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EFFECTS OF H-7 ARE NOT EXCLUSIVELY MEDIATED THROUGH PROTEIN KINASE C OR THE CYCLIC NUCLEOTIDE-DEPENDENT KINASES

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Culturing murine T cell tumor lines in the presence of the protein kinase inhibitor H-7 for 4 days led to their dependence on H-7 for maximal constitutive proliferation. Withdrawal of H-7 from H-7-conditioned cells led to inhibition of proliferation and cell death. The mechanism underlying this H-7 dependence does not appear to be related to clonal selection or to effects on protein kinase C or the cyclic nucleotide-dependent kinases. This suggests that all the effects of the widely used H-7 may not be completely understood, and that H-7 may be useful in the dissection of the complex patterns of growth regulation in T cell malignancies.

Protein kinase C has been shown to subserve numerous functions in T lymphocytes. In normal T cells, it phosphorylates a number of different proteins and contributes to cell activation and proliferation (1-12). The role of protein kinase C in the proliferation of T cell malignancies or T cell hybridomas is less clear. Recently, it has been demonstrated that constitutive proliferation decreases when T cell hybridomas are stimulated with their specific antigen and MHC determinants (13,14). To investigate the role of protein kinase C in hybridoma proliferation, the protein kinase C inhibitor H-7 was utilized. Recently H-7 has been used to determine the contribution of protein kinase C to diverse T cell events ranging from activation to HIV infection (15-17). We now demonstrate that although H-7 initially inhibits tumor growth, tumor cells rapidly adapt to manifest significant dependence on H-7 for optimal viability and proliferation. This dependence appears to be related to a novel effect of H-7.

MATERIALS AND METHODS

Cell lines. BW5147 is an AKR murine thymoma and JJ15.4.3 is a hybridoma generated by electrofusion of BW5147 and a myelin-basic protein-reactive SJL/J T cell line.

Preculture of tumor cells with inhibitors. 1-(5-Isoquinolinesulfonyl-2-methylpiperazine dihydrochloride (H-7) (Seigaku Inc, St. Petersburg, FL) is a potent inhibitor of both protein kinase C (inhibition constant [Ki] = 6.0 uM) and the cyclic nucleotide dependent kinases (C-

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Abbreviation used in this paper: CM - Complete Media.

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GMP-dependent protein kinase Ki = 5.8 uM or C-AMP-dependent protein kinase Ki = 3.0 uM). N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004) (Seigaku Inc, St. Petersburg, FL) is a potent inhibitor of cyclic nucleotide-dependent protein kinases (C-GMP-dependent protein kinase Ki = 1.3 uM; C-AMP-dependent protein kinase Ki = 2.3 uM), but is a significantly weaker inhibitor of protein kinase C than H-7 (Ki = 40 uM) (18). HA1004 therefore can serve as a control in studies of protein kinase C inhibition. 5×10^4 cells of either BW5147 or the hybridoma JJ15.4.3 were plated in a 24-well plate in 1 ml of complete medium (CM) (RPMI-1640 [Gibco, Grand Island, NY] 10% fetal calf serum, 5×10^{-5}M 2-mercaptoethanol) in the presence or absence of various concentrations of H-7 or HA1004. On the second day, an additional 1ml of appropriate media was added to each well.

Proliferation Assay. Precultured cells were tested on the fourth day of the preculture. Precultured cells were washed twice in HBSS and 5×10^3 cells were plated in 96-well plates in 200 ul of medium in the presence or absence of inhibitors. After 24 hours, the wells were pulsed with 2 uCi of [^{3}H]thymidine and the cells harvested onto filter paper 18 hours later with a semi-automatic cell harvester. Proliferation was expressed as the mean counts per minute (CPM) of [^{3}H]thymidine incorporated, as measured by a beta-scintillation counter.

Viability. Cells were cultured under the same conditions as in the proliferation assay, and their viability was determined by trypan blue dye exclusion.

Doubling Time. Tumor cells were cultured in CM or 20 uM or 50 uM H-7 for greater than one month. In order to investigate the rate of proliferation of these cells, 1×10^5 cells per ml were plated in 24-well plates in the presence or absence of 20 uM or 50 uM H-7. Cells were resuspended daily, and live cells counted on a hemocytometer using trypan blue exclusion. Wells never contained more than 2×10^6 cells before they were split, and fresh media was given every 2 days. Doubling time was determined by regression analysis.

