & CEROTTINI J.C. (1979) Limiting dilution analysis of alloantigen-reactive T lymphocytes. II. Effect of cortisone and cyclophosphamide on cytolytic precursor frequencies in the thymus. *Thymus*, **1,** 119.

TEH H.S. & YU M. (1983) Activation of nonspecific killer cells by interleukin 2-containing supernatants. *J. Immunol.* **131,** 1827.