Biochemical Pharmacology, Vol. 33, No. 21, pp. 3439–3443, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

MODULATION BY A HUMAN INTERFERON OF ANTITUMOR EFFECTS OF CYCLOPHOSPHAMIDE AGAINST A LYMPHOSARCOMA IN HAMSTERS

SANG HE LEE,* HENRY CHIU,* KEN W. RENTON† and NOWELL STEBBING*‡
*Genentech, Inc., South San Francisco, CA 94080, U.S.A.; and † Dalhousie University, Dalhousie, Halifax, Nova Scotia, Canada

(*Received* 28 *June* 1983; *accepted* 8 *March* 1984)

Abstract—Administration to hamsters of a highly purified human leukocyte interferon subtype, IFN-αA, obtained by recombinant DNA methods, abolished the efficacy of high doses of cyclophosphamide (2.5 mg/hamster) against the TBD 932 lymphosarcoma. The effect was more pronounced with concomitant than with sequential treatments and did not occur with melphalan. Antagonistic effects occurred at high interferon doses (10^5 I.U./treatment), but an additive or synergistic positive effect occurred at lower interferon doses (10^3 I.U./treatment) and at lower, non-protective, doses of cyclophosphamide. These effects may be due to immunomodulatory responses induced by the drugs involved.

Recombinant DNA-derived interferons have been shown to have immunological and pharmacological effects similar to those of natural interferon preparations [1–3]. Several effects of interferons on immune parameters are antagonistic [4, 5], and the known direct and indirect mechanisms of action of interferons are likely to cause complex drug interactions. Clinical studies with interferons are beginning to show some potential utility [6], but the magnitude of the effects observed to date is not great. Combination therapies involving interferons and existing chemotherapeutic agents might be more beneficial but require some understanding of the mechanisms involved if beneficial effects are to be optimized.

A negative interaction has been observed between cyclophosphamide and a purified recombinant DNA derived hybrid leukocyte interferon when administered to L1210 tumor bearing mice [7]. Cyclophosphamide is converted to the active antiproliferative metabolite, a phosphamide mustard, after metabolism to 4-hydroxycyclophosphamide by the hepatic cytochrome P-450 oxygenase system [8, 9], and interferons have been shown to inhibit P-450 metabolism [10]. Because cyclophosphamide shows greater antitumor activity against the TBD 932 lymphosarcoma of hamsters than against the L1210 lymphoma of mice, we studied interactions between cyclophosphamide and interferon in the hamster system. The present studies were designed to determine the involvement, if any, of P-450 effects and define the nature of the factors causing interactive effects between interferons and cyclophosphamide.

MATERIALS AND METHODS

Female Golden Syrian hamsters were obtained at 6–8 weeks of age from Simonsen, Gilroy, CA, and maintained on an unlimited diet of Purina Chow with constant access to water. The animals used in the present study weighed 100 ± 5 g. The hamster lymphosarcoma cell line, TBD 932, was obtained from EG & G Mason Research Institute, Worcester, MA, and was maintained as an ascitic tumor in hamsters by weekly intraperitoneal inoculation of approximately 5×10^5 cells/hamster. Experimental studies involved intraperitoneal administration of 10^4 or 10^5 cells/hamster, and these tumor doses routinely caused death of at least 90% of animals within 40 days, the mean time of death being about 12 days. Cyclophosphamide and melphalan (Sigma Chemical Co., St. Louis, MO) were administered in 0.5 ml of phosphate-buffered saline (PBS) intraperitoneally for 3 days after tumor inoculation. Dose-dependent protection was observed with cyclophosphamide between 50 μg and 2.5 mg, and with melphalan between 10 and 50 μg. Significance of differences in mean survival time between treatments was assessed by a logrank χ^2 method [11] which takes account of surviving animals.

