Brief Definitive Report

CYTOTOXIC T LYMPHOCYTES PRODUCE IMMUNE INTERFERON IN RESPONSE TO ANTIGEN OR MITOGEN*

By JOHN R. KLEIN, DAVID H. RAULET, MARK S. PASTERNACK, AND MICHAEL J. BEVAN

From the Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Interferons (IFN) are a group of polypeptides that have in common the ability to induce in cells resistance to a broad spectrum of viral infections. Immune or γ-IFN differs in several distinct ways from α-IFN (leukocyte derived) and β-IFN (fibroblast derived) on the basis of both biologic and physicochemical criteria (1–3). Leukocyte and fibroblast IFN are released when cells become infected by virus; immune IFN, on the other hand, is produced by lymphoid cells during the course of an immune response. There are a number of correlations between the stimulation of T cells and the release of immune IFN. For example, culturing lymphoid cells with T cell mitogens or with antigens that stimulate a strong T cell response leads to the production of immune IFN (4–9). Recently, there have been reports that a human T cell line (10) and a number of murine T cell clones (11) release immune IFN upon culture with PHA or Con A. Although these cell lines were probably of the T cell lineage, they lacked any functionally defined T cell characteristics.

Kirchner et al. (8) reported the provocative finding that in murine mixed lymphocyte culture (MLC), differences in class I (H-2K or D) antigens induced more IFN production than differences in the I region or the Mls locus. This was true even though I region or Mls differences were better stimulators of T cell proliferation. This finding suggested to us that immune IFN may be released by cytotoxic T lymphocytes (CTL) that are H-2K, D region specific. Additionally, treatment of antigen-stimulated spleen cells with anti-Lyt-2,3 antisera plus complement (C′) has been shown to greatly reduce the level of IFN, although in these experiments anti-immunoglobulin, anti-Ia, and anti-Lyt-1 sera also had the same effect (12). Moreover, a correlation has been demonstrated between IFN release and the development of CTL activity in MLC in which the responders came from primed or normal mice (9). Farrar and colleagues (13) have pointed to the correlation between immune IFN release and CTL activation, and we have also found a strong correlation between CTL activity and IFN production (unpublished observations).

In this communication, we show that clones of murine antigen-specific, H-2-restricted CTL release high levels of immune IFN when they are co-cultured with the mitogen Con A or with allogeneic cells presenting antigen. IFN release in the latter case is antigen specific and H-2 restricted. These results add a new, important aspect to our understanding of T cell immunity to foreign membrane antigens in showing

* Supported by grants AI-14269 and CA-14501 from the U. S. Public Health Service.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/82/04/1198/06 $1.00
Volume 155 April 1982 1198–1203

that CTL can deliver a "one-two punch" at a local site: death to a target cell and the local release of immune IFN.

Materials and Methods

Mice. BALB/cAnN (H-2^d), BALB.B (H-2^b), and (B10.BR × B10.D2)F_1 (H-2^k × H-2^d) mice were bred at the Center for Cancer Research, Massachusetts Institute of Technology. B10.D2/nSnJ and B10.BR/SgSnJ mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All animals were used at 8–20 wk of age.

Antisera Treatment. Thy-1 positive cells were removed from normal spleen cells by treatment with two monoclonal anti-Thy-1 antibodies followed by rabbit C′. Normal spleen cells were suspended at 10^7 cells/ml in Hanks' balanced salt solution containing ascites fluid from mice bearing hybridoma clone 13.4 (14) plus T24 culture supernatant (15) at final dilutions of 1:100 and 1:50, respectively. After 30 min at 4°C, cells were resuspended at 5 × 10^6/ml in a 1:20 dilution of a selected rabbit C′ and incubated for 45 min at 37°C.

Cloned CTL. The history and specificity of the cr15 cloned CTL line has been described (16). The cells derive from a (B10.BR × B10.D2)F_1 mouse and are specific for a BALB background minor histocompatibility antigen plus H-$2D^d$. The cells were cloned by limiting dilution (at 0.3 cells/well) and maintained as described by Glasebrook and Fitch (17) by weekly subculture in medium containing IL-2 plus irradiated BALB/c spleen cells. The medium used was RPMI 1640 (Microbiological Associates, Walkersville, MD) containing 5% fetal calf serum, 5 × 10^{-5} *M* 2-mercaptoethanol (MLC medium), supplemented with 50 *mM* α-methyl mannoside, and 20% medium from spleen cells (5 × 10^6/ml) cultured for 24 h with 2 μg/ml concanavalin A (Con A).

The spleen Con A supernatant can be replaced by the supernatant from AOFS 21.10.9 T hybridoma cells cultured with 10 μg/ml Con A for 24 h (18). Most of the Con A was removed from AOFS Con A supernatant (AOFS Sup) by adsorption to Sepharose 4B coupled with *p*-amino-phenyl-α-D-mannopyranoside (D. C. Parker, personal communication).

Assay for Cytotoxic Activity. cr15 cells that had been subcultured in medium with AOFS Sup without stimulator cells for 5 d were titrated against a constant number (4 × 10^4) of ^{51}Cr-sodium chromate lipopolysaccharide-induced B cell blasts from various mouse strains (16).

IFN Production. cr15 cells were harvested 7–12 d after the previous subculture and washed twice in RPMI 1640 medium containing 5% FCS. 2 × 10^5 to 5 × 10^5 cells/ml were cultured in 2 ml volumes in Costar wells (Costar, Data Packaging, Cambridge, MA) in MLC medium supplemented with 20% AOFS Sup with 2 × 10^6/ml irradiated (1,300 rad), T cell-depleted spleen cells, or Con A. Usually, after 2 d incubation at 37°C, the cultures were harvested, and centrifuged at 500 *g* for 10 min. The supernatants were passed through a 0.2 μm filter and stored frozen. 0.2 ml cultures in Costar 3596 wells were pulsed on day 2–3 for 4 h with 2 μCi [^{3}H]thymidine for 4 h to assess the rate of DNA synthesis.

IFN Assay. IFN activity was determined in a conventional cytopathic effect (CPE) inhibition assay of vesicular stomatitis virus (VSV) on L cells (1). L cells were seeded into 96-well microtiter plates (Costar 3596) in a volume of 100 μl/well. To this was added 50 μl of serially diluted test supernatants. 24 h later, 50 μl of 200 $TCID_{50}$ VSV was added to each well. 48 h post-infection, the tissue monolayer was scored visually for virus-induced CPE. The IFN titer is defined as the reciprocal of the highest dilution showing at least 50% inhibition of CPE in triplicate cultures.

IFN Neutralization. Supernatant from antigen or mitogen-induced cr15 cells was dialyzed for 24 h against 0.1 M glycine-HCl, 0.15 M NaCl, pH 2.0 buffer, followed by dialysis for 48 h against phosphate-buffered saline, pH 7.2, and RPMI 1640 medium. Rabbit anti-mouse immune IFN serum was generously provided by Dr. Howard M. Johnson, University of Texas, Galveston, TX, and was used according to his protocol (19). Briefly, diluted antiserum (1:30) was mixed with an equal volume of serially diluted IFN supernatant and incubated at room temperature for 1 h. 50 μl of each sample was then added to L cell monolayers and tested for VSV inhibition as described above.

Results

Characteristics of Cloned CTL. Clones of CTL isolated and maintained with antigen

TABLE I

H-2^d-restricted Cytotoxic Activity of cr15 CTL

Target cell	Percent specific lysis at CTL/target ratio	
	10:1	1:1
(B10.BR × B10.D2)F_1, H-2^k/H-2^d	−5	−2
B10.D2, H-2^d	−4	0
BALB/c, H-2^d	38	22
BALB.B, H-2^b	−2	−1
(BALB.B × B10.D2)F_1, H-2^b/H-2^d	20	15

cr15 CTL were assayed for 4 h for lysis of ^{51}Cr-labeled, 3-d LPS-induced spleen cells from the various strains listed.

TABLE II

Production of Immune IFN by cr15 CTL in Response to Antigen Stimulation

	Cells cultured	IFN Titer following treatment				[^{3}H]TdR incorporation
		None	Dialysis pH 7.2	Dialysis pH 2.0	Anti-immune IFN	
Experiment 1	BALB.B$_x$ alone	<6	ND*	ND	ND	40
	BALB/c$_x$ alone	<6	ND	ND	ND	520
	cr15 plus BALB.B$_x$	96	96	<6	ND	2,156
	cr15 plus BALB/c$_x$	768	768	12	ND	15,440
Experiment 2	cr15 alone	<6	ND	ND	ND	1,440
	cr15 plus B10.D2$_x$	96	ND	ND	ND	800
	cr15 plus BALB/c$_x$	3,072	ND	ND	96	6,660

2.6×10^5 cr15 cells/ml (experiment 1) or 5×10^5 cr15 cells/ml (experiment 2) were cultured in 20% AOFS Sup containing medium with 2×10^6/ml spleen cells that had been treated with anti-Thy 1 plus C′ and irradiated. Supernatants were harvested on day 2 of culture for the IFN assay. Triplicate 0.2-ml cultures were pulsed with [^{3}H]thymidine on day 3. Stimulator cells, when cultured with Con A, 2 μg/ml, produced no detectable amounts of IFN (<6).

* Not done.

and growth factors remain dependent on both antigenic stimulation and IL-2 for optimum growth. The cytotoxic specificity of cr15 (B10.BR × B10.D2)F_1 CTL for a BALB background minor histocompatibility antigen recognized in the context of H-2^d is illustrated by the data in Table I: the CTL lyse BALB/c(H-2^d) targets, but not BALB.B(H-2^b) targets, because of H-2 restriction, or B10.D2(H-2^d) targets, which lack the foreign minor antigen; they do lyse the (BALB.B × B10.D2)F_1 target, which carries the BALB minor histocompatibility antigen plus H-2^d (16). The cr15 CTL used in the following experiments were typed with a battery of antisera and shown to be 100% sensitive to lysis with anti-H-2^d, anti-H-2^k, anti-Thy-1.2, and anti-Lyt-2.2 antibodies and C′ (data not shown).

IFN Production by cr15 in Response to Antigens. When cultured in the presence of T cell-depleted, irradiated allogeneic stimulator cells in IL-2 supplemented medium free of IFN (i.e., AOFS Sup), cr15 cells were found to make significant levels of IFN in 2-d cultures (Table II). IFN produced by these cultures was of the immune type based on its sensitivity to pH 2.0 and on its inactivation by anti-mouse immune IFN serum (>98% loss of IFN activity in each case). cr15 cultured alone, i.e., in the absence of antigen but in IL-2 supplemented medium, as above, failed to produce IFN. Additionally, cr15, when cultured with semisyngeneic cells or H-2 mismatched BALB.B cells, produced substantially less immune IFN (1:96) than that made in response to alloantigenic stimulation (1:768 and 1:3,072). The possibility that immune IFN was produced by the T cell-depleted, irradiated stimulator cells in our culture system was

TABLE III

Con A Induced Release of IFN from cr15 CTL

Cells cultured	Dose of Con A	IFN Titer		[^{3}H]TdR incorporation
		No treatment	Anti-immune IFN	
cr15	0	<6	ND*	1,440
cr15	0.4 μg/ml	6,144	ND	150
cr15	1.0 μg/ml	12,288	ND	160
cr15	2.0 μg/ml	12,288	384	118
cr15	4.0 μg/ml	6,144	ND	110
cr15	8.0 μg/ml	1,530	ND	122
BALB/c spleen, 5×10^6/ml	2.0 μg/ml	768	48	ND

cr15 cells were washed twice in medium lacking IFN and cultured at 5×10^5/ml with the indicated dose of Con A in MLC medium supplemented with 20% AOFS Sup. Culture medium was collected on day 2 for the IFN assay.

* Not done.

effectively ruled out because Con A stimulation of these cells alone failed to induce IFN production; Con A is known to promote IFN production in normal whole spleen cell cultures (see Table III). Thus, the cloned CTL line described here makes immune IFN in an antigen-dependent fashion with the same specificity that regulates its CTL activity.

As previously described, cr15 require both antigen-specific stimulation and IL-2 for growth. In both sets of experiments (Table II), thymidine incorporation as well as IFN production was maximal in cr15 cultures stimulated with antigen-bearing cells.

Effect of Con A Stimulation on IFN Production by cr15. cr15 were tested for their ability to make immune IFN in the absence of antigen when stimulated with the T cell mitogen, Con A. Cells cultured with Con A produced high levels of IFN in a dose-dependent fashion; in the same experiments there was a fourfold greater IFN titer (12,288 vs. 3,072) in Con A-stimulated cr15 cells as compared with antigen-stimulated cells. The ability of cr15 cells to make IFN upon addition of Con A further confirms the source of IFN to be the CTL themselves, because no additional cells were added. Of interest in this experiment was the sharp reduction in proliferative activity by Con A-stimulated cr15 cells, despite large amounts of secreted IFN.

The IFN activity induced by Con A was also shown to be >94% susceptible to overnight dialysis at pH 2.0 (data not shown) and treatment with anti-immune IFN serum. The IFN titer of a typical 24-h Con A induced spleen cell supernatant is also shown in Table III. Finally, we have shown that an alloreactive, cloned CTL line, G4, derived from a BALB.B mouse, specific for H-2^d antigens, also releases IFN in response to Con A or antigen stimulation.

Discussion

The results presented here demonstrating antigen- or mitogen-induced release of immune IFN by cloned CTL lines explain the correlations that have been noted between CTL stimulation and IFN production (7–9, 13, 20). In what way immune IFN participates in CTL function remains to be determined. However, there are several possible ways in which IFN could be of importance. IFN could contribute to the killing of target cells recognized by the CTL, i.e., it could provide the "lethal-hit" to the target. Immune IFN may also be an interleukin which, when released by CTL,

acts in some immunoregulatory capacity on other immune cells responding to the same antigen; there is a substantial body of information supporting this role for immune IFN (1, 3). However, the most straightforward interpretation of the finding that CTL release IFN on binding antigen is that IFN contains the spread of antigen. For example, CTL that recognize and kill virus-infected cells would help prevent the spread of infection if, in addition, they released IFN at the local site of infection. IFN may also inhibit tumor growth or enhance the activity of other killer mechanisms at the site of a CTL-target recognition event (21, 22). Finally, it should be kept in mind that our results demonstrating IFN production by CTL do not rule out the possibility that immune IFN is made by other lymphoid cells as well.

Summary

Interferon (IFN) was found to be secreted by cloned lines of murine cytotoxic T lymphocytes in response to mitogenic or antigen specific stimulation. The IFN produced was shown to be of the immune type based on its sensitivity to pH 2.0 and on the ability of an antiserum to immune IFN to neutralize the antiviral activity.

Note added in proof. Since submission of this manuscript, a report of antigen-induced secretion of IFN by a virus-specific CTL line has appeared (23).

Received for publication 28 December 1981.

References

1. Stewart, W. E. 1981. The Interferon System. Springer-Verlag, Vienna. 493.
2. 1980. Interferon nomenclature. *Nature (Lond.)* **286:**110.
3. DeMaeyer, E., and J. DeMaeyer-Guignard. 1979. Interferons. *Compr. Virol.* **15:**205
4. Green, J. A., S. R. Cooperband, and S. Kibrick. 1969. Immune specific induction of interferon synthesis in cultures of human blood lymphocytes. *Science (Wash. D. C.).* **164:** 1415.
5. Langford, M. P., J. A. Georgiades, G. J. Stanton, F. Dianzani, and H. M. Johnson. 1979. Large-scale production and physicochemical characterization of human immune interferon. *Infect. Immun.* **26:**36.
6. de Ley, M., J. von Damme, H. Claeys, H. Weening, J. W. Heine, A. Billiau, C. Vermylen, and P. de Somer. 1980. Interferon induced in human leukocytes by mitogens: production, partial purification and characterization. *Eur. J. Immunol.* **10:**877.
7. Gifford, G. E., A. Tibor, and D. L. Peavy. 1971. Interferon production in mixed lymphocyte cultures. *Infect. Immun.* **3:**164.
8. Kirchner, H., R. Zawatzky, and V. Schirrmacher. 1979. Interferon production in murine mixed lymphocyte culture. I. Interferon production caused by differences in the H-2 K and H-2 D regions but not by differences in the I region or the Mls locus. *Eur. J. Immunol.* **9:**97.
9. Perussia, B., L. Mangoni, H. D. Engers, and G. Trinchieri. 1980. Interferon production by human and murine lymphocytes in response to alloantigens. *J. Immunol.* **125:**1589.
10. Nathan, J., J. E. Groopman, S. G. Quan, N. Bersch, and D. W. Golde. 1981. Immune (γ) interferon produced by a human T-lymphoblast cell line. *Nature (Lond.).* **292:**842.
11. Marcucci, F., M. Waller, H. Kirschner, and P. Krammer. 1981. Production of immune interferon by murine T-cell clones from long-term cultures. *Nature (Lond.).* **291:**79.
12. Sonnenfeld, G., A. D. Mandel, and T. C. Merigan. 1979. *In vitro* production and cellular origin of murine type II interferon. *Immunology.* **36:**883.
13. Farrar, W. L., H. M. Johnson, and J. J. Farrar. 1981. Regulation of the production of immune interferon and cytotoxic T lymphocytes by interleukin 2. *J. Immunol.* **126:**1120.

14. Marshak-Rothstein, A., P. Fink, T. Gridley, D. H. Raulet, M. J. Bevan, and M. L. Gefter. 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* **122:**2491.
15. Dennert, G., R. Hyman, J. Lesley and I. S. Trowbridge. 1980. Effects of cytotoxic monoclonal antibody specific for T200 on functional lymphoid populations. *Cell. Immunol.* **53:**350.
16. Hünig, T., and M. J. Bevan. 1982. Antigen recognition by cloned cytotoxic T lymphocytes follows rules predicted by the altered-self hypothesis. *J. Exp. Med.* **155:**111.
17. Glasebrook, A. L., and F. W. Fitch. 1980. Alloreactive cloned T cell lines I. Interactions between cloned amplifier and cytolytic T cell lines. *J. Exp. Med.* **151:**876.
18. Harwell, L., B. Skidmore, P. Marrack, and J. Kappler. 1980. Concanavalin A-inducible, interleukin-2 producing T cell hybridoma. *J. Exp. Med.* **152:**893.
19. Osborne, L. C., J. A. Georgiades and H. M. Johnson. 1980. Classification of interferons with antibody to immune interferon. *Cell. Immunol.* **53:**65.
20. Ennis, F. A., and A. Meager. 1981. Immune interferon produced to high levels by antigenic stimulation of human lymphocytes with influenza virus. *J. Exp. Med.* **154:**1279.
21. Gresser, I. 1977. On the varied biologic effects of interferon. *Cell. Immunol.* **34:**406.
22. Bloom, B. R. 1980. Interferons and the immune system. *Nature (Lond.).* **284:**593.
23. Morris, A. G., Y.-L. Lin, and B. A. Askonas. 1982. Immune interferon release when a cloned cytotoxic T cell line meets its correct influenza-infected target cell. *Nature (Lond.).* **295:**150.