Quantification of Cellular Protein Kinase C Levels. Cells were collected by centrifugation at 500 x g, washed, resuspended at 5×10^7/ml in extraction buffer (2-mercaptoethanol, 50 mM; phenylmethylsulfonyl fluoride, 1 mM; EGTA, 2 mM; Tris-HCl, 50 mM, pH 7.2) with 1 mM leupeptin, sonicated twice for 30 seconds, and centrifuged at 1000 x g for 15 minutes to remove nuclei and whole cells. The supernatant was centrifuged at 100,000 x g for one hour at 4°C. The supernatant was stored on ice and the membrane proteins extracted by shaking 30 minutes at 4°C in one volume of extraction buffer with 2% Triton X-100 and 0.1 mM leupeptin. The mixture was centrifuged at 100,000 x g for one hour at 4°C and the supernatant saved. The nonextractable membrane pellet never showed protein kinase C activity and was discarded. Both supernatants were loaded on a DEAE cellulose column to remove cytosolic inhibitor of protein kinase C, and fractions were eluted with increasing concentrations of NaCl. Those fractions eluted with 60 mM and 80 mM salt contained most of the protein kinase C activity and were pooled for use in the histone phosphorylation assay. The reaction mixture contained 5×10^6 cell equivalents of partially purified cytosol or membrane, 0.2 mM PMSF, 0.4 mM EGTA, 10 mM $MgCl_2$, 5 mM ATP, 2×10^6 CPM of [^{32}P]ATP, 10 mM 2-mercaptoethanol, 0.01% Triton X-100, 100 ng/ml PMA, 400 ug/ml histone III-s and 35 mM Tris-HCl, pH 7.2. To this reaction mixture was added either H_2O, 4 mM $CaCl_2$, 20 ug/ml phosphatidylserine, or both calcium and phosphatidylserine to a total reaction volume of 200 ul. Cytosol reactions were 10 minutes at 20°C, whereas membrane extractable activity was measured for 30 minutes at 20°C. The reaction was stopped by spotting 30 ul of the reaction solution onto nitrocellulose filters and then washing off the excess radioactivity with deionized water. The filters were dried and the CPM determined on a beta-scintillation counter. Activity was expressed as picomoles of phosphate per milligram of total cytosolic protein incorporated into histone per minute.

RESULTS

Proliferation of BW5147 and JJ15.4.3. As can be seen in Table 1(a), the thymoma BW5147 precultured for 4 days in complete media (CM) showed little decrease in proliferation when tested with 20 uM H-7, but a significant decrease when tested with 50 uM H-7; there was no inhibition when tested with 20 uM or 50 uM HA1004, the control cyclic nucleotide-dependent kinase inhibitor. However, we were surprised to find that BW5147 precultured in the presence

TABLE 1 PROLIFERATIVE RESPONSES OF PRECULTURED TUMOR CELLS

	TEST CONDITIONS	PRECULTURE CONDITION				
		CM	20uM H-7	50uM H-7	20uM HA1004	50uM HA1004
(A)	CM	106,122 ± 4043	23,934 ± 597	3,892 ± 38	93,962 ± 1035	157,085 ± 3418
	20uM H-7	100,604 ± 1860	128,324 ± 1591	119,046 ± 3204	77,264 ± 1002	141,067 ± 2749
	50uM H-7	52,257 ± 1811	98,606 ± 3566	125,722 ± 222	39,643 ± 3574	40,507 ± 591
	20uM HA1004	108,882 ± 2341	28,511 ± 917	3,719 ± 126	86,310 ± 522	162,976 ± 845
	50uM HA1004	119,173 ± 2749	31,127 ± 611	4,768 ± 574	85,438 ± 1316	149,657 ± 1194
(B)	CM	45,421 ± 3696	12,094 ± 274	2,321 ± 314	28,887 ± 1248	31,982 ± 1065
	20uM H-7	32,302 ± 598	35,921 ± 1892	24,108 ± 948	18,076 ± 474	21,437 ± 682
	50uM H-7	1,455 ± 126	24,460 ± 806	23,082 ± 192	2,029 ± 25	2,390 ± 126
	20uM HA1004	45,050 ± 1576	23,649 ± 818	2,120 ± 103	27,939 ± 2054	29,256 ± 1074
	50uM HA1004	32,525 ± 1104	24,537 ± 644	2,125 ± 102	30,924 ± 1703	30,976 ± 537

5×10^4 cells/ml of BW5147 (A) or JJ15.4.3 (B) were precultured for 4 days in the presence or absence of various concentrations of H-7 or HA1004, as described in "Materials and Methods". 5×10^3 precultured cells were plated in 96 well plates in 200ul of medium in the presence or absence of H-7 or HA1004. After 24 hours, 2 uCi of [^{3}H]thymidine was added to each well, and cells were harvested 18 hours later. Proliferation is expressed as the mean CPM ± standard deviation of [^{3}H]thymidine incorporation of triplicate cultures.