The human leukocyte interferon subtype, IFN-αA, was purified to homogeneity as assessed by polyacrylamide gel electrophoresis as described elsewhere [12] and titrated on human WISH cells using Vesicular Stomatitis virus challenge with calibration against the NIH leukocyte interferon standard (G-023-901-527). The interferon doses are given in International Units (I.U.). The interferon preparation used in these studies had a specific activity of 2×10^8 I.U./mg protein. Interferon treatments were by the intraperitoneal route, and control animals were treated with PBS in place of interferon or other treatments, as appropriate.

The activity of the hepatic cytochrome P-450 monooxygenase system was assessed as follows. At 1 or 3 days following interferon doses the animals were killed, and hepatic microsomes were prepared as described by El Defrawy El Masry *et al.* [13]. All preparations were used on the day they were prepared. Microsomal protein levels were deter-

‡ Send all correspondence to: Dr. N. Stebbing, Amgen, Inc., 1900 Oak Terrace Lane, Thousand Oaks, CA 91320, U.S.A.

mined by the method of Lowry *et al.* [14] using bovine serum albumin as a standard. Cytochrome P-450 and cytochrome b_5 levels in microsomes were determined by the method of Omura and Sato [15]. Microsomal N-demethylation was determined as described by Sladek and Mannering [16]. Benzo[*a*] pyrene hydroxylase was determined as described by Nebert and Gelboin [17]. The formation of alkylating metabolites from cyclophosphamide was determined as described by Christian *et al.* [18]. Alkylating activity was standardized using nitrogen mustard (HN_2) as a reference alkylating compound and is expressed as HN_2 equivalents.

RESULTS

Cyclophosphamide, at 2.5 mg/hamster daily for 3 days following TBD 932 tumor inoculation, limited tumor development and death of hamsters. All treated animals survived with no signs of tumors up to 60 days post tumor inoculation. Treatments with the human leukocyte interferon subtype, IFN-αA [12], at doses of 10^5 I.U./hamster on days 1–12 or 3–12 post tumor inoculation had no antitumor effects and all treated animals died (see Fig. 1). Combined treatments with cyclophosphamide and IFN-αA at 10^5 I.U./hamster/treatment, caused significantly lower incidence of survival than cyclophosphamide alone, and the effect was more pronounced with the treatment schedule involving concomitant IFN-αA and cyclophosphamide during the first 3 days post tumor inoculation (Fig. 1). Thus, at the doses tested, the interferon alone showed no antitumor effects but dramatically decreased the efficacy of cyclophosphamide. With inoculation of 10^5 cells/hamster, quantitatively similar effects were observed although at this tumor dose cyclophosphamide alone at doses of 2.5 mg/hamster generally caused only about 80% survival. The hamsters with the combined treatments of interferon and cyclophosphamide died of ascitic tumors as rapidly as those in the PBS-treated control group. Therefore, the effect of these high doses of interferon seems to be abrogation of the efficacy of cyclophosphamide. However, it remained possible that the combination of cyclophosphamide and IFN-αA was highly toxic and was the cause of death.

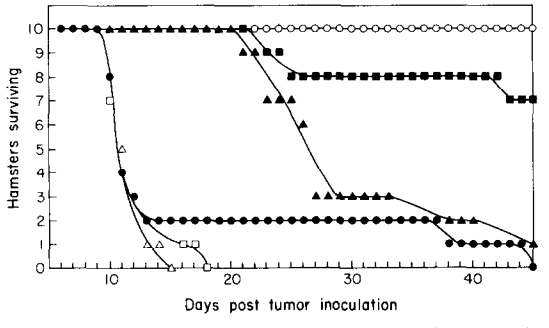

Fig. 1. Effects of cyclophosphamide and interferon on the TBD 932 lymphosarcoma in hamsters. Interferon treatments: IFN-αA 10^5 I.U./hamster daily on days 1–12 (△), or days 3–12 (□). Cyclophosphamide treatments: 2.5 mg/hamster daily on days 1–3 (○). Both cyclophosphamide (days 1–3) and interferon, days 1–12 (▲) and days 3–12 (■). Untreated (PBS) controls, 10^4 TBD 932 cells, i.p. (●).

