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Control of immune interferon release by cytotoxic T-cell clones specific for influenza

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Summary. We have studied the release of immune interferon (IFN-γ) by influenza-specific cytotoxic T-cell (Tc) clones. IFN-γ release is entirely dependent on specific antigen recognition or mitogen treatment and correlates inversely with the growth rate of the clone, while no differences in cytotoxic activity can be discerned at the different stages of Tc maturation. Although the mitogen Con A provides a more powerful stimulus for IFN release by Tc clones, specific antigen leads to a more rapid secretion, starting within 2 hr of contact with Tc clones and their specific targets. This may be of significance in an infection, providing a quick, but localized, mechanism to prevent viral spread.

We also examined whether ligand interactions with T-cell surface glycoproteins Lyt-2 or LFA-1, important in Tc recognition, affected IFN release. Monoclonal antibodies to both Lyt-2 and LFA-1 block specific target cell lysis of Tc clone BA4, but do not affect Tc clone T9/5. This latter finding adds LFA-1 to the list of T-cell surface components which are not always essential for target cell recognition. Antibody to Lyt-2 blocked antigen-induced IFN-γ release by all Tc clones studied, whilst two monoclonal antibodies to LFA-1 had little or no effect. Thus, the Lyt-2 molecule plays a role in the regulation of IFN secretion.

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INTRODUCTION

Recent studies with activated T cells and their clones have shown that functionally different T cells, both in man and mouse, can be stimulated by antigen or T-cell mitogens to release interferon (IFN) (e.g. Krammer *et al.*, 1983; Kasahara *et al.*, 1983; Morris, Lin & Askonas, 1982). The T-cell derived IFN has been defined as immune IFN, except in the case of a human T-helper cell line which appeared to secrete acid-labile IFN neutralized by a polyclonal antibody to α/β IFN (Balkwill *et al.*, 1983). IFN-γ has an important role in the regulation of the immune system, in addition to its antiviral activity; for example, it affects macrophage phenotype and function (e.g. Nathan *et al.*, 1984) and may act as a B-cell maturation factor (Leibson *et al.*, 1984; Sidman *et al.*, 1984). In our hands, IFN has no effect on cytotoxic T-cell (Tc) generation or cytolytic activity (Askonas & Pala, 1985).

In view of its many regulatory functions, we have further studied several aspects of IFN-γ release by Tc clones specific for type A influenza virus. As previously shown with a similar Tc clone (Morris *et al.*, 1982), release of IFN-γ follows contact with syngeneic cells infected with type A influenza virus. This phenomenon thereby reflects the pattern of recognition of target cells by Tc in being MHC-restricted and virus type-specific. The addition of concanavalin A can overcome these rules.

We had previously found heterogeneity in IFN

release by a series of highly cytotoxic Tc clones (Taylor & Askonas, 1983), thereby showing that the two functions do not always coincide. Whether or not this reflected differences in Tc-target cell interaction or in the differentiation stage of Tc cells was not clear.

In the present study, we have characterized the kinetics of IFN-γ release by several Tc clones and have found an inverse correlation between cell proliferation and IFN release, while cytotoxic activity was constant. Furthermore, we have used monoclonal antibodies directed to the Tc cell surface glycoproteins Lyt-2 and LFA-1 to investigate the role of these membrane components in IFN-γ release and/or target cell lysis. Previous studies using monoclonal antibodies have implicated a role for both molecules in Tc-target cell recognition (Pierres, Goridis & Golstein, 1982; Springer *et al.*, 1982). In this study, anti-Lyt-2 inhibited antigen-induced IFN release, although anti-Lyt-2 did not inhibit clone T9/5-mediated cytotoxicity. In contrast, anti-LFA-1 had no significant effect on IFN-γ release by the Tc clones, only one of which was inhibited by the antibody.

MATERIALS AND METHODS

Mice

BALB/c mice were bred at this Institute under SPF conditions.

Influenza virus strains

Influenza virus strains A/X31 and B/HK were grown in the allantoic sac of 11-day embryonated chicken eggs, and infectious allantoic fluid was stored at $-70°$.

Cytotoxic T-cell clones

Influenza-specific Tc clones were selected and maintained as previously described (Lin & Askonas, 1980; Townsend *et al.*, 1982). In brief, BALB/c mice were primed intranasally by infection with A/X31 influenza virus and, after 1–5 months, spleen cells were restimulated several times *in vitro* every 8–10 days with A/X31-infected spleen stimulator cells. The medium was RPMI-1640 + 10% fetal calf serum with added penicillin and streptomycin. Cloning and recloning was on infected spleen cells in 96 flat microwell plates, according to the method of Braciale, Andrew & Braciale (1981). Con A-stimulated rat spleen cell supernatant, containing 20 mg/ml methyl-α-D-mannoside, was added at this stage at 10–20% (v/v) as a source of TCGF (Wraith, Holtkamp & Askonas, 1983). The three Tc clones used for this study (T9/5, T9/13 and BA4) were maintained in tissue culture for many months in TCGF. They were restimulated every 2 weeks with A/X31-infected thioglycollate-induced syngeneic macrophages (Wraith, 1984).

Cytotoxicity assay

Cytotoxicity assays were as previously described (Zweerink *et al.*, 1977). Targets were A/X31-infected, ^{51}Cr-labelled P815 mastocytoma cells at 2×10^4 microwell. For the experiment shown in Fig. 2, the targets were used at 5×10^4/microwell (K/T = 1). This increased number of target cells was found to improve IFN yields (results not shown). B/HK-infected target cells served as control. The ^{51}Cr released by the Tc over a 6-h period was assayed.

Monoclonal antibodies to LFA-1 and Lyt-2

Anti-Lyt 2. Culture supernatant from 53-6.7 hybridoma cells were used at 1/4–1/20. This is an IgG2a rat antibody not cytotoxic for Lyt-2$^+$ cells (Ledbetter & Herzenberg, 1979).

Anti-LFA-1. Two anti-LFA-1 antibodies were tested: culture supernatant from H35-89.9 hybridoma cells (rat IgG2a) was a kind gift of Dr M. Pierres (Pierres *et al.*, 1982). Culture supernatant from hybridoma M17-5.2 (rat IgG2b) was kindly donated by Dr T. Springer (Springer *et al.*, 1982). Tc were preincubated with the appropriate monoclonal antibody for 30 min before the addition of target cells.

Interferon assay

In order to assay the secretion of immune IFN, cloned Tc cells at 5×10^5/ml were incubated for 18–22 hr, unless otherwise indicated, with P815 target cells infected with A/X31 influenza virus (K/T = 1) in flat-bottomed wells. B/HK-infected or uninfected P815 cells served as specificity controls. Supernatant was harvested and stored in two sterile aliquots at $-70°$. IFN was assayed essentially according to the method of Atkins *et al.* (1974) for chick cells. Briefly, L929 cells (4×10^4/well in 96-well flat-bottomed microwell plates) were treated overnight with dilutions of IFN-γ containing supernatants. After removal of the supernatant, the cells were infected with Semliki Forest virus (multiplicity 250 PFU/cell) in the presence of 3 μg/ml actinomycin D for 2·5 hr. The incorporation of 1 μCi [^{3}H]uridine/well (Amersham, Amersham, Bucks; specific activity 5 Ci/mmol) into the cells during the next 2·5 hr was measured. The IFN titre represents that dilution of sample which gives a

50% reduction in uridine incorporation compared to the virus controls. IFN values were only calculated from titrations where all points showed a linear relationship (correlation coefficient $>0{\cdot}98$) and were compared only within a single assay. Each point was in triplicate with four dilutions/sample. Since no mouse γ-IFN standard was available, results are expressed as U/ml IFN in relation to the NIH α/β mouse standard (G002904511).

Capping of Tc cell surface markers

We wished to test whether capping and modulation of the Tc cell surface markers Lyt-2 and LFA-1 could induce IFN-γ production. Tc clone BA4 was treated with antibodies on ice for 30 min, washed and incubated with fluorescein-conjugated rabbit anti-mouse IgG for 1 hr at 37°. Cells were resuspended to 10^6/ml, incubated for 3 hr, and supernatants were collected for IFN assay. The cells were checked for capping on the fluorescent microscope at various times throughout the incubation.

RESULTS

Proliferation and IFN release by Tc

For this study, we selected three BALB/c Tc clones (T9/5, BA 4 and T9/13) which lysed syngeneic target cells infected with any type A influenza virus, but not type B influenza infected targets. The Tc clones described here require a source of IL-2 and regular antigenic stimulation for optimal growth. The clones divide rapidly up to 4 days post-antigenic stimulation and then slow down, but their viability and cytotoxicity can be successfully maintained for up to 16 days with TCGF alone (Fig. 1a), after which time they start to die.

Figure 1b shows that samples of cells taken from the rapid growth phase do not release IFN on contact with appropriate target cells, but cells taken 6–17 days post-antigenic stimulation release high levels of immune IFN. These results indicate an inverse correlation between proliferation of Tc and their antigen-induced IFN release.

Antigen-specific release of IFN occurs rapidly within 2 hr of contact with A/X31-infected cells, and rises for 6 hr in parallel to cytotoxicity levels (Fig. 2a & b). After 6 hr no further increase in cytotoxicity occurs and levels of IFN-γ in the supernatant decline slightly.

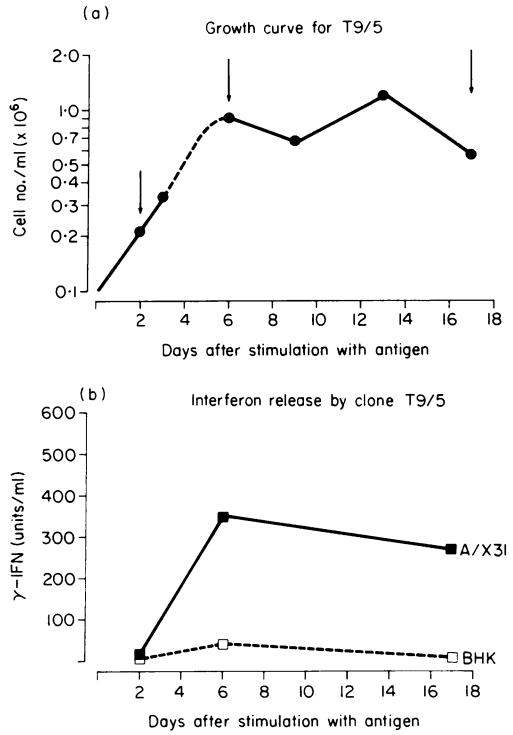

Figure 1. IFN secretion correlates inversely with the growth rate of cloned Tc. (a) Growth curve of T9/5 cells at various times following stimulation with an equal number of A/X31-infected thioglycollate-induced macrophages in the presence of IL-2-containing medium. Cells were split 1:1 on Day 6 in the IL-2 medium. Fifty μl of undiluted TCGF was added on Days 3, 9 and 13. Points represent the average of three triplicate 1-ml cultures in 24-well Costar plates. Line is broken between Days 3 and 6, since cell counts on Days 4 and 5 were not available. Arrows indicate times of IFN secretion assays [see (b)]. (b) Tc (5×10^5/ml) at 2, 6 and 17 days following antigenic stimulation were incubated for 24 hr with P815 target cells infected for 90 min with A/X31 or B/HK (as control); K/T was 1. IFN content of the supernatants was assayed as described in the Materials and methods.

Kinetics of IFN release by TC in the presence of Con A

The difference between Con A and antigen induction of IFN-γ is not clear. In studies with anti-influenza Tc clones (Taylor & Askonas, 1983; our unpublished observations), Con A can stimulate the release of higher levels of IFN-γ than contact with appropriately infected target cells, since secreted IFN levels continue to rise between 6 and 22 hr, while antigen-induced IFN does not (Fig. 3). Con A leads to aggregation of the cells and gradual death.

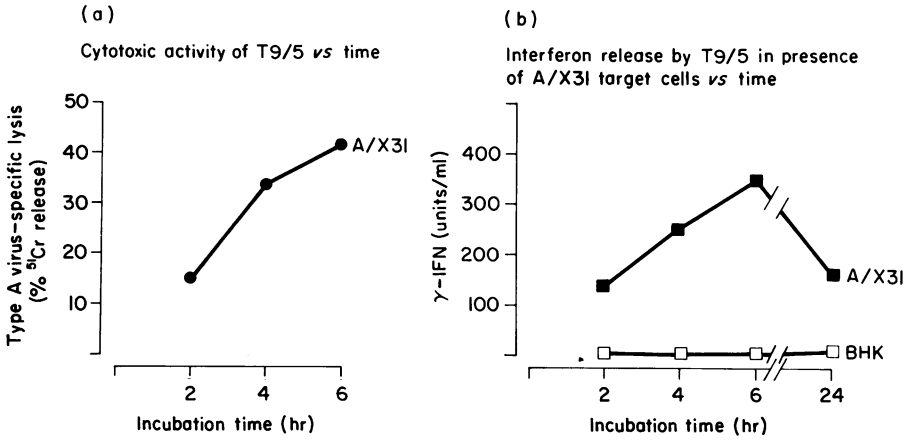

Figure 2. Kinetics of IFN release by Tc in the presence of antigen. Tc clone T9/5, at 5×10^5 cells/ml, K/T = 1. (a) Target cells were P815 cells infected overnight with A/X31 virus at 33°, and labelled with ^{51}Cr for 1 hr before use (^{51}Cr-release assay over a period of 6 hr). The 24-hr time point was not assayed because of high spontaneous ^{51}Cr-release after prolonged infection. Low non-specific lysis of B/HK-infected targets was subtracted. (b) Target cells as above. IFN content was determined for supernatants harvested at 2, 4, 6 and 24 hr after addition of target cells.

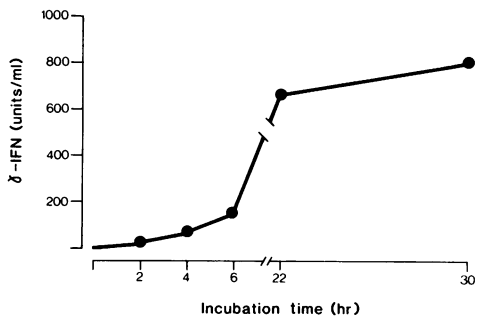

Figure 3. Kinetics of Con A-induced IFN release by Tc clone T9/13. 5×10^5/ml cloned Tc (T9/5) (3 days post-antigenic stimulation) were incubated with 5 μg/ml Con A for various time intervals. IFN content of the supernatant was assayed (see Materials and Methods).

Release of IFN continues on repeated antigenic stimulation

Some receptor functions are down-regulated following interaction with ligands, as in the case of PMA activation of macrophages (Berton & Gordon, 1983). We wished to see whether Tc cells maintain their ability to produce IFN on repeated contact with antigen. IFN release was assayed over 6 hr in the presence of target cells. After that, clone T9/5 was maintained in IL-2 overnight and then IFN release measured again after addition of target cells. Target lysis is complete within a few hours under our conditions. Table 1 shows that repeated antigenic stimulation did not result in loss of the ability to secrete IFN by T9/5 Tc. There was only a small loss of viable cells (27% by Day 4) and the short 6 hr antigen pulses did not result in significant T-cell proliferation. No IFN was found when Tc were incubated with uninfected P815 cells.

Interferon secretion following antibody interactions with surface LFA-1 or Lyt-2 of killer cells

Lyt-2 and LFA-1 molecules have previously been implicated in Tc-target cell recognition (for reviews, see Golstein *et al.*, 1982; Springer *et al.*, 1982). We wished to investigate whether antibody interactions with these membrane proteins affected IFN release. Table 2 shows that, while cytotoxicity of Tc clone BA 4 is inhibited by the three mAbs, this is not so for Tc clone T9/5. While variability in sensitivity by Lyt-2^+ T-cell clones to anti-Lyt-2 has been previously reported (e.g. MacDonald, Thiernesse & Cerottini, 1981), we show here that LFA-1 is also not always essential for target cell recognition. Cytotoxicity of the monoclonal antibodies can be excluded by lack of inhibition of clone T9/5.

Treatment of Tc with monoclonal antibody 53-6.7 (anti-Lyt-2) resulted in profound inhibition of IFN secretion by all three Tc clones tested, whether or not

Table 1. Antigen-induced IFN release by Tc clone T9/5 does not down-regulate with repeated antigenic stimulation

Number of exposures to A/X31 targets	γ-IFN release (U/ml)	% viable cell recovery
–	0	
1	149	100
2	150	ND*
3	166	85
4	123	73

Tc cells (clone T9/5) 7 days after antigenic stimulation at 5×10^5 ml were incubated with A/X31 infected P815 cells (K/T = 1) for 6 hr in a flat-bottomed microtitre plate. Supernatants were removed for γ-IFN assays. Cells were then fed with IL-2 containing medium for 18 hr. The supernatants were discarded, and A/X31 target cells added for 6 hr to estimate IFN induction. This was repeated two more times. P815 target cells were infected overnight with A/X31 (Zweerink *et al.*, 1977).

* ND, not done.

target cell lysis was inhibited. This inhibition was reproducible, and Table 3 illustrates one of the experiments done with each clone. The antibody was also inhibitory for Con A-induced IFN secretion by T9/5 (Table 3). We checked that anti-Lyt-2 did not destroy γ-IFN by titrating an IFN-γ preparation before and after incubation with 1/4 anti-Lyt-2 (not illustrated). In contrast to anti-Lyt-2, two monoclonal antibodies to LFA-1 essentially did not affect IFN secretion by clone T9/5 or BA4 in the presence of antigen. If anything, a slight enhancement of the IFN level was detected (Table 4).

We then examined whether treatment of Tc with the same monoclonal antibodies, followed by addition of fluorescent anti-Ig to produce capping of LFA-1 or Lyt-2, induced IFN release. The surface components capped within 90 min, but this did not result in release of IFN-γ after a 4-hr incubation (Table 5). Even so, Con A treatment of Tc clone BA4 resulted in significant IFN release.

DISCUSSION

It is clear that IFN-γ secretion by influenza-specific cloned Tc depends on recognition of syngeneic influenza-infected target cells and, hence, is H-2 restricted and antigen-dependent (Morris *et al.*, 1982, and this study). However, we found that not all such highly cytotoxic Tc clones are able to secrete IFN-γ on antigen contact, while Con A treatment results in IFN secretion (Taylor & Askonas, 1983). This anomaly led us to further investigate (i) whether Tc cells release IFN at all stages of growth and maturation, and (ii) whether ligand interactions with T-cell surface proteins other than the antigen receptor influence IFN release.

Our results with Tc clone T9/5 show that cell proliferation correlates inversely with induction of IFN-γ by antigen contact. Similarly, Hecht, Longo & Matis (1983) studying T-helper cell lines or clones have

Table 2. Heterogeneity in the inhibition of Tc clone cytotoxicity by anti-LFA-1 or anti-Lyt-2

Tc clone	% target lysis K/T = 5:1	Antibody	Specificity	% inhibition of cytotoxicity AB: 1/8	1/16
BA4	49	M17-5.2	LFA-1	44	52
	54	H35-89.9	LFA-1	82	83
	37	53-6.7	LYT-2	87	80
T9/5	62	M17-5.2	LFA-1	16	8
	58	H35-89.9	LFA-1	14	13
	78	53-6.7	LYT-2	10	6

Targets were ^{51}Cr-labelled P815 cells infected for 3 hr with A/X31 virus. Cytotoxicity assay was for 3 hr. Tc were pretreated with antibodies for 30 min.

Table 3. Anti-Lyt-2 inhibits Ag-induced or Con A-mediated IFN release by Tc

Tc clone	Stimulant	Anti-Lyt-2*	γ-IFN (U/ml)
T9/5	A/31+H-2^d†	–	304
T9/5	A/X31+H-2^d	+	<10
T9/5	Con A‡	–	453
T9/5	Con A	+	115
BA4	A/X31+H-2^d	–	103
BA4	A/X31+H-2^d	+	<10
T9/13	A/X31+H-2^d	–	204
T9/13	A/X31+H-2^d	+	75

* Anti-Lyt-2 is culture supernatant of hybridoma 53-6.7 at 1/4–1/10 final dilution.

† Targets for stimulation of γ-IFN release were A/X31 virus infected P815 cells, killer to target ratio = 1.

‡ Con A was at 5 μg/ml.

Table 4. Anti-LFA-1 does not inhibit γ-IFN release by Tc cell clones BA4 and T9/5

Tc clone + A/X31 P815 cells	Antibody	Dilution	IFN (U/ml)
BA4	–	–	93
BA4	M17-5.2	1/20	115
BA4	–	–	93
BA4	H35-89.9	1/20	105
T9/5	–	–	152
T9/5	M17-5.2	1/20	262
T9/5	–	–	127
T9/5	H35-89.9	1/10	109

MAbs against LFA-1 were culture supernatants from hybridomas M17-5.2 and M35-89.9. Cells were stimulated to secrete γ-IFN by the addition of A/X31-infected P815 cells for 6 hr, K/T = 1.

Table 5. Capping of Tc cell surface markers does not induce γ-IFN secretion by clone BA4

Cell treatment	γ-IFN (U/ml)
Con A*	858
None	<10
Anti-Lyt-2†/RAMIg‡	<10
None	<10
Anti-LFA-1§/RAMIg‡	<10

* Con A was at 5 μg/ml.

† Anti-Lyt-2 was culture supernatant of hybridoma 53-6.7 at 1/10 final dilution.

‡ Clone BA4 was treated with antibodies on ice for 30 min, washed, and treated with fluorescein-conjugated rabbit anti-mouse IgG for 1 hr at 37°. Cells were resuspended to 10^6/ml, incubated for 3 hr, and capping checked.

§ Anti-LFA-1 was culture supernatant of hybridoma M17-5.2 or M35-89.9.

shown some dissociation between proliferation and IFN-γ secretion. Thus, the ability of Tc to secrete IFN on contact with antigen may reflect a T-cell maturation stage and may have special significance in viral infections to prevent further spread of virus particles. Cell agglutination by Con A appears to give a stronger stimulus for IFN-γ release at T-cell stages when antigen does not induce IFN (Taylor & Askonas, 1983). On the other hand, it must be noted that some of our Tc clones totally lose their ability to produce IFN-γ after long-term culture (not illustrated) as previously reported by Guerne, Piguet & Vassalli (1984) for some Lyt-1$^+$2$^-$ or Lyt-1$^-$2$^+$ T-cell clones. The powerful stimulus for IFN secretion by Con A suggests that membrane perturbations and/or transmembrane signalling events are important for IFN-γ release. Tc clones on their own do not show IFN release, even in the presence of IL-2.

Capping of LFA-1 or Lyt-2 on the cloned Tc did not lead to IFN secretion. The cytotoxic T-cell clones differed in their susceptibility to inhibition by the monoclonal antibodies. Target cell lysis by Tc clone T9/5 was not significantly inhibited by the anti-Lyt-2 or anti-LFA-1 antibodies, while clone BA4 was strongly inhibited (~80%) by these antibodies. Lack of inhibition of T9/5 by anti-LFA-1 adds LFA-1 to the list of T-cell membrane components not essential for target cell interaction. Regardless of its inhibitory power of target cell lysis, the antibody to Lyt-2 strongly supressed antigen-induced IFN release by both Tc clones, as well as Con A-induced IFN. Glasebrook, Kelso & MacDonald (1983) found a 60–70% suppression of IFN production by alloreactive Tc clones not inhibited by anti-Lyt-2. We noted no significant effects on antigen-dependent secretion of IFN following anti-LFA treatment of both Tc clones T9/5 and BA4, but even BA4 cytotoxicity is not completely inhibited by the anti-LFA.

It is of interest to note that, of the two Tc clones,

BA4 consistently gave lower levels of target cell lysis than T9/5, possibly reflecting a lower affinity of the clone for its target. Cytotoxic activity of this clone was more readily inhibited by either anti-LFA-1 or anti-Lyt-2 antibodies, indicating, as previously suggested (MacDonald *et al.*, 1982), a role for these molecules in stabilizing target cell binding by Tc clones. Lyt-2 may play a further role in the transmembrane signalling leading to IFN-γ release, especially as monoclonal antibody 53-6.7 was capable of inhibiting release after Con A treatment.

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