of 20 uM or 50 uM H-7 for four days demonstrated significantly decreased proliferation when tested in CM alone. Furthermore, this decreased proliferation could be reversed by adding 20 uM or 50 uM H-7 to the H-7 pretreated cells, but could not be reversed by the addition of 20 uM or 50 uM HA1004. Preculture of BW5147 in either 20 uM or 50 uM HA1004 resulted in test responses that were essentially similar to those seen after preculture in CM alone. The pattern of proliferative responses was essentially the same for the hybridoma JJ15.4.3 as for the parent thymoma BW5147 [Table 1(b)].

Viability. Viability of the cells under the same conditions used in the preculture and proliferation assays was determined by trypan blue exclusion (Table 2). Cells precultured in CM showed a slightly decreased viability when further cultured for 2 days in 20 uM H-7, but a significantly decreased viability in 50 uM H-7. Paradoxically, cells precultured for 4 days in either 20 uM or 50 uM H-7 showed a decreased viability when cultured for the last 2 days in CM alone, requiring further culture in H-7 to maintain optimal viability.

Doubling Time. When cells were cultured with H-7 for a month or more, their doubling rate became slower than that of cells cultured with media alone. Doubling times of both the parent BW5147 and the hybridoma JJ15.4.3 were very similar. Cells cultured in medium without H-7 doubled approximately every 14 hours. 20 uM H-7-cultured cells proliferated more slowly than

TABLE 2 PERCENT VIABILITY OF PRECULTURED TUMOR CELLS TESTED IN THE PRESENCE OR ABSENCE OF H-7

PRE-CULTURE[a]	TEST CONDITIONS[b]					
	CM		20uM H-7		50uM H-7	
	JJ15.4.3	BW5147	JJ15.4.3	BW5147	JJ15.4.3	BW5147
CM	95.5 ± 0.7	95.6 ± 1.2	89.8 ± 9.0	90.4 ± 7.0	23.5 ± 9.9	20.7 ± 7.5
20uM H-7	72.8 ± 6.4	81.5 ± 3.9	95.7 ± 2.1	98.3 ± 3.2	91.9 ± 3.2	95.1 ± 1.3
50uM H-7	3.8 ± 1.6	4.8 ± 1.3	96.7 ± 0.3	96.6 ± 1.4	97.4 ± 1.5	98.9 ± 0.3

[a] [b] Cells were precultured as described in Table 1 legend.
Precultured cells were cultured for an additional 2 days in the presence or absence of H-7. Viability was determined by trypan blue exclusion and expressed as a percent of viable cells. Results represent the mean and standard deviation of 3 experiments.

TABLE 3 DOUBLING TIMES OF JJ15.4.3 AND BW5147

PRECULTURE	Doubling Time (hrs)[a] JJ15.4.3	BW5147
CM	13.96 ± 1.00	13.65 ± 1.11
20uM H-7	19.76 ± 0.65	19.50 ± 1.51
50uM H-7	25.1 ± 3.7	28.2 ± 2.6

1×10^5 cells per ml were plated in 24 well plates in the presence or absence of H-7. Cells were resuspended daily and viable cells counted by trypan blue exclusion. Doubling time was determined by regression statistics.

those in H-7-free medium, taking 5-6 hours longer to divide. Cells that were adapted to growth in 50 uM H-7 proliferated even more slowly, having a doubling time of about 25 to 28 hours (Table 3). This decline in proliferation was only occasionally seen in short-term, H-7-precultured cells (Table 1b). Although this discrepancy between the doubling time and the thymidine-incorporation proliferation assays cannot be definitively explained, we believe it relates to the long-term culture used in the doubling time studies versus the short-term culture used in the thymidine-incorporation assays.

Determination of Protein Kinase C Levels. To determine if the preculture of cells in H-7 resulted in alterations in protein kinase C activity, we assayed cellular levels of protein kinase C activity before and after the preculture. As can be seen in Table 4, there was no difference in the cytosolic protein kinase C activity of either BW5147 or JJ15.4.3 when activity of H-7-precultured and CM-precultured populations was compared. No significant protein kinase C activity was found associated with the extractable membrane-fraction from these cells under any of the conditions tested (data not shown).