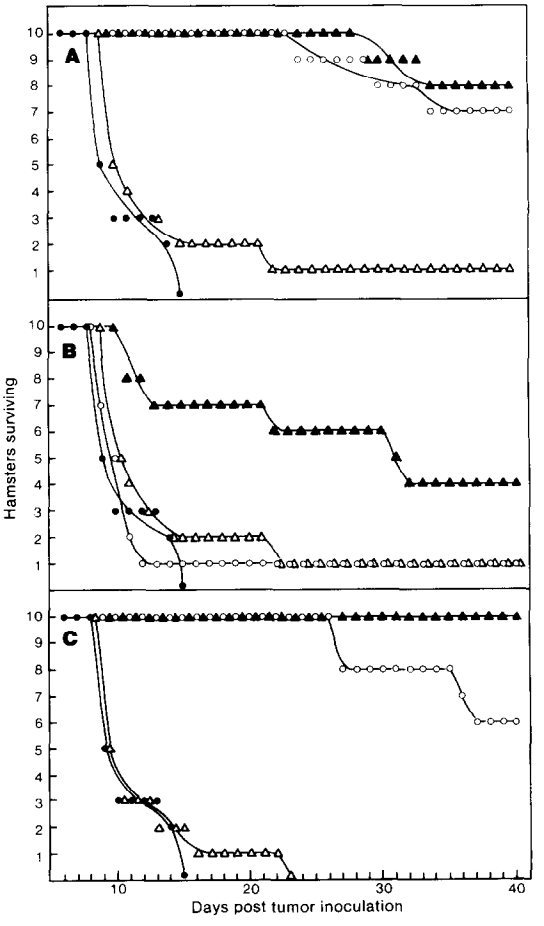

Fig. 2. Effect on survival of hamsters inoculated with the TBD 932 lymphosarcoma after treatments with: (A) melphalan, 50 μg/hamster, and IFN-αA, 10^5 I.U./hamster; (B) cyclophosphamide, 50 μg/hamster, and IFN-αA, 10^5 I.U./hamster; and (C) cyclophosphamide, 2.5 mg/hamster, and IFN-αA, 10^3 I.U./hamster. Key: (○) all drug treatments given on days 1–3 post tumor inoculation; (△) interferon (IFN-αA) treatments given daily on days 1–12 post tumor inoculation; (▲) combination of drug and interferon treatments; and (●) untreated (saline) controls, 10^5 TBD 932 cells.

This possibility was eliminated by examining the combined effect of cyclophosphamide and interferon treatments in hamsters which were not inoculated with tumor cells. No such treated animals died or showed any obvious adverse effects (data not shown).

The possible abrogation of efficacy of another chemotherapeutic agent by interferon was investigated using doses of 50 μg/hamster of melphalan. An identical protocol was used for melphalan as for the cyclophosphamide studies. The results, in Fig. 2A, showed no obvious effect on the efficacy of melphalan in the combined regimen with interferon.

Because melphalan is not metabolized by the hepatic P-450 oxygenase system, it seemed possible that abrogation of the efficacy of cyclophosphamide by interferon treatment could be due to a decrease in activity of P-450 metabolism. We therefore measured directly the effect of IFN-αA on hepatic P-450 in

Table 1. Effect of interferon on cytochrome P-450 levels and cyclophosphamide metabolism in the hamster liver*

Treatment	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine *N*-demethylase (nmoles HCHO/mg protein/hr)	Benzo[*a*]pyrene hydroxylase (nmoles 3-OHBP/ mg protein/hr)	Cyclophosphamide alkylating species (μg HN_2/mg protein/hr)
24 hr					
Control	0.66 ± 0.06	0.34 ± 0.04	577 ± 43	9.2 ± 0.8	4.65 ± 0.08
IFN-αA (10^4 units)	0.65 ± 0.03	0.35 ± 0.01	501 ± 10	7.5 ± 0.2	4.48 ± 0.16
IFN-αA (10^5 units)	0.55 ± 0.04	0.33 ± 0.03	475 ± 26	6.1 ± 0.3†	3.88 ± 0.09†
5 days					
Control	0.63 ± 0.07	0.30 ± 0.05	393 ± 9	6.3 ± 0.1	5.20 ± 0.24
IFN-αA (10^5 units)	0.72 ± 0.03	0.35 ± 0.02	408 ± 14	6.0 ± 0.2	4.44 ± 0.16†

* Animals were treated with one or five daily doses of interferon and killed 24 hr after the final dose. Control animals received a corresponding volume of sterile saline at the same time.

