tized rabbit RBCs were prepared as described by T. A. E. Platt-Mills and K. Ishizaka [*J. Immunol.* **113**, 348 (1974)].
6. G. J. Kotwal and B. Moss, *Virology* **167**, 524 (1988); G. J. Kotwal *et al.*, *Defense Molecules: UCLA Symp. Mol. Cell. Biol. New Ser.* **121**, 149 (1990).
7. Plasmid pGK10, containing the entire gene for the 35-kD protein within the Hind III–Bam HI portion of the Hind III C fragment of vaccinia virus DNA cloned in pUC19, was cleaved with either Hinc II (for vSIGK1) resulting in the excision of a 2-kb region containing the entire open reading frame (ORF) for the 35-kD gene along with the end regions of the adjacent ORFs or with Eco RV (for vSIGK3) resulting in the excision of only 70 bp within the ORF of the 35-kD gene. A *gpt* cassette (containing the *Escherichia coli gpt* gene under the control of the vaccinia P7.5 promoter) was excised from plasmid pTK61-*gpt* [F. G. Falkner and B. Moss, *J. Virol.* **62**, 1849 (1988)] by digestion with Eco RI and inserted in place of the 35-kD gene or within the 35-kD gene after filling in the ends with the Klenow fragment of DNA polymerase, to yield plasmids pGK11 and pGK12, respectively. CV-1 cells infected with wild-type virus were transfected with pGK11 or pGK12. Recombinant vaccinia viruses were isolated by three rounds of plaque purification in selective medium. The resulting plaque-purified mycophenolic acid–resistant viruses, vSIGK1 and vSIGK3, were amplified to high titer.
8. G. J. Kotwal, A. W. Hugin, B. Moss, *Virology* **171**, 579 (1989).
9. T. A. Gaither, C. H. Hammer, M. M. Frank, *J. Immunol.* **123**, 1195 (1979).
10. R. McKenzie, unpublished data.
11. L. P. Chung, D. R. Bentley, K. B. M. Reid, *Biochem. J.* **230**, 133 (1985).
12. A. Ferreira, M. Takahoshi, V. Nuzzenzweig, *J. Exp. Med.* **146**, 1001 (1977); J. Scharfstein, A. Ferreira, I. Gigli, V. Nussenzweig, *ibid.* **148**, 207 (1978); J. Burge, A. Nicholson-Weller, K. F. Austen, *J. Immunol.* **126**, 232 (1981).
13. I. Gigli, T. Fujita, V. Nussenzweig, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6596 (1979).
14. D. M. Lublin *et al.*, *J. Exp. Med.* **168**, 181 (1988).
15. M. E. Medof *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2007 (1987); I. W. Caras *et al.*, *Nature* **325**, 545 (1988).
16. B. Moss, *Virology* (Raven, New York, 1990), pp. 2079–2112.
17. S. N. Isaacs, unpublished data.
18. G. J. Kotwal and A. Hügin, unpublished data.
19. We thank L. Maloy for peptides and N. Cooper for CV-1 and BSC-1 cells.

27 April 1990; accepted 6 August 1990

# Expression of Interleukin-10 Activity by Epstein-Barr Virus Protein BCRF1

DI-HWEI HSU, RENE DE WAAL MALEFYT, DAVID F. FIORENTINO, MINH-NGOC DANG, PAULO VIEIRA, JAN DEVRIES, HERGEN SPITS, TIMOTHY R. MOSMANN,* KEVIN W. MOORE†

Cytokine synthesis inhibitory factor (CSIF; interleukin-10), a product of mouse $T_H2$ T cell clones that inhibits synthesis of cytokines by mouse $T_H1$ T cell clones, exhibits extensive sequence similarity to an uncharacterized open reading frame in the Epstein-Barr virus BCRF1. Recombinant BCRF1 protein mimics the activity of interleukin-10, suggesting that BCRF1 may have a role in the interaction of the virus with the host's immune system.

INTERLEUKIN 10 (IL-10) IS A CYTOKINE produced by one class of mouse helper T cell clone (TH2) that inhibits synthesis of cytokines [notably interferon-γ (IFN-γ)] by activated TH1 clones (*1*). Because TH1 cells preferentially mediate delayed type hypersensitivity (DTH) and macrophage activation (*2*), whereas TH2 cells provide superior help for B cell (antibody) responses (*3*), IL-10 may represent a mechanism whereby TH2 cells can inhibit the effector functions of TH1 cells. This possibility could help explain why DTH responses and antibody responses are often mutually exclusive (*4*).

Complementary DNA clones that encode mouse IL-10 (mIL-10) (*5*) reveal that the mature, secreted IL-10 polypeptide has approximately 70% amino acid identity to an uncharacterized open reading frame in the Epstein-Barr virus (EBV) BCRF1 (*6*). We therefore cloned and expressed the *BCRF1* gene, and demonstrated that the expressed BCRF1 protein, like IL-10, inhibits IFN-γ synthesis by activated lymphoid cells.

As a source of BCRF1 DNA, we used either whole EBV genomic DNA prepared from infectious virus isolated from the marmoset cell line B95-8 (*7*), or plasmid subclones of the EBV Bam HI C fragment (*6*). The predicted protein-coding region of the *BCRF1* gene (*5, 6*) was amplified by polymerase chain reaction (PCR) with oligonucleotide primers that also contained Eco RI sites for subsequent cloning into a modified form of the pcDSRα296 expression vector (*8*). The BCRF1 insert in the expression plasmid used in these experiments was derived from the Bam HI C fragment subclones, but EBV genomic DNA isolated from infectious virus also gave a PCR-amplified fragment of the expected size. The complete DNA sequence of the resulting BCRF1 insert was determined and was identical to the published sequence (*6*). COS-7 cells transiently transfected with this plasmid were cultured in the presence of [$^{35}$S]methionine, with or without tunicamycin $B_2$ ($TcB_2$) (*5*), and supernatants were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The $^{35}$S-labeled supernatant from BCRF1-expressing cells contained an ~17 kD polypeptide not present in supernatants from mock-transfected cells. The BCRF1 polypeptide is approximately the same size as the unglycosylated form of mIL-10. The mobility of BCRF1 in SDS-PAGE was not altered when $TcB_2$ was included in the culture, suggesting that, unlike mIL-10 (*5*), BCRF1 contains little or no N-linked oligosaccharide. Because BCRF1 lacks the N-linked glycosylation site at Asn[11] of mIL-10 (*5*),

**Table 1.** BCRF1 inhibits IFN-γ synthesis by antibody to CD3 (anti-CD3)–stimulated PBMC. Occasionally enhancement by IFN-γ synthesis by COS-7 (mock) supernatant was observed (experiments 2 and 3). This result was not uniformly obtained among various donors (experiment 1).

| Stimulation | IFN-γ (ng/ml) | | |
|---|---|---|---|
| | Experiment 1 | Experiment 2 | Experiment 3 |
| None | <0.30 | <0.30 | <0.30 |
| Anti-CD3 | 18.65 | 3.95 | 7.87 |
| Anti-CD3 + BCRF1 | <0.30 | 1.04 | <0.30 |
| Anti-CD3 + mock | 19.29 | 9.00 | 14.67 |

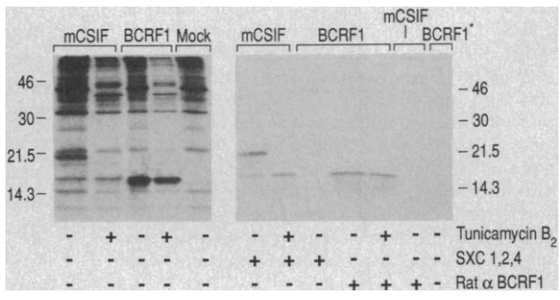


**Fig. 1.** Expression of the BCRF1 gene (*22*). Lanes show either total $^{35}$S-labeled COS-7 supernatants (mCSIF, BCRF1, mock) or immunoabsorbed mCSIF or BCRF1 as indicated. *, Immunoabsorption was carried out with preimmune rat serum (*23*). SXC 1,2,4 are monoclonal antibodies to rat mIL-10 (*9*).

D.-H. Hsu, D. F. Fiorentino, M.-N. Dang, P. Vieira, T. R. Mosmann, K. W. Moore, Department of Immunology, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304.
R. de Waal Malefyt, J. deVries, H. Spits, Department of Human Immunology, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304.

*Present address: Department of Immunology, University of Alberta, Edmonton, Alberta, Canada.
†To whom correspondence should be addressed.

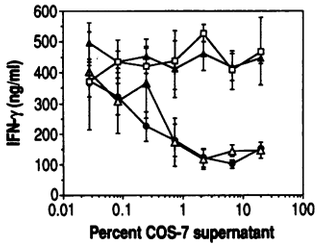


**Fig. 2.** BCRF1 inhibits IFN-γ synthesis by an antigen-stimulated mouse TH1 clone. Mouse IFN-γ concentration (nanograms per milliliter) is shown as a function of the amount of BCRF1 (△), mIL-10 (●), or mock (□, ▲) supernatants introduced into the culture. The mIL-10 cDNA clone F115 was used for expression of the mouse cytokine in COS-7 cells. The TH1 clone (HDK.1) was cultured with trinitrophenol-modified keyhole limpet hemocyanin and syngeneic (BALB/c) spleen cells for 36 to 44 hours; supernatants were harvested and tested for IFN-γ by ELISA as described (*1*). Error bars show standard deviations of triplicate samples. These data have been reproduced in more than a dozen experiments.

these results suggest that $Asn^{11}$ may be the principal site of attachment of N-linked carbohydrate in mIL-10.

Rat monoclonal antibodies specific for mIL-10 (*5, 9*) did not recognize BCRF1 in a solid-phase radioimmunoabsorption assay (Fig. 1). However, a polyclonal rat antiserum against COS-7–BCRF1 supernatant contained antibodies that immunoabsorbed BCRF1, but lacked detectable cross-reaction with mIL-10 in this system. Thus, despite the substantial similarity between BCRF1 and mIL-10, no antibodies that cross-react with the two cytokines have yet been identified.

Mouse IL-10 inhibits IFN-γ synthesis by an antigen-stimulated mouse TH1 clone in the presence of syngeneic antigen-presenting cells (*1, 5*). Like mIL-10, BCRF1 inhibited IFN-γ synthesis in this assay (Fig. 2). The slopes of the titration curves and maximum extents of inhibition by supernatants containing mIL-10 and BCRF1 were generally similar. BCRF1 therefore has IL-10–like activity on mouse cells.

Whether BCRF1 could exert a similar effect on IFN-γ synthesis by human cells was tested by stimulating human peripheral blood mononuclear cells (PBMC) with either phytohemagglutinin (PHA), antibodies against the T cell receptor for antigen (CD3), or IL-2 in the presence of COS-7 supernatants containing BCRF1, mouse IL-10, or control supernatants from mock transfections (*5*). BCRF1 inhibited IFN-γ synthesis in cultures of PHA- and IL-2–stimulated human PBMC (Fig. 3). BCRF1 also inhibited IFN-γ synthesis induced by antibodies to CD3 (anti-CD3) (Table 1). In contrast, mIL-10 did not have activity on human cells in this system at the concentrations tested (*10*). Similar results are also observed with the RNA (Fig. 3B): BCRF1 inhibited the amount of IFN-γ mRNA that was detected in stimulated human PBMC. Control experiments in which an actin probe was used established that the amount of RNA remained relatively constant in these cultures (Fig. 3B). Thus, BCRF1 has IL-10–like activity on both mouse and human cells.

Natural killer (NK) cells, rather than T cells are the major source of IFN-γ in human PBMC stimulated with either PHA or IL-2 (*11*). Therefore, the ability of BCRF1 to inhibit IFN-γ synthesis by a mouse T cell clone, anti-CD3–stimulated human PBMC, and by PHA- or IL-2–stimulated human PBMC suggests that this cytokine may inhibit IFN-γ synthesis not only by T cells, but also by NK cells.

The results reported here and elsewhere (*5, 10*) suggest that BCRF1 may represent a processed viral homolog of the cellular IL-10 gene. The observation that BCRF1 has functional IL-10 cytokine activity suggests that it could participate in the interaction of the virus with the host's immune system. Whereas the control of persistent EBV infection is probably mediated principally by classical MHC-restricted cytotoxic T cells (*12*), IFN-γ inhibits the early stages of generation and outgrowth of EBV-infected cells in vitro (*13*). NK cells also appear to participate in the response to EBV in the early stages of infection (*14*); therefore the possibility that BCRF1 may inhibit IFN-γ synthesis by NK cells is suggestive. We therefore propose that BCRF1 may have a functional role during the acute stage of EBV infection of the target cell. In fact, the *BCRF1* gene is transcribed in the late phase of the lytic virus cycle (*15*). Whereas BCRF1 expression is not detected by RNA blot in latently infected cells (*15–17*), we have detected BCRF1 RNA by PCR in four of seven EBV-transformed B cell lines (*17–19*). This could be caused by a small proportion of latently infected cells spontaneously entering the lytic cycle. Thus, BCRF1 may exert a protective effect during the lytic cycle when both the early and late class of viral proteins are produced by the infected cell (*20*).

These results, along with others (*5, 21*) suggest the possibility that expression of captured genes encoding immunoregulatory proteins could be a mechanism used by other viruses, microbial pathogens, or parasites in their interactions with the host's immune system.

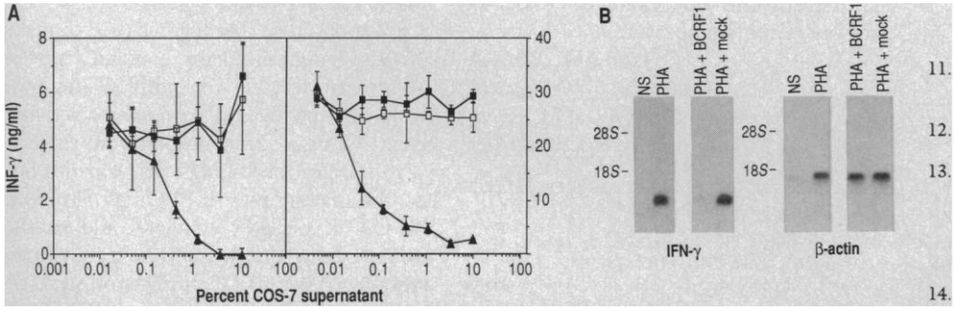


**Fig. 3.** BCRF1 inhibits IFN-γ synthesis by activated human PBMC. (**A**) Effects of BCRF1 (▲), mIL-10 (■), or mock (□) COS-7 supernatants on IFN-γ synthesis in human PBMC cultures stimulated with PHA (left) or IL-2 (right) (*24*). Error bars and numbers of experiments are as in Fig. 2. (**B**) BCRF1 inhibits the amount of IFN-γ mRNA present in PHA-stimulated human PBMC. The figure shows the same RNA blot filter probed with an IFN-γ cDNA probe (left) or mouse β-actin (right) (*25*).

## REFERENCES AND NOTES


1. D. F. Fiorentino, M. W. Bond, T. R. Mosmann, *J. Exp. Med.* **170**, 2081 (1989).
2. D. J. Cher and T. R. Mosmann, *J. Immunol.* **138**, 3688 (1987); R. D. Stout and K. D. Bottomly, *ibid.* **142**, 760 (1989).
3. W. H. Boom, D. Liano, A. K. Abbas, *J. Exp. Med.* **167**, 1352 (1988); L. Killar, G. MacDonald, J. West, A. Woods, K. Bottomly, *J. Immunol.* **138**, 1674 (1987).
4. Y. Katsura, *Immunology* **32**, 227 (1977); C. R. Parish, *Transplant. Rev.* **13**, 35 (1972).
5. K. W. Moore *et al.*, *Science* **248**, 1230 (1990).
6. R. Baer *et al.*, *Nature* **310**, 207 (1984).
7. G. Miller and M. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 170 (1973).
8. Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988).
9. T. R. Mosmann *et al.*, *J. Immunol.*, in press.
10. Analysis of human CSIF cDNA clones isolated from a human T cell clone (P. Vieira *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press) has revealed that the human cytokine cDNA is homologous to its murine counterpart throughout the coding sequence and 3′ untranslated region, but is similarly related to BCRF1 only in the mature protein coding sequence.
11. G. Trinchieri *et al.*, *J. Exp. Med.* **160**, 1147 (1984); H. A. Young and J. R. Ortaldo, *J. Immunol.* **139**, 724 (1987).
12. S. F. Slovin, R. T. Schooley, D. A. Thorley-Lawson, *J. Immunol.* **130**, 2127 (1983).
13. J. Gosselin, J. Menezes, G. Mercier, G. Lamoureux, D. Oth, *Cell. Immunol.* **122**, 440 (1989); F. Hasler, H. G. Bluestein, N. J. Zvaifler, L. B. Epstein, *J. Exp. Med.* **157**, 173 (1983); M. Lotz, C. D. Tsoukas, S. Fong, D. A. Carson, J. H. Vaughan, *Eur. J. Immunol.* **15**, 520 (1985).
14. M. G. Masucci, M. T. Bejarano, G. Masucci, E. Klein, *Cell Immunol.* **76**, 311 (1983); G. Klein and E. Klein, *Prog. Med. Virol.* **30**, 87 (1984).
15. G. S. Hudson, A. T. Bankier, S. C. Satchwell, B. G. Barrell, *Virology* **147**, 81 (1985).
16. J. Sample *et al.*, *ibid.* **139**, 1 (1984).
17. D.-H. Hsu *et al.*, unpublished data.

18. PCR on reverse-transcribed RNA was as described (*5, 19*). Control reactions in which reverse transcriptase was omitted were done for each sample. Because of the presence of closed circular EBV episomes in some samples, reverse transcription was preceded by treatment with ribonuclease-free deoxyribonuclease [F. A. W. Fuqua, S. A. Fitzgerald, W. L. McGuire, *Biotechniques* **9**, 206 (1990)]. Under these conditions, BCRF1 RNA was detected in the JDV and NPR (H. Yssel, H. Gascan, P. Schneider, H. Spits, J. E. de Vries, in preparation), Daudi, and RPMI8866 cell lines.
19. S. O. Gollnick *et al.*, *J. Immunol.* **144**, 1974 (1990).
20. J. Dillner and B. Kallin, *Adv. Cancer Res.* **50**, 95 (1988).
21. C. A. Smith *et al.*, *Science* **248**, 1019 (1990).
22. PCR was done as described (*19*) with the following primers: 5′-ACAGGAATTCGATGGAGCGAAGGTTAGTGGTCACTCTGCAG-3′ (sense) and 5′-GTAGGAATTCCTCACCCTGGCTTTAATTGTCATGTATGCTTCTAT-3′ (antisense), where the Eco RI sites for cloning are indicated by underlining and the translation start and stop sites are bold. The PCR product was extracted with phenol and ethanol precipitated, and subjected to a second round of amplification (40 cycles) under the same conditions. After phenol extraction and ethanol precipitation, the amplified fragment was digested with Eco RI to generate cohesive ends for cloning. After purification by agarose gel electrophoresis, the BCRF1 fragment was cloned into a modified form of the pcDSRα296 plasmid (*8*).
23. Rats were immunized with 50-fold concentrated, serum-free COS-7–BCRF1 transfection supernatant in complete Freund's adjuvant, then boosted 1 month later with the same immunogen in incomplete Freund's adjuvant. Sera were collected before the first immunization and 10 days after the second. COS-7 cells on day 3 after transfection were cultured in methionine-free medium supplemented with $^{35}$S-methionine at 1 mCi/ml (Amersham) for 4 hours at 37°C. Solid-phase radioimmunoabsorption was done (*9*) with the following modifications in cases where rat antisera were used. Each well of a round-bottom 96-well microtiter plate was coated with 20 μl of goat antibody to rat immunoglobulin G (100 μg/ml) (Jackson Immunoresearch) overnight at 4°C. After blocking in 10% fetal calf serum, (FCS), the plates were washed, incubated with immune or control rat serum (1%) for 1 hour at 37°C, and washed again. A mixture of 50% of a 3-day mock COS-7 transfection supernatant and a 1:4 dilution of $^{35}$S-methionine-labeled transfection supernatant in 5% FCS, 0.02% Tween-20 was then incubated in each well for 1 hour at 37°C. After subsequent washing, samples were processed as described (*9*) for SDS-PAGE and autoradiography for 1 to 10 days.
24. Human PBMC were isolated from buffy coats from healthy donors by centrifugation over Ficoll-Hypaque and cultured at $10^6$per milliliter with varying amounts of COS-7–BCRF1 or COS-7 (mock) supernatants and PHA (0.1 μg/ml) for 3 days, or with recombinant IL-2 (20 unit/ml) for 5 days in Yssel's medium with 1% human AB$^+$ serum [H. Yssel, J. E. de Vries, M. Koken, W. van Blitterswijk, H. Spits, *J. Immunol. Methods* **72**, 219 (1984)]. Cultures were done in triplicate in 96-well plates, 200 μl per well. IFN-γ in culture supernatants was measured by enzyme-linked immunosorbant assay (ELISA) [C. Favre *et al.*, *Mol. Immunol.* **26**, 17 (1989)]. The limit of sensitivity of the ELISA was 0.3 ng/ml. Because COS-7 supernatant may contain transforming growth factor–β (TGF-β), which can inhibit IFN-γ production (*1*), experiments were done with a neutralizing monoclonal antibody to TGF-β (anti–TGF-β) (10 μg/ml) [J. R. Dasch, D. R. Pace, W. Waegell, D. Inenaga, L. Ellingsworth, *J. Immunol.* **142**, 1536 (1989)] included in the cultures. The profile of inhibition was unchanged in the presence of anti–TGF-β, showing that inhibition of IFN-γ synthesis in the cultures was primarily the effect of BCRF1.
25. PBMC (*24*) were cultured in the presence of the anti-CD3 monoclonal antibody SPV-T3b [H. Spits *et al.*, *Hybridoma* **2**, 423 (1983)] at a 1:3000 dilution of ascites fluid. Where indicated, COS-7–BCRF1 and COS-7 (mock) supernatants were included at 10% of culture volume. Cells were harvested after 24 hours and total RNA was prepared as described [H. Yssel, J. P. Aubrey, R. de Waal Malefyt, J. E. de Vries, H. Spits, *J. Immunol.* **139**, 2850 (1987)]. RNA blot analysis was performed with 10 μg per lane. The 1.1-kb Pst I–Hinc II fragment of pcD-H-IFN-γ [T. Yokota *et al.*, *Lymphokines* **13**, 1 (1987)] and a mouse β-actin cDNA were used as probes. Hybridization conditions were substantially as described (*5, 19*).
26. The authors thank D. Hayward, W. Sugden, and E. Kieff for the Bam HI C fragment subclone used in this study, H Yssel for EBV-transformed cell lines and EBV-containing supernatant from the B95-8 cell line, F. Vega and D. Robison for synthetic oligonucleotides, and E. Mocarski, E. Kieff, J. Sixbey, J. Banchereau, and J. Luka for helpful discussions.

4 May 1990; accepted 7 September 1990

## Technical Comments

# Does Diphtheria Toxin Have Nuclease Activity?

The report by M. P. Chang *et al.* (*1*) proposing a "second pathway" of cell killing by diphtheria toxin (DT) by means of a putative toxin nuclease activity contradicts genetic evidence concerning the toxin killing mechanism. Biochemical studies (*2*) demonstrate that DT inhibits cellular protein synthesis by adenosine diphosphate (ADP)–ribosylating and thus inactivating protein synthesis elongation factor 2 (EF-2). Mutations in the EF-2 gene producing amino acid substitutions near the site of ADP-ribosylation of the factor render the cell resistant to the biologic effects of the toxin (*3*). Cellular resistance to DT is also caused by mutations in the pathway of enzymes that synthesize diphthamide, the unique posttranslational histidine derivative in EF-2 that is ADP-ribosylated (*4*). Cells bearing these mutations are unaffected by the intracellular production of otherwise lethal levels of the catalytic DT fragment A (*5*). In these mutant cells toxin resistance results from alterations in EF-2 that prevent its ADP-ribosylation by toxin. Conversely, a mutation producing a single amino acid substitution in DT abolishes its ability to ADP-ribosylate EF-2 and yields a nontoxic molecule (*6*). Thus, cell killing by DT requires ADP-ribosylation of EF-2, and failure to ADP-ribosylate EF-2 prevents killing. The genetic evidence therefore demonstrates that there is only one biologically relevant pathway of cell killing by DT.

JAMES W. BODLEY
*Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455*

REFERENCES

1. M. P. Chang, R. L. Baldwin, C. Bruce, B. J. Wisnieski, *Science* **246**, 1165 (1989).
2. A. M. Pappenheimer, Jr., *Ann. Rev. Biochem.* **46**, 69 (1977).
3. J. M. Moehring and T. J. Moehring, *Somatic Cell Genet.* **5**, 453 (1979); R. S. Gupta and L. Siminovich, *ibid.* **6**, 361 (1980); K. Kohno, T. Uchida, M. Mekada, Y. Okada, *Somatic Cell Mol. Genet.* **11**, 421 (1985); K. Kohno and T. Uchida, *J. Biol. Chem.* **262**, 12298 (1987).
4. B. G. Van Ness, J. B. Howard, J. W. Bodley, *J. Biol. Chem.* **255**, 10710 (1980); J. M. Moehring, T. J. Moehring, D. E. Danley, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1010 (1980); J.-Y. Chen, J. W. Bodley, D. M. Livingston, *Mol. Cell. Biol.* **5**, 3357 (1985); J. M. Moehring and T. J. Moehring, *J. Biol. Chem.* **263**, 3840 (1988); J.-C. Chen and J. W. Bodley, *ibid.*, p. 11692.
5. J. P. Perentesis *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8386 (1988).
6. T. Uchida, A. M. Pappenheimer, Jr., A. A. Harper, *Science* **175**, 901 (1972); T. Uchida, A. M. Pappenheimer, Jr., R. Greany, *J. Biol. Chem.* **248**, 3838 (1973).

29 January 1990; accepted 6 July 1990

The potent toxicity of diphtheria toxin (DT) is widely attributed to its ability to catalyze the adenosine diphosphate (ADP)–ribosylation of elongation factor 2 (EF-2) resulting in the inhibition of protein synthesis (*1*). Recently, M. P. Chang *et al.* (*2*) proposed a second cytotoxic pathway for DT. They reported that DT exhibits a nuclease activity that is stimulated by $Ca^{2+}$ and $Mg^{2+}$, is susceptible to inhibition by antitoxin, and migrates with the A subunit of the toxin. They further suggest that DT-induced cell lysis is not simply a consequence of protein synthesis inhibition, but may instead involve direct chromosomal attack by intracellular toxin molecules. While this is an intriguing proposal, it does not explain why cells that cannot be ADP-ribosylated because of mutations in EF-2 are resistant to DT (*3*).

It was observed that the DT used to make DT-based immunotoxins contained nuclease activity, whereas the purified immunotoxins had no detectable nuclease activity. Since DT-based immunotoxins are subjected to purification by high-performance liquid chromatography (HPLC) gel filtration, the loss of nuclease activity could be the result of either the chemical linkage with an antibody or the HPLC purification step. To test this hypothesis, we obtained DT from one of the same sources used by Chang *et al.* (*2*) and chromatographed it on a TSK-3000 gel filtration HPLC column, collected the DT peak, and evaluated the nuclease activi-