DISCUSSION

In this report we have examined the effects of protein kinase inhibitors on the proliferation of the murine thymoma BW5147 and a T cell hybridoma JJ15.4.3. (derived from

TABLE 4 PROTEIN KINASE C ACTIVITY OF TUMOR CELLS PRECULTURED WITH OR WITHOUT H-7

	Protein Kinase C Activity[a] CM PRECULTURE	20uM H-7 PRECULTURE[b]
JJ15.4.3	1,784 ± 390	1,640 ± 115
BW5147	839 ± 107	799 ± 185

[a] 5×10^7 tumor cells were suspended in 1ml extraction buffer, disrupted and separated by centrifugation into cytosolic and membrane fractions. Both samples were purified by elution from a DEAE-cellulose column by 60-80 mM salt and activity tested by incorporation of ^{32}P into histone as described in "Materials and Methods". No activity was ever found in the extractable or nonextractable membrane fractions. Results are expressed as picomoles ATP incorporated per mg of total cytosolic protein per minute of reaction at 20°C.

[b] Cells were precultured for 4 days with or without 20uM H-7, as described in "Materials and Methods".

BW5147). H-7 inhibited the constitutive proliferation of naive tumor cells, but to a lesser extent than that reported for the (activated) proliferation of normal T cells. Surprisingly, however, we have found that a four-day preculture in the presence of H-7 yielded a population of BW5147 or JJ15.4.3 cells that were paradoxically dependent on the continued presence of H-7 for optimal constitutive proliferation. After preculture with H-7, maximal proliferation could only be restored by the addition of H-7. This was not simply a short-term phenomenon, as cells grown under these conditions for months still gave similar experimental results. The mechanism by which this dependence on H-7 develops is unclear. As discussed below, it does not appear to be related to protein kinase inhibition or selection of a separate subpopulation of pre-existing H-7-dependent cells, but rather it is related to some novel effect of H-7.

Examination of T cell receptor beta-chain gene rearrangements of the populations before and after preculture in H-7 demonstrated that no obvious clonal selection was occurring as a result of the preculture (data not shown). In addition, doubling time studies of the tumor cells indicate that it is unlikely that H-7-dependent cells were present as a small subpopulation within the initial populations in that the slowly proliferating H-7-dependent cells would soon be overgrown when cultured with the rapidly dividing "naive" cells.

H-7's inhibition of protein kinase C does not seem to be the factor that causes H-7 dependence, nor does the level of protein kinase C activity seem to be important in the constitutive proliferation of these cells. We could not demonstrate any significant difference in total protein kinase C activity extracted from H-7 and non-H-7-treated tumor cells, nor could we find any shift from the cytosol to membrane compartment. However, subtle changes in the level of protein kinase C activity beyond the limitations of our detection methods cannot be ruled out.

It has been previously demonstrated that high dose phorbol myristate acetate (PMA) treatment of cells for greater than 18 hours can down-regulate protein kinase C activity through degradation of the enzyme (20-22). Four-day preculture of our cells with 100 ng/ml of PMA did not cause any decrease in constitutive proliferation (data not shown). Further, the proliferation of PMA-precultured cells was inhibited by H-7 to the same extent as non-PMA-precultured cells. Coupled with HA1004's lack of inhibition of these protein kinase C-depleted cells, this indicates that the inhibition of our tumor cell proliferation by H-7 and the proliferation-dependence that develops after growth in H-7 may be caused by a property unrelated to H-7's known ability to inhibit protein kinases.

It is likely that the tumor cells may be adapting to an as yet unidentified, non-protein kinase C-related effect of H-7 that is involved with constitutive proliferation. Consistent with this hypothesis, the protein kinase C inhibitor, staurosporine (23,24), unlike H-7, is unable to induce tumor cell dependency during a 4-day preculture (data not shown). Overall, our results suggest that all of the effects of the widely used H-7 may not be completely understood and furthermore, that H-7 may be useful in the dissection of the complex patterns of growth regulation in T cell malignancies.

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REFERENCES

1. Shackelford, D.A., and Trowbridge, I.S. (1986) J. Biol. Chem. 261, 8334-8341.
2. Cantrell, D.A., Davies, A.A., and Crumpton, M.J. (1985) Proc. Natl. Acad. Sci. USA. 82, 8158-8162.
3. Taguchi, M., Thomas, T.P., Anderson, W.B., and Farrar, W.L. (1986) C. Biochem. Biophys. Res. Commun. 135, 239-247.
4. Isakov, N., Scholz, W., and Altman, A. (1986) Immunol. Today. 7, 271-277.
5. Weiss, A., Imboden, J., Hardy, K., Manger, B., Terhorst, C., and Stobo, J. (1986) Ann. Rev. