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Production of interferon and tumour necrosis factor by cloned human natural cytotoxic lymphocytes and T cells

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SUMMARY

Cell lines and clones, derived from natural killer (NK) cell-enriched ($B73.1^+$) peripheral blood lymphocytes (PBL) from several human donors, that expressed distinct surface phenotypes and were cytolytically active against K562 target cells were tested for their capacity to produce interferon (IFN) and tumour necrosis factor (TNF). IFN and TNF were measured firstly in biological assays and secondly in specific immunoassays for α-IFN, γ-IFN and tumour necrosis factor (TNFα). It was found that the majority of NK-derived lines and clones were highly cytotoxic towards K562, but generally produced relatively low or undetectable levels of γ-IFN and TNFα following stimulation with phytohaemagglutinin. No α-IFN was detected in supernatants from these cells. In comparison, cell lines and clones, derived from T lymphocyte ($B73.1^-$) enriched PBL from the same donors were poorly cytotoxic towards K562, but generally produced higher levels of γ-IFN and TNF than NK-derived cells. Thus, neither γ-IFN nor TNF production were shown to correlate well with the capacity of NK-derived or T cell clones to effect cytotoxic action towards K562 *in vitro*. These results suggest that the co-production of γ-IFN and TNF is not indicative of cytotoxic potential.

Keywords γ interferon tumour necrosis factorα NK cells T cells

INTRODUCTION

Human natural killer (NK) cells are capable of carrying out a variety of cytotoxic and immunoregulatory functions *in vitro*. As well as being able to kill certain tumour cell types they are also active in regulating proliferative or functional responses of a range of normal cell types including B cells, thymocytes and certain haematopoietic stem cells (Herberman & Ortaldo, 1981; Trinchieri & Perussia, 1984). NK cells are a heterogeneous population of lymphoid cells, morphologically identified as large granular lymphocytes (LGL), with respect to (i) a number of cell-surface antigens, such as Fcγ receptors, a variety of T lymphocyte markers (e.g. CD1, CD8, CD10, CD11) and the monocyte-macrophage marker OKM1 and (ii) target cell specificity (Trinchieri & Perussia, 1984).

γ-Interferon (γ-IFN) appears to be mainly produced by different classes of functionally active T cells following antigenic or mitogenic stimulation (Wilkinson & Morris, 1984; Palacios, Martinez-Maza & De Ley, 1983; Kasahara *et al.*, 1983b). However, cells of NK cell phenotype and LGL morphology can apparently also produce γ-IFN following stimulation with lectins (O'Malley *et al.*, 1982; Kasahara *et al.*, 1983a), viruses (Djeu *et al.*, 1982) or IL-2 (Ortaldo *et al.*, 1984; Trinchieri *et*

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al., 1984). In addition, stimulated LGL have also been reported to produce α-IFN (Djeu *et al.*, 1982; Kasahara *et al.*, 1983a).

NK cells have also been demonstrated to be a source of cytotoxic factors (NKCF; Wright & Bonavida, 1982) which exert cytotoxic or cytostatic activity towards certain tumour cells. The biochemical identity of NKCF has recently been associated with tumour necrosis factor (TNFα) (Degliantoni *et al.*, 1985; Svedersky *et al.*, 1985a). Stimulated non-adherent PBL (Nedwin *et al.*, 1985) and cloned T cells (Leopardi & Rosenau, 1984) produce mainly lymphotoxin (LT), but one report (Green *et al.*, 1985) has shown that cloned human cytotoxic T lymphocytes (CTL) produced cytotoxins, possibly including TNFα, which are mostly antigenically unrelated to LT.

The B73.1 (CD16) monoclonal antibody (MoAb) which binds to the Fcγ receptor (Perussia *et al.*, 1983), is highly selective for lymphocytes with the morphology of large granular lymphocytes (LGL) and which mediate virtually all of the NK activity in non-adherent PBL preparations (Trinchieri & Perussia, 1984). Therefore, using the B73.1 MoAb to sort non-adherent PBL from several human donors, we have prepared purified, IL-2 dependent, NK-derived lines and clones, along with T cell controls. We have studied the production of α-IFN, γ-IFN and αTNF, estimated both by biological assays and specific immunoassays, following mitogenic stimulation with phytohaemagglutinin (PHA) in an attempt to characterize NK cells further with respect to cytokine production and to determine the relationship if any, between this production and cytotoxicity. Our results suggest that NK cell cytokine production is not correlated with their ability to lyse typical NK tumour cell targets such as K562.

MATERIALS AND METHODS

Cells

Peripheral blood was obtained from normal donors by venepuncture. Mononuclear cells were isolated by layering heparinized blood onto Lymphocyte Separation Medium (Flow Laboratories, Scotland) and centrifuging for 20 min at 400 ***g***. Cells accumulating at the interface were washed twice in phosphate buffered saline (PBS) before use. Unseparated mononuclear cells were used as feeder cells, whereas cells to be used for cloning were incubated in plastic flasks in RPMI 1640 + 10% autologous plasma for 2 h at 37°C to remove adherent cells.

The B lymphoblastoid cell line BSM (Roberts & Moore, 1985) was maintained in RPMI 1640 + 10% heat inactivated fetal calf serum supplemented with 200 μg/ml streptomycin and 100 μg/ml ampicillin (RPMI-CS). The erythroleukaemic cell line K562 was grown in the same medium except that heat inactivated newborn calf serum was used in place of fetal calf serum. Cell lines were routinely screened for the presence of mycoplasma contamination and found to be negative.

Cell cloning

Peripheral blood T cells and NK cells were purified and cloned as previously described (Roberts & Moore, 1985). Briefly, non-adherent mononuclear cells were labelled with the B73.1 monoclonal antibody, the gift of Dr G. Trinchieri, Wistar Institute, Philadelphia, USA, which binds to the Fcγ receptor on NK cells (Perussia *et al.*, 1983). Cells were washed twice in ice-cold PBS and labelled with fluorescein-conjugated rabbit anti-mouse antiserum (Dakopatts, Copenhagen). After further washing, cells were sorted into B73.1$^+$ (NK cell) and B73.1$^-$ (T cell) populations using a FACS IV (Becton Dickinson, California). While B73.1$^-$ populations were routinely >99% pure, B73.1$^+$ populations regularly contained 1–5% contamination with B73.1$^-$ cells and so were re-sorted, normally resulting in populations which were >99% B73.1$^+$ cells.

Both B73.1$^+$ and B73.1$^-$ cells were cloned by limiting dilution (Roberts & Moore, 1985) in RPMI 1640 + 10% autologous plasma, 2×10^5 autologous or allogeneic peripheral blood mononuclear cells/ml and 10^5 BSM/ml (all given 50 Gy gamma irradiation) supplemented with 1 μg/ml indomethacin, 1 μg/ml and interleukin 2 (IL-2). The latter was either in the form of culture supernatant from the MLA 144 cell line (Roberts & Moore, 1985) or recombinant material (Biogen) and in either case the amount of IL-2 added was that giving almost maximal proliferation of a human T cell line. B73.1$^+$ cells were plated in 96-well plates over a concentration range of 1–10 cells/

well while B73.1$^-$ cells were plated at 0·3 and 0·5 cells/well. After 14–16 days incubation at 37°C in an atmosphere of 5% CO_2, plates were examined and colonies scored. Colonies which, by Poisson statistical analysis, had a probability of >95% of being clones were transferred to fresh feeder mixture as above and grown for a further 14–21 days in IL-2, with regular feeding, before use. As previously reported (Roberts & Moore, 1985) of the order of 10^6–10^7 cells could be obtained per clone of cells before blast crisis. For B73·1$^+$ cells proliferative frequencies were between 1 in 10 and 1 in 20 while for B73.1$^-$ cells these were between 1 in 1 and 1 in 2. It was therefore of great importance to use highly purified starting populations of B73.1$^+$ cells to avoid T cell outgrowth (cf donor M.M.).

Bulk cultures of B73.1$^+$ and B73.1$^-$ cells were initiated and maintained under the same conditions.

Stimulation of cytokine production

Bulk cultures or cloned cells which had been incubated overnight in RPMI-CS were incubated for 48 h in 96-well round-bottomed plates in RPMI-CS + 2·5 μg/ml PHA at a concentration of 10^6 cells/ml (Allavena *et al.*, 1985). Supernatants were harvested and stored at −20°C for up to 14 days before assay for cytokine content. Supernatants from cells incubated in RPMI-CS alone served as controls.

IFN bioassay

The antiviral effect of IFN was estimated in human HEp2/c cells challenged with encephalomyocarditis virus as previously described (Ennis & Meager, 1981). A laboratory standard of γ-IFN calibrated against the NIH (USA) γ-IFN standard, Gg23-901-530, (4000 IU/ml) was included in all assays and all γ-IFN titres are expressed in international units (IU).

TNF bioassay

TNF content of culture supernatants was estimated in a subline clone 10, of L-A9 cells which was highly sensitive to the cytotoxic action of TNF and LT. Cells were seeded at 5×10^4 cells per well in microtitre trays and incubated overnight at 37°C. Serial dilutions of a α-TNF laboratory standard and culture supernatants were added in medium containing actinomycin D. The plates were stained with amido blue black following 24 h further incubation at 37°C. The dye was extracted with 50 mM NaOH and the plates read in a Titertek Multiscan set at 620 nm. The end-point was defined as the dilution of standard or supernatant giving an OD_{620} measurement halfway (50%) between the positive and negative controls. Titres, expressed as units/ml, were equivalent to the reciprocals of the dilutions giving the end-point. A laboratory standard of α-TNF (T250, Knoll Ag, West Germany) was used to calibrate each assay, and to correct titres obtained from assays performed on different occasions.

Enzyme-linked immunosorbent assays (ELISA)

For γ-IFN. This was carried out according to the method described by Berg (1986). Briefly, purified LO-22 monoclonal antibody (MoAb) was coated at 10 μg/ml in PBS on microELISA plates, 50 μl per well, for 1 h at 37°C. Two percent (2%) heat inactivated newborn calf serum (HINCS) in PBS, 150 μl per well, was added to block remaining protein-binding sites and the plates stored at 4°C before use. MoAb and HINCS solution was removed, wells washed extensively with 0·05% Tween 20-PBS (Tw20-PBS) and then serial dilutions of an α-IFN laboratory reference preparation, calibrated against the 69/19 International reference preparation for human leucocyte IFN, and undiluted culture supernatants, 50 μl per well in duplicate, added and incubated for 1 h at 37°C. Following this, the α-IFN containing fluids were removed and wells washed thoroughly with Tw20-PBS before adding a 1:4000 dilution of calf-antiα-IFN-horseradish peroxidase complex in 10% HINCS-PBS, 50 μl per well, and further incubating the plates for 30 min at 37°C. Finally, the wells were washed three times with Tw20-PBS and twice with 0·1 M citrate/phosphate buffer, pH 5·0, and colour developed in the dark upon addition of substrate, orthophenylenediamine, 1 mg/ml, in 0·1 M citrate/phosphate buffer containing 0·006% H_2O_2. The reaction was stopped after 30 min by adding 50 μl 1 M H_2SO_4 to each well and optical density was read at 450 nm in a Titertek Multiscan.

The titres of α-IFN present in the culture supernatants were interpolated from the α-IFN standard calibration curve. This ELISA had a detection limit of approximately 20 IU/ml.

For γ-IFN. Purified rabbit anti-γ-IFN (Leist *et al.*, 1985) diluted 1:100 in PBS was coated on U-bottomed polyvinyl chloride microtitre plates (Dynatech), 50 μl per well, for 2 h at 37°C. Remaining sites in wells were blocked with 2% bovine serum albumin (BSA)-PBS, 150 μl per well, overnight at 4°C. Excess antibodies and blocking buffer were then removed and wells washed four times with 0·05% Tw20-PBS. Following the last wash, samples and serial dilutions of a γ-IFN standard, 50 μl per well, were added and incubated at 37°C for 1 h. The wells were washed again four times with 0·05% Tw20-PBS followed by (i) addition of purified 4S.B3 MoAb against γ-IFN (Meager *et al.*, 1984), 1:100 in 2% BSA-PBS, 50 μl per well, and incubation for 1 h at 37°C, (ii) washing with 0·05% Tw20-PBS and addition of biotinylated sheep anti-mouse immunoglobulin (Amersham International), 1:500 in 2% BSA-PBS, 50 μl per well, and incubation for 1 h at 37°C, (iii) washing with 0·05% Tw20-PBS and addition of streptavidin-biotinylated horseradish peroxidase complex (Amersham International), 1:500 in 2% BSA-PBS, 50 μl per well and incubation for 30 min at 37°C, (iv) processing the assays for colour development as described for the α-IFN ELISA. Levels of γ-IFN present in the culture supernatants were interpolated from the γ-IFN standard calibration curve. This ELISA had a detection limit of approximately 1 IU/ml.

For TNFα. The ELISA was carried out in a manner similar to that described above for the γ-IFN ELISA except that rabbit and mouse antibodies against γ-IFN were replaced by TNFα specific antibodies. Both rabbit polyclonal anti-TNFα and mouse MoAb against TNFα were prepared using highly purified TNFα ($>2\times10^7$ units/mg protein) generously provided by Knoll Ag, West Germany. For this ELISA, the MoAb designated 33.F3 (A.M., unpublished results), giving a detection limit of approximately 3 units/ml, was used.

All three ELISAs were highly specific for their respective cytokines and no significant cross-reactivity was observed.

Cytotoxicity assays

Effector cells were incubated overnight in RPMI-CS before assay. Cytotoxicity assays against the erythroleukaemic target cell line K562 were performed as previously described (Roberts & Moore, 1985). Briefly, K562 cells were labelled with 0·2–0·3 mCi ^{51}Cr sodium chromate (Amersham International) and added to effector cells at an E:T ratio of 2·5:1 in triplicate samples in a volume of 0·2 ml RPMI-CS. After centrifugation at 200 ***g*** for 10 min, cells were incubated together for 4 h at 37°C and the assay was terminated by removing 0·1 ml of supernatant. This and the remaining supernatant and cell pellet were counted using a gamma counter. Spontaneous release was obtained by incubating target cells with RPMI-CS alone and maximum release was measured using 1% Triton X-100. Cytotoxic activity were calculated as follows:

% specific cytotoxicity =

$$\frac{\text{Test } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{Maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100\%$$

Spontaneous release was always < 15% and maximum release was between 90 and 105%.

Phenotypic analysis

Cytocentrifuge smears were made using a Cytospin 2 (Shandon, Runcorn) and were air dried and stored for up to 14 days at −20°C before use. Smears were fixed for 1 min in acetone and stained for the presence of lymphocyte membrane antigens using the immunoalkaline phosphatase technique (Cordell *et al.*, 1984). Smears having > 20% of cells staining with a particular monoclonal antibody were regarded as positive. Primary antibodies used were: UCHTI (CD3), T3-10 (CD4) and Tu102 (CD8) (detailed in Ghosh, Spriggs & Mason, 1985).

Statistical analysis

The significance of differences in cytokine titres between groups of clones was compared nonparametrically using the Kruskal–Wallace one-way analysis of variance or, in the case of paired

Table 1. Cytokine production and cytotoxic capacity for bulk cultures derived from B73.1$^+$ and B73.1$^-$ cells

Donor		B73.1$^+$ derived	B73.1$^-$ derived
S.C. (1)	IFN bioassay*	< 10	200
	γ-IFN ELISA*	< 9	124
	% cytotoxicity†	75·5 ± 2·1	21·4 ± 0·4
S.C. (2)	IFN bioassay	< 10	144
	γ-IFN ELISA	< 3	85
	TNF bioassay*	< 4	12
	TNFα ELISA*	< 4	12
	% cytotoxicity	43·8 ± 3·7	15·6 ± 1·4
A.S.	IFN bioassay	8	700
	γ-IFN ELISA	9	760
	% cytotoxicity	83·6 ± 2·3	31·0 ± 1·1
M.D.	IFN bioassay	< 10	63
	γ-IFN ELISA	< 9	46
	TNF bioassay	< 2	20
	TNFα ELISA	NT	5
	% cytotoxicity	9·0 ± 1·0	3·5 ± 0·9
B.D.	IFN bioassay	< 4	61
	γ-IFN ELISA	< 2	60
	TNFα ELISA	< 4	24
	% cytotoxicity	39·1 ± 1·6	12·3 ± 3·2
J.G.	IFN bioassay	< 10	63
	γ-IFN ELISA	13	62
	TNF bioassay	< 4	< 4
	TNFα ELISA	< 2	5
	% cytotoxicity	17·6 ± 1·6	25·2 ± 1·3
M.M.	IFN bioassay	128	281
	γ-IFN ELISA	121	285
	TNF bioassay	16	96
	% cytotoxicity	37·7 ± 1·6	3·8 ± 1·4
D.H.	IFN bioassay	< 10	180
	γ-IFN ELISA	3	170
	TNF bioassay	6	48
	TNFα ELISA	< 4	20
	% cytotoxicity	54·7 ± 4·3	11·5 ± 1·1

NT, Not tested.

* Units/ml

† K562 cells were used as targets at an effector/target ratio of 2·5:1 and results are expressed as mean % specific cytotoxicity ± s.d.

groups, the Mann–Whitney *U*-test. Values below the background level of detection were assigned an arbitrary cytokine titre of 1 unit/ml.

RESULTS

Cytokine production and cytotoxic capacity of bulk cultures. Bulk cultures of purified B73.1$^+$ and B73.1$^-$ cells from seven donors were studied in eight experiments as shown in Table 1. In all cases, B73.1$^-$ derived cells produced higher levels of IFN than B73.1$^+$ derived cells from the same donor. The IFN was shown to be predominantly γ-IFN, the amounts detected by bioassays and the γ-IFN ELISA being generally very similar. In only one instance (donor M.M.) did B73.1$^+$ derived cells

Table 2. Cytokine production and cytotoxic activity of T cell clones

	CD4+	CD8+	CD3+
γ-IFN			
n	16	6	27
Range	8–500	21–100	8–500
TNFα			
n	11	4	19
Range	<2–70	2–22	<2–70
% cytotoxicity*			
n	16	6	27
Range	0–5·5†	8·0–36·5†	0–44·5

* E:T = 2·5:1.

† $P < 0{\cdot}01$ for difference between CD4+ and CD8+ cells.

Clones are grouped according to the presence of CD4 and CD8 antigens; five clones were not tested for the presence of CD4/8 and are included in the CD3+ column. Differences between cytokine titres for CD4+ and CD8+ cells were not statistically significant.

produce relatively high-titred γ-IFN and this was the only experiment in which the purity of the B73.1+ starting population was below 99% (in fact, 98%). No α-IFN was detected using an α-IFN specific ELISA in the supernatants from either B73.1− or B73.1+ derived cells from any donor. However, in all six experiments (Table 1) in which supernatants were assayed for TNF content, B73.1− derived cells produced low, but significant amounts whereas, with the exception of donor M.M., B73.1+ derived cells failed to produce detectable levels. Some of the TNF activity of the B73.1−-derived cells was attributable to the presence of α-TNF, since this was detected by α-TNF specific ELISA in the supernatants of cells obtained from four donors (Table 1). In no instance was either IFN or TNF activity detected in the supernatants derived from unstimulated cells. In 7/8 cases B73.1+ derived cells were more highly cytotoxic towards the NK target K562 than were B73.1− derived cells. In general, therefore, there was an inverse relationship between cytotoxic activity of bulk cultures and their ability to produce γ-IFN or TNF.

Cytokine production by T cell clones. All 27 T cell clones tested from eight donors, produced significant titres of γ-IFN ranging from 8 to 500 IU/ml (Table 2) while supernatants from unstimulated cells were without activity. In general, CD4+ clones produced higher amounts than CD8+ clones but this was not statistically significant. As before, IFN bioassays gave similar results to γ-IFN ELISAs and no α-IFN production was detected (data not shown). When the same supernatants were tested for TNF production, CD4+ clones again generally produced higher amounts than CD8+ clones but this was again not statistically significant. In most cases, TNFα ELISAs gave similar results to bioassays (data not shown). Linear regression analysis revealed a significant positive correlation between γ-IFN and TNFα production by T cell clones ($r = 0{\cdot}64$; $P < 0{\cdot}05$). All CD4+ clones tested showed negligible cytotoxic activity against K562 targets while CD8+ clones showed low to moderate levels of cytotoxicity (Table 2).

Cytokine production by B73.1+ derived clones. A panel of 90 clones derived from B73.1+ cells from six donors was tested for IFN production. Clones were grouped according to T cell antigen phenotype as shown in Table 3. Most clones produced considerably lower amounts of γ-IFN than did T cell clones from the same set of donors (Table 2). Clones which were CD3− generally gave lower titres of γ-IFN than did CD3+ clones ($P < 0{\cdot}05$). The possibility that at least some of the

Table 3. γ-IFN titres (IU/ml) produced by B73.1$^+$ derived clones grouped according to T cell antigen phenotype; mean cytotoxic activities against K562 targets (E:T = 2·5:1) are also shown

Donor	*n*	$CD3^-4^-8^-$	$CD3^+$	$CD3^+8^+$	$CD3^-CD8^+$	$CD3^+4^+$
S.C. (1)	11	<9(×7)	<9,49	<9	<9	—
A.S.	7	10, 19, 20, 20, 49	—	27	20	—
M.M.	11	9, 10, 24	54	26	—	14, 19, 32, 159, 224,900
M.D.	11	<9(×5)	—	—	<9(×3), 10, 19, 23	—
B.D.	6	—	<2, 43, 100	—	4, 11	5
J.G.	11	<2, 8, 8, 16, 17, 21	—	14, 150	10, 10, 20	—
S.C. (2)	20	<3, 8, 11, 15, 19, 24, 27, 28, 33, 42, 52, 62	36	—	6, 17, 20, 25, 26, 30, 54	—
S.C. (3)	13	<3, 5, 6, 7, 7, 16, 30	11	14	<3, <3, 15, 19	—
Range		<2–62	<2–100	<9–150	<3–54	5–900
n		45	8	6	24	7
Significance ($P<$)*		0·001	0·02	0·03	0·001	ns
% specific cytotoxicity Mean ± s.d.		54·2 ± 27·4	51·6 ± 27·9	44·8 ± 29·9	48·8 ± 26·3	1·5 ± 1·1
(Range)		4·0–83·9	5·0–76·9	11·6–73·3	3·3–87·7	0·6–3·2

* As compared to values for T cell clones in Table 2.
NS, not significant.

$CD3^+$ clones were a result of T cell contamination of the B73.1$^+$ starting population was suggested by the results for donor M.M.; in contrast to other experiments, most clones obtained had the phenotype of classical T cells. Several of these clones produced high levels of γ-IFN, comparable to those produced by T cell clones. As with supernatants from T cell clones, IFN bioassays gave similar results to γ-IFN ELISAs and no α-IFN was detected in any of the supernatants.

In contrast to T cell clones, the majority (> 80%) of B73.1$^+$ derived clones were highly cytotoxic towards the NK cell target K562. When γ-IFN production by panels of clones was compared with their cytotoxic capacity no obvious relationship between the two parameters was noted. Figure 1 illustrates a representative result for a panel of 13 B73.1$^+$ derived clones from donor S.C. Linear regression analysis of this and a further seven experiments revealed no significant correlation, either positive or negative, between a clone's cytotoxic capacity and its ability to produce γ-IFN.

A panel of 56 of the clones detailed in Table 3, derived from five different donors, was assayed for TNF production. All of the 50 $CD4^-$ clones tested produced very low or undetectable amounts of TNF (< 8 u/ml; $P < 0·001$ as compared to T cell clones) while the six $CD3^+4^+$ clones from donor M.M. produced levels ranging from 3–74 u/ml, comparable to those produced by $CD4^+$ T cell clones (Table 2), again suggesting that these clones were derived from T cells contaminating the B73.1$^+$ starting population.

DISCUSSION

We have used the B73.1 MoAb to obtain highly purified NK cell populations from which IL-2 dependent lines and clones have been derived. The majority of NK-derived clones (~80%) did not express the mature T cell marker CD3. A minority did, however, express CD3 and this finding is in agreement with a recent report by Schmidt *et al.* (1986). It is also likely that some B73.1$^+$ cells which

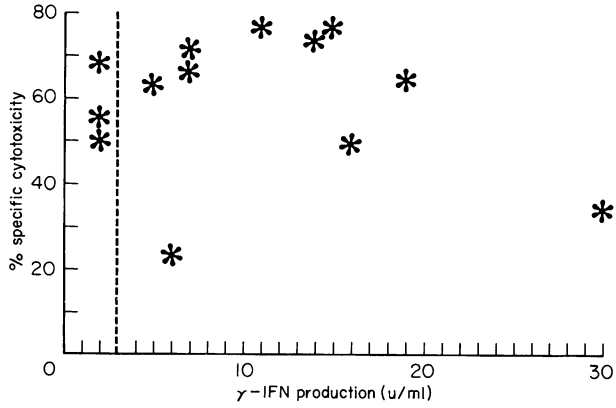

Fig. 1. Relationship between cytotoxic activity against K562 target cells (E:T = 2·5:1) and γ-IFN production for a panel of 13 B73.1$^+$ derived clones from donor S.C. (3). The dashed line represents background for the γ-IFN ELISA.

are initially CD3$^-$ acquire this marker during culture in IL-2 containing medium (Burns, Trigla & Werkmeister, 1984; Roberts & Moore, 1985; Allavena *et al.*, 1985). It is also possible that at least some of these CD3$^+$ clones were derived from contaminating T cells in the original B73.1$^+$ cell population. However, only in one case was there evidence to support this possibility. Six out of 11 B73.1 selected clones from donor M.M. were shown to have the classic T helper cell phenotype, CD3$^+$,4$^+$ suggesting that contaminating T cells, which, in the presence of PHA and IL-2, have a proliferative advantage compared to LGL (Roberts & Moore, 1985), outgrew NK cells. This probably occurred because the B73.1$^+$ starting population was in this instance only 98% as compared with the >99% purity of this population achieved with the PBL of all other donors. Significant numbers of both CD3$^-$ and CD3$^+$ NK clones co-expressed CD8, a marker associated with cytotoxic T lymphocytes (CTL) and some NK cells (Trinchieri & Perussia, 1984; Schmidt *et al.*, 1986).

Analyses of IFN and TNF production by B73.1$^+$ and B73.1$^-$-derived populations showed that PHA-stimulated B73.1$^+$ derived cultures responded relatively poorly in this regard compared to stimulated B73.1$^-$ (T cell) cultures. Use of specific immunoassays for α-IFN and γ-IFN showed that the IFN produced by T cell cultures was predominantly γ-IFN; no α-IFN was ever detected. A TNFα-specific ELISA strongly indicated that a significant proportion of the TNF produced by such cultures was TNFα. The latter is believed to be primarily a product of stimulated monocytes (Carswell *et al.*, 1975), whilst LT (or TNFβ) is produced primarily by cells contained in the non-adherent cell subset of PBL (Stone-Wolff *et al.*, 1984; Nedwin *et al.*, 1985). However, no study to date rules out the possibility that some T cells can produce either TNFα or TNFβ or both, and one recent report strongly suggests that the TNF produced by cloned human CTL contains both (Green *et al.*, 1985).