† Significantly different from corresponding control, $P < 0.05$, $N = 4$.

hamsters. Following treatment of hamsters with single doses of IFN-αA (10^4 or 10^5 I.U./treatment) or with five daily doses (10^5 I.U./treatment), the levels of cytochrome P-450, cytochrome b_5, and aminopyrine *N*-demethylase were unchanged compared to control animals treated with saline (Table 1). Benzo[*a*]pyrene hydroxylase and the formation of alkylating species from cyclophosphamide were only slightly depressed in microsomes prepared from animals treated with 10^5 I.U. of IFN-αA for 1 or 5 days. In the case of the alkylating species, the reduction was only 15–16%.

Although cyclophosphamide is well tolerated in hamsters, 2.5 mg/treatment is a high dose. We therefore investigated the effects of lower doses in combination with interferon and also examined the effect of the high cyclophosphamide doses with lower interferon doses. The results, in Fig. 2B, show that cyclophosphamide doses of 50 μg/hamster on days 1–3 post tumor inoculation did not affect survival of lymphosarcoma bearing hamsters, but in combination with IFN-αA at 10^5 I.U./hamster on days 1–12 post tumor inoculation there was enhanced survival, which was significant. Moreover, with the higher dose of cyclophosphamide there was no abrogation of efficacy when the interferon dose was reduced to 10^3 I.U./hamster on days 1–12 post tumor inoculation (see Fig. 2C).

Because cyclophosphamide itself is known to suppress cytochrome P-450 metabolism as well as being metabolized by this system [8], it seemed possible that IFN-αA could simply accentuate this effect and thereby abrogate the antitumor activity of cyclophosphamide, which is dependent upon metabolism by the hepatic mixed function oxidase system. We therefore compared the effects of IFN-αA and cyclophosphamide, each alone and together, on various substrate pathways of the cytochrome P-450 system. The results, shown in Table 2, indicate that the combination treatment did not cause a greater suppression of P-450 parameters, including the formation of alkylating species from cyclophosphamide, than either treatment alone. Pharmacological effects of an interferon, IFN-αA, without detectable antitumor activity, indicates that the various known effects of interferons [4] may arise from distinct molecular domains within the interferon. IFN-αA is known to have only trivial antiviral effects in hamster cell cultures [19], and other studies have shown that this interferon does not have significant antiviral effects against encephalomyocarditis or herpes simplex type 2 virus infections of hamsters (P. K. Weck, E. N. Fish and N. Stebbing, unpublished results). Moreover, IFN-αA does not affect NK cell activity of hamsters or lymphocyte oligo 2′5′A synthetase activity in this species (S. H. Lee and E. N. Fish, unpublished results). It remains possible that other cellular immune systems are involved in abrogation of the antitumor activity of cyclophosphamide described here.

Table 2. Effects of interferon cyclophosphamide on cytochrome P-450 levels in the hamster liver*

	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine *N*-demethylase (nmoles HCHO/mg protein/hr)	Benzo[*a*]pyrene hydroxylase (nmoles 3-OHBP/ mg protein/hr)	Cyclophosphamide alkylating species (μg HN_2/mg protein/hr)
Control	0.48 ± 0.02	0.20 ± 0.03	722 ± 47	8.3 ± 0.5	3.92 ± 0.32
IFN-αA (10^5 units)	0.44 ± 0.02	0.26 ± 0.02	716 ± 30	9.1 ± 0.5	4.50 ± 0.44
Cyclophosphamide	0.46 ± 0.02	0.27 ± 0.03	807 ± 46	9.1 ± 0.4	4.76 ± 0.40
IFN-αA plus cyclophosphamide	0.45 ± 0.01	0.28 ± 0.03	641 ± 27	9.9 ± 0.7	3.66 ± 0.20

* Animals were treated with four daily doses of interferon (IFN-αA, 10^5 units/hamster) and three daily doses of cyclophosphamide (2.5 mg/hamster) and killed 24 hr after the final treatment. Control animals received a corresponding volume of sterile saline at the same times.

DISCUSSION

Abrogation of the efficacy of high dose cyclophosphamide was particularly apparent in hamsters bearing the TBD 932 lymphosarcoma because cyclophosphamide was otherwise well tolerated and very effective in preventing tumor development and death. The effect in hamsters was more pronounced with concomitant treatments than with sequential treatments and did not occur with melphalan which was unaffected by P-450. Although interferons suppress hepatic P-450 metabolism [10], abrogation of the efficacy of cyclophosphamide by interferon would not seem to be due to suppression of P-450, for several reasons. Direct determinations showed no effect on cytochrome P-450 or representative drug biotransformation pathways. Moreover, at low doses of cyclophosphamide, the combination with interferon caused increased antitumor activity. In addition, in rats, variation in the rate of P-450 metabolism has been shown to have no effect on antitumor activity of cyclophosphamide, apparently because the total dose × time (area under the clearance curve) of the drug is unaffected [8]. Although the formation of alkylating metabolites from cyclophosphamide was depressed by about 15% in interferon-treated animals, it is highly unlikely that a reduction of this small magnitude in the formation of these active metabolites could account for the large decrease in cyclophosphamide efficacy.

The mechanism whereby IFN-αA suppresses the efficacy of cyclophosphamide would not seem to be accentuation of the effects of cyclophosphamide itself on cytochrome P-450 metabolism or on the formation of alkylating species from cyclophosphamide itself (Table 2). Moreover, mechanisms essential for the antitumor or antiviral activity of the interferon would not seem to be involved because antitumor and antiviral effects have not been observed in hamsters. Also, no effects have been observed on NK cell activation or oligo 2′5′A synthetase in hamsters. However, it remains possible that the efficacy of cyclophosphamide is due to effects on immune parameters. Cyclophosphamide is known to stimulate some immune responses by inhibiting suppressor cell activity [20] so that direct cytotoxic effects on tumor cells and indirect effects on host antitumor immunity may cooperate in eradication of tumors [21]. Interferons, in mice at least, can cause suppression of antibody synthesis [22] so that abrogation of the efficacy of cyclophosphamide could result simply from reduction in antitumor immunity. Because of various immunomodulatory effects arising from interferon treatments, there is a possibility that clinical efficacy of cyclophosphamide may be modulated by certain interferon concentrations in plasma produced by interferon therapy or even adventitious viral infections causing induction of interferon.

Other studies, in mice, involving combinations of natural IFN-α preparations and cyclophosphamide, have indicated either no interactive effects or positive effects [23–27], such as those reported here for low doses of IFN-αA in hamsters. It is noteworthy that the one mouse study showing a negative interactive effect with cyclophosphamide involved a single molecular species of interferon [7] as do the current hamster studies. It is known that the various molecular subtypes of IFN-α that are present in natural preparations have distinct properties which are dose dependent [1–3, 19]. Thus, it is possible that the different molecular subtypes of IFN-α have opposing effects so that a marked effect, such as that observed in our studies, is only manifest when using a single molecular species of IFN-α. These factors are currently under investigation.

Although suppression of cytochrome P-450 metabolism does not seem to be involved in the drug interaction effects described here, with other drugs predictable effects have been observed [3, 28], and these effects would seem to warrant attention in clinical situations. Studies in mice have shown a correlation between antiviral activity of interferons and their ability to suppress cytochrome P-450 metabolism [10]. In the case of IFN-αA in hamsters, there is little suppression of cytochrome P-450 metabolism and no antitumor activity, but significant pharmacological activity of the interferon is apparent from the modulatory effect exerted on efficacy of cyclophosphamide against the TBD 932 lymphosarcoma.

REFERENCES

1. M. G. Masucci, R. Szigeti, E. Klein, G. Klein, J. Gruest, L. Montagnier, H. Tair, A. Hall, S. Nagata and C. Weissmann, *Science* **209**, 1413 (1980).
2. S. H. Lee, S. Kelly, H. Chiu and N. Stebbing, *Cancer Res.* **42**, 1312 (1982).
3. S. H. Lee, P. K. Weck, J. Moore, S. Chen and N. Stebbing, in *Chemistry and Biology of Interferons: Relationship to Therapeutics* (Eds. T. Merigan and R. Friedman), pp. 341–51. Academic Press, New York (1982).
4. I. Gresser, *Cell Immun.* **34**, 406 (1977).
5. W. H. Fridman, I. Gresser, M. T. Bandu, M. Aguet and C. Neauport-Sautes, *J. Immun.* **124**, 2436 (1980).
6. E. C. Borden and L. A. Ball, *Prog. Hemat.* **12**, 299 (1982).
7. N. Stebbing, P. K. Weck, J. T. Fenno, S. Apperson and S. H. Lee, in *The Biology of the Interferon System* (Eds. E. De Maeyer, G. Talosso and H. Schellekens), pp. 25–33. Elsevier/North-Holland Biomedical Press, Amsterdam (1981).
8. N. E. Sladek, *Cancer Res.* **32**, 535 (1972).
9. J. H. Hipkens, R. F. Struck and H. L. Gurtoo, *Cancer Res.* **41**, 3571 (1981).
10. G. Singh, K. W. Renton and N. Stebbing, *Biochem. biophys. Res. Commun.* **106**, 1256 (1982).
11. R. Peto and M. C. Pike, *Biometrics* **29**, 579 (1973).
12. R. Wetzel, J. Perry, D. A. Estell, N. Lin, H. L. Levine, B. Slinker, F. Fields, M. J. Ross and J. J. Shively, *Interferon Res.* **1**, 381 (1981).
13. S. El Dawfry El Masry, G. M. Cohen and G. J. Mannering, in *Drug. Metab. Dispos.* **2**, 267 (1974).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
16. N. E. Sladek and G. Mannering, *Molec. Pharmac.* **5**, 174 (1969).
17. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243**, 6242 (1968).
18. R. A. Christian, S. K. Chaffee, C. J. Hovick and W. J. Steele, *Life Sci.* **27**, 2595 (1980).
19. P. K. Weck, S. Aperson, L. May and N. Stebbing, *J. gen. Virol.* **57**, 233 (1981).

20. L. Polak and J. L. Turk, *Nature, Lond.* **249**, 654 (1974).
21. J. C. D. Hengst, M. B. Mokyr and S. Dray, *Cancer Res.* **41**, 2163 (1981).
22. B. R. Brodeur and T. C. Merigan, *J. Immun.* **114**, 1323 (1975).
23. I. Gresser, C. Maury and M. Tovey, *Eur. J. Cancer* **14**, 97 (1978).
24. L. M. Slater, M. W. Wetzel and J. Cesano, *Cancer, N.Y.* **48**, 5 (1981).
25. F. R. Balkwill, E. M. Moodie, S. Mowshowity and K. H. Fantes, in *The Biology of the Interferon System* (Eds. E. De Maeyer and H. Schellekens), pp. 443–8. Elsevier/North-Holland Biomedical Press, Amsterdam (1983).
26. J. G. Bekesi and J. Roboz, *Tex. Rep. Biol. Med.* **41**, 614 (1982).
27. M. Tozowa, T. Kodowaki, T. Tanaka, T. Sawada, T. Kusunoki, T. Oku and T. Kishida, *Cancer Treat. Rep.* **66**, 1575 (1982).
28. J. A. Moore, B. J. Marafino and N. Stebbing, *Res. Commun. Chem. Path. Pharmac.* **39**, 113 (1983).