NOTES

Effects of Pure Interferons on Epstein-Barr Virus Infection In Vitro

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Received 12 September 1984/Accepted 14 January 1985

Pure human gamma-interferon as well as alpha-interferon inhibited induction of immunoglobulin synthesis by Epstein-Barr virus but not by pokeweed mitogen in B lymphocytes from adult but not from newborn humans. The interferons inhibited the infected B lymphocytes directly, irrespective of the Epstein-Barr virus immune status of the donor, and their inhibitory effect was synergistic.

T lymphocytes that suppress the outgrowth of autologous Epstein-Barr virus (EBV)-infected B lymphocytes produce both alpha-interferon (IFN- α) (1, 25), and gamma-interferon (IFN- γ) (3), suggesting a possible role of IFNs in the regulation of herpesvirus infections. EBV transformation of lymphocytes from adults can be inhibited by exogenous human IFN- α (HuIFN- α) (16, 18, 28), but not that of umbilical cord blood lymphocytes (20, 28). Furthermore, passive addition of HuIFN- γ suppresses EBV-induced effects in lymphocytes from adults (2, 15).

Previous investigators have used impure IFN preparations. In this report, we have used highly purified natural HuIFN- γ , *Escherichia coli*-derived recombinant DNA HuIFN- γ (rec. HuIFN- γ), and highly purified natural HuIFN- α and studied their influence on EBV infection of human mononuclear cells (MNC), making use of the fact that EBV is a direct B-cell activator (5). As a control, we determined the effect of IFNs on a nonviral activator of human T and B cells (17), pokeweed mitogen (PWM).

Mononuclear cells (MNC) were separated from the blood of EBV-seropositive and EBV-seronegative healthy adults and from umbilical cord blood samples on a Ficoll-Isopaque gradient (6). The cells were cultured at 10^6 MNC per ml of medium in polystyrene test tubes (12 by 75 mm; no. 2058, Falcon Plastics, Oxnard, Calif.). Purified natural HuIFN- α (24), purified natural HuIFN- γ (8), or rec. HuIFN- γ (11) was added 1 h before the cells were exposed to EBV B95-8 (21) or PWM (17), and the cells were incubated at 37°C in serum-free RPMI 1640 medium supplemented with antibiotics (100 IU of penicillin and 100 μ g of streptomycin per ml). The cells were then exposed to EBV or PWM for 1 h, followed by addition of 10% heat-inactivated human AB serum to the cultures. All cultures were incubated at 37°C for 7 days in a humidified atmosphere containing 5% CO₂. B lymphocytes were separated by a previously described procedure (29) in which macrophages are removed by carboxyl-iron treatment and T cells are removed by passing the cells through a nylon-wool column followed by two cycles of E rosetting. This yields a B-cell fraction of 99% purity, as judged by immunoglobulin surface staining. HuIFN- α was prepared from human buffy-coat leukocytes and purified on

an NK-2 monoclonal antibody column (24) to a final specific activity of 10^8 IU/mg of protein.

Natural HuIFN- γ was produced by stimulating fresh buffy-coat leukocytes with the streptococcal preparation OK-432 (8). After various purification steps (8), the preparation had a specific activity of $10^{7.5}$ U/mg of protein and was found to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions.

Rec. HuIFN- γ was produced by Genentech, Inc. (11), and had a specific activity of 1.2×10^7 U/mg of protein.

The mean number of immunoglobulin M (IgM)-secreting cells (plaque-forming cells; PFC) and the percentage of EBV nuclear antigen (EBNA)-positive cells (23) after EBV and PWM exposure, respectively, are shown in Table 1. These data were the basis for the subsequent calculations of the inhibitory effects of HuIFNs. Pretreating MNC from adults and newborns with HuIFN- α (Fig. 1), HuIFN- γ (Fig. 1), or *E. coli*-produced rec. HuIFN- γ (Fig. 2) inhibited EBNA expression and polyclonal IgM secretion, whereas no inhibition of IgM secretion was noted in PWM-stimulated MNC except with very high HuIFN- α doses (exceeding 1,000 U/ml). On the contrary, both HuIFN- γ and rec. HuIFN- γ augmented the PWM-induced IgM response in MNC from adults and also in the two of eight cord blood cultures which responded to PWM by immunoglobulin secretion (data not shown). The inhibitory effect of IFN on EBV-stimulated MNC was dose dependent for all three IFN preparations but could not be demonstrated over a broad dose range in cord blood lymphocytes.

MNC from EBV-seronegative and EBV-seropositive donors were equally sensitive to the effects of exogenous HuIFN- γ and HuIFN- α (data not shown). However, it was noted regularly that the number of EBNA-positive cells after 7 days of exposing non-IFN-treated cells to EBV was higher in the MNC from EBV-nonimmune individuals (Table 1).

Both HuIFN- α and HuIFN- γ had a direct effect on the infected B cells, since enriched B-cell cultures (>99% immunoglobulin surface-positive cells) from adult donors were inhibited in induction of IgM synthesis to the same extent as were unseparated MNC from the same donors when pretreated with IFN before EBV exposure (Fig. 3). This finding was true also for inhibition of EBNA expression and could

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TABLE 1. Effects of EBV and PWM on PFC and EBNA in human blood cells^a

Cells (no. of expts)	Material added	No. (mean $\pm$ SEM) of PFC/ 10^6 cells	% (mean $\pm$ SEM) EBNA-positive cells/ 10^3 cells counted
Adult MNC (14)	EBV	5,600 $\pm$ 800	18 $\pm$ 3.2 ^b
	PWM	7,200 $\pm$ 1,050	0
	None	0	0
Adult B lymphocytes (4)	EBV	12,300 $\pm$ 3,500	61 $\pm$ 6
	PWM	ND ^c	ND
	None	0	0
Newborn MNC (8)	EBV	4,300 $\pm$ 1,100	49 $\pm$ 4
	PWM	900 $\pm$ 500 ^d	0
	None	0	0

^a MNC or purified B lymphocytes were exposed to EBV or PWM for 7 days.

^b Two seropositive and two seronegative donors tested on the same occasion yielded 8.5 $\pm$ 0.1 and 27.2 $\pm$ 1.2% EBNA-positive cells, respectively.

^c ND, Not determined.

^d Only two of eight responded.

be demonstrated for both HuIFN- γ and HuIFN- α (data not shown).

The effects of adding HuIFN- α and HuIFN- γ simultaneously to MNC from adults before EBV exposure were greater than their individual inhibitory effects. These synergistic effects were most pronounced at doses between 1 and 100 U/ml (Fig. 4).

In principle, the observed effects of IFNs on in vitro EBV infection as well as on immune regulation as a whole have been shown earlier for both HuIFN- α and HuIFN- γ (2, 15,

FIG. 1. Effect of IFNs on EBV-induced IgM production. Ficoll-separated lymphocytes (10^6 /ml of serum-free medium) from adult or umbilical cord blood were exposed to various doses of highly purified HuIFN- γ (—) or HuIFN- α (....) for 1 h at 37°C. They were then exposed to 0.1 ml of EBV B95-8 supernatant (21) containing 10^6 infectious units per ml for 1 h. The cultures were then supplemented with heat-inactivated human AB serum (10%) and incubated in 5% CO₂ at 37°C. After 1 week the number of PFC was determined by the protein A plaque assay (13). The percent inhibition was calculated from the number of PFC per 10^6 cells in cultures of the same donor cells not preexposed to IFN. Data are means $\pm$ standard errors of the mean for 14 adult and 8 cord experiments.

FIG. 2. Lymphocytes from adults were exposed to rec. HuIFN- γ for 1 h and then to EBV. In addition, after rec. HuIFN- γ exposure, one part of the cells was mixed with PWM (20 μ g/ 10^6 cells per ml), which is a nonviral T cell-dependent activator of immunoglobulin synthesis (17). After 1 week of culture, the number of PFC (----) and of EBNA-positive cells per 10^3 acetone-fixed cells counted (—) were determined. Data are means $\pm$ standard errors of the mean for eight experiments.

18, 28). However, previous data were generated with impure HuIFN preparations. We therefore undertook a study of these parameters with pure IFNs. We used HuIFN- α that was 100- to 1,000-fold purer than that used in the earlier studies and HuIFN- γ that had been purified to homogeneity or prepared by recombinant DNA technology. These purified, or pure, IFNs all affect EBV infection in vitro, whether expressed as development of EBNA or as induction of IgM synthesis, two strictly virus-dependent phenomena. EBNA appears within 24 h of in vitro EBV exposure (9), and secretion of IgM starts within 5 days post-virus exposure (4).

FIG. 3. HuIFN- γ or HuIFN- α was added to highly purified human B lymphocytes and MNC from the same donors for 1 h before exposure of the cells to EBV. The number of PFC was assayed on day 7. Data represent means $\pm$ standard errors of the mean for four different experiments. HuIFN- α produced the same inhibition, which was equally demonstrable when the number of EBNA-positive cells was calculated.

FIG. 4. Exogenous HuIFN- γ and HuIFN- α , separately and in combinations, inhibited EBNA expression in lymphocytes from adult donors exposed for 1 week to EBV. At both 10 and 100 U of IFN, the combination of HuIFN- γ and HuIFN- α was significantly more inhibitory ($P < 0.05$, Student's t test) than either IFN alone. Data represent means $\pm$ standard errors of the mean for nine separate experiments. IgM secretion was inhibited similarly.

Exogenous HuIFNs inhibited both of these effects, and there was a close correlation between the inhibitory effect, measured as EBNA expression or IgM secretion (Fig. 2), for all HuIFNs tested.

These inhibitory effects could not be explained merely by the well-documented nonspecific, antiproliferative influence of IFNs (27), since PWM-activated MNC from the same blood donors were not at all inhibited (Fig. 2), nor were EBV-infected lymphocytes from umbilical cord blood (Fig. 1).

EBNA expression and IgM secretion on day 7 of culture probably result from the additive effect of primary infection and proliferation of infected cells (5). However, no IgM synthesis could be seen in mitomycin-treated EBV infected cultures (data not shown), whereas EBNA appears only at a low frequency, which is evident as early as day 1 or 2 of culture (14). Since both EBNA expression and IgM PFC were inhibited by HuIFN- α and HuIFN- γ in all experiments, we postulate that the antiviral effects of both of these preparations are due to their effect on primary EBV infection of susceptible B cells, as well as on subsequent virus-induced proliferation, irrespective of the EBV immune status of the donor.

The initial total number of EBNA-positive cells and IgM PFC after 7 days of culture was three times higher in MNC from EBV-seronegative donors than in those from EBV-seropositive donors (Table 1). This observation indicated to us that under these in vitro conditions, EBNA expression and IgM formation were under some control in the immune individual, perhaps through rapid specific cellular production of HuIFN- γ . Indeed, several reports indicate that both HuIFN- γ and HuIFN- α can be produced in lymphocytes from immune individuals, whereas only HuIFN- α is synthesized in lymphocytes from nonimmune donors (3, 12, 23, 27) as a result of virus exposure.

Potential of the antiviral activities of IFN- β with IFN- γ has been described previously (10). An indication of such dual importance of HuIFN- α and HuIFN- γ was the finding that combinations of the two preparations synergistically inhibited EBV-induced activation of MNC from adults (Fig.

4). One explanation for the synergistic effects could be that B cells carry separate receptors for HuIFN- α and HuIFN- γ (7).

We believe that the IFN-mediated in vitro effects are brought about by a direct effect on the B lymphocytes, since highly enriched B lymphocyte preparations (>99% immunoglobulin surface-positive cells) were inhibited as effectively as unseparated MNC from the same donors (Fig. 3).

We do not know whether the unresponsiveness of B lymphocytes from cord blood to IFNs is caused by a lack of receptors for IFNs or because cord blood B lymphocytes show a different intracellular response to IFN. However, we have previously shown that umbilical cord lymphocytes synthesize and excrete IFN- γ spontaneously at a high rate (19).

This work was supported by grants from the Swedish Medical Research Council, Swedish Medical Association, the Swedish Association against Rheumatism, and the Swedish Cancer Society. M.D.L. is Bevoegdverklaard Navorsers of the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek.

We thank Erik Lundgren, University of Umeå, for providing highly purified HuIFN- α , Boehringer-Ingelheim for supplying recombinant IFN- γ , and Kerstin Falk and Lena Svensson for excellent technical assistance.

LITERATURE CITED

1. Adams, A., B. Lidin, H. Strander, and K. Cantell. 1975. Spontaneous interferon production and Epstein-Barr virus antigen expression in human lymphoid cell lines. *J. Gen. Virol.* **28**: 219-223.
2. Andersson, U., S. Britton, M. DeLey, and G. Bird. 1983. Evidence for the ontogenetic precedence of suppressor T cell functions in the human neonate. *Eur. J. Immunol.* **13**:6-13.
3. Andersson, U., O. Martinez-Maza, J. Andersson, S. Britton, H. Gadler, and S. Modrow. 1984. Secretion of gamma-interferon at the cellular level. Induction by Epstein-Barr virus. *Scand. J. Immunol.* **20**:425-432.
4. Bird, G., and S. Britton. 1979. A new approach to the study of human B lymphocyte function using an indirect plaque assay and a direct B cell activator. *Immunol. Rev.* **45**:41-45.
5. Bird, G., S. Britton, I. Ernberg, and K. Nilsson. 1981. Characteristics of Epstein-Barr virus activation of human B lymphocytes. *J. Exp. Med.* **154**:832-839.
6. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Lab. Invest. Suppl.* **97**:31-33.
7. Branca, A. A., and C. Bagolini. 1981. Evidence that types I and II interferons have different receptors. *Nature (London)* **294**: 768-770.
8. DeLey, M., and H. Claeys. 1984. Streptococcal preparation OK-432-induced interferon in human leukocytes: purification and characterization. *Int. Arch. Allergy Appl. Immunol.* **74**: 21-28.
9. Einhorn, L., and I. Ernberg. 1978. Induction of EBNA precedes the first cellular s-phase after EBV-infection of human lymphocytes. *Int. J. Cancer* **21**:157-160.
10. Fleishman, W. R., J. A. Georgiades, L. C. Osborne, and H. M. Johnson. 1979. Potentiation of interferon activity by mixed preparations of fibroblast and immune interferon. *Infect. Immun.* **26**:248-253.
11. Gray, P. W., D. W. Leung, D. Pennica, E. Yelverton, R. Najarian, and D. Goeddel. 1982. Expression of human immune interferon cDNA in *E. coli* and monkey cells. *Nature (London)* **295**:503-508.
12. Green, J. A., T. J. Yeh, and J. C. Overall. 1981. Sequential production of IFN- α and immune-specific IFN- γ by human mononuclear leukocytes exposed to herpes simplex virus. *J. Immunol.* **127**:1192-1196.
13. Gronowicz, E., A. Coutinho, and F. Melchers. 1976. A plaque assay for cells secreting Ig of a given type or class. *Eur. J.*

- Immunol. 6:588-590.
14. **Hansson, M., K. Falk, and I. Ernberg.** 1983. Epstein-Barr virus transformation of human pre-B cells. *J. Exp. Med.* **158**:616-622.
 15. **Hasler, F., H. G. Bluestein, N. J. Zvaifler, and L. B. Epstein.** 1983. Analysis of the defects responsible for the impaired regulation of Epstein-Barr virus-induced B cell proliferation by rheumatoid arthritis lymphocytes. *J. Exp. Med.* **157**:173-188.
 16. **Hendersson, E. E., P. W. Doetsch, R. Charubala, W. Pleiderer, and R. J. Suhadolnik.** 1982. Inhibition of Epstein-Barr virus-associated nuclear antigen (EBNA). Induction by (2',5') oligo-adenylate and the cordycepin analog: mechanism of action for inhibition of EBV-induced transformation. *Virology* **122**:198-201.
 17. **Keightley, R. G., M. D. Cooper, and A. R. Lawton.** 1976. The T cell dependence of B cell differentiation induced by pokeweed mitogen. *J. Immunol.* **117**:1538-1544.
 18. **Lvovsky, E., P. H. Levine, D. Fucillo, D. V. Ablashi, Z. H. Bengali, G. R. Armstrong, and H. B. Levy.** 1981. Epstein-Barr virus and herpesvirus saimiri: sensitivity to interferons and interferon inducers. *J. Natl. Cancer Inst.* **66**:1013-1019.
 19. **Martinez-Maza, O., U. Andersson, J. Andersson, S. Britton, and M. DeLey.** 1984. Spontaneous production of interferon-gamma in adult and newborn humans. *J. Immunol.* **132**:251-255.
 20. **Menezes, J., P. Patel, H. Dussault, J. Joncas, and W. Leibold.** 1976. Effect of interferon on lymphocyte transformation and nuclear antigen production by Epstein-Barr virus. *Nature (London)* **260**:430-432.
 21. **Miller, G., and M. Lipman.** 1973. Comparison of the yield of infectious virus from clones of human and simian lymphoblastoid lines transformed by Epstein-Barr virus. *J. Exp. Med.* **138**:1398-1402.
 22. **Nakayama, T.** 1983. Immune-specific production of gamma interferon in human lymphocyte cultures in response to mumps virus. *Infect. Immun.* **40**:486-492.
 23. **Reedman, B., and G. Klein.** 1973. Cellular localization of an Epstein-Barr virus (EBV)-associated complement fixing antigen in producer lymphoblastoid cellines. *Int. J. Cancer* **11**:499-520.
 24. **Secher, D. S., and D. C. Burke.** 1980. A monoclonal antibody for large-scale purification of human leukocyte interferon. *Nature (London)* **285**:446-450.
 25. **Slovin, S. F., R. T. Schooley, and D. A. Thorley-Lawson.** 1983. Analysis of cellular immune response to EBV by using cloned T cell lines. *J. Immunol.* **130**:2127-2132.
 26. **Starr, S. E., B. Dalton, T. Garrabrandt, K. Paucker, and S. Plotkin.** 1980. Lymphocyte blastogenesis and interferon production in adult human leukocyte cultures stimulated with cytomegalovirus antigen. *Infect. Immun.* **30**:17-22.
 27. **Stewart, W. E.** 1981. The interferon system, 2nd ed., p. 238-244. Springer-Verlag, New York.
 28. **Thorley-Lawson, D. A.** 1981. The transformation of adult but not newborn human lymphocytes by Epstein-Barr virus and by phytohemagglutinin is inhibited by interferon: the early suppression by T-cells of Epstein-Barr virus infection is mediated by interferon. *J. Immunol.* **126**:829-833.
 29. **Yefenof, E., T. Bakacs, L. Einhorn, I. Ernberg, and G. Klein.** 1978. Epstein-Barr virus (EBV) receptors, complement receptors, and EBV infectivity of different lymphocyte fractions of human peripheral blood. *Cell. Immunol.* **35**:34-42.