When stimulated with PHA, all T cell clones produced significant amounts of γ-IFN (Table 2). These findings are in agreement with a report of γ-IFN production by PHA stimulated human CTL clones (Matsuyama *et al.*, 1982). In our study, T cell clones of the helper CD4$^+$ phenotype generally produced more γ-IFN than CD8$^+$ T cell clones although this was not statistically significant. In addition to γ-IFN, many of these T cell clones produced TNF, the amounts again being generally less for CD8$^+$ clones than CD4$^+$ clones (Table 2). In accord with our results for TNF produced by T cell cultures, most, if not all, of the TNF produced by T cell clones was defined as α-TNF by α-TNF specific ELISA. That our T cell clones produced α-TNF is in contrast to the reported (Leopardi & Rosenau, 1984) production of αLT, presumably LT, by human T cell subsets, but is in agreement with the findings of Green *et al.* (1985) who demonstrated that, whilst unfractionated PBL produced 'classical' LT, human CTL clones produced a TNF that was antigenically unrelated to classical LT. Co-production of γ-IFN and LT in non-adherent human PBL is well established (Stone-Wolff *et al.*,

1984; Svedersky *et al.*, 1985b) and linear regression analysis has revealed a significant positive correlation between γ-IFN and TNFα production by our T cell clones. All CD4$^+$ clones tested showed negligible cytotoxic activity towards K562 while CD8$^+$ clones showed low to moderate levels of cytotoxicity (Table 2). It is possible that clones with the capacity to produce high titres of γ-IFN may have induced resistance of K562 target cells to lysis (Moore, White & Potter 1980; Gronberg *et al.*, 1983).

In comparison to T cell clones, B73.1$^+$ derived clones from the same donors produced markedly less γ-IFN ($P < 0{\cdot}02$) and TNF ($P < 0{\cdot}001$). CD3$^+$ clones produced more γ-IFN than CD3$^-$ clones ($P < 0{\cdot}05$), and in both cases levels of TNF were very low (< 8 u/ml) to undetectable. Most B73.1$^+$ derived clones were highly cytotoxic against K562 whereas most T cell clones were only weakly so. Allavena *et al.* (1985) and Nocera *et al.* (1985) have both reported the preparation of NK clones which upon stimulation produced high levels of γ-IFN or both γ-IFN and α-IFN. However, the majority of NK clones established by these groups were CD3$^+$, which is present only on a small percentage of fresh NK cells (Schmidt *et al.*, 1986) and only about a quarter of our clones. Whether subtle differences in the starting populations are responsible for these discrepancies is unclear.

We have demonstrated no obvious correlation between cytotoxic capacity and γ-IFN production by our NK-derived clones in agreement with previous reports (Allavena *et al.*, 1985; Nocera *et al.*, 1985; Matsuyama *et al.*, 1982). The very low to undetectable levels of TNFα in supernatants of highly cytotoxic B73.1$^+$ derived clones would suggest that its production is not indicative of cytotoxic potential. However, it remains a possibility that the ability of a particular clone to produce IFN and TNF following stimulation with PHA differs from that following contact with a susceptible target cell.

If only very low levels (< 8 u/ml) of α-TNF are required to effect target cell lysis by NK cells, then this might explain why in our study no correlation between TNF production and cytotoxicity was revealed. However, the question remains as to why T cell clones which produced appreciable amounts of TNF following PHA stimulation showed only low levels of cytotoxic activity towards K562. Either T cell clones did not make TNF on contact with K562 or TNF alone was insufficient to effect lysis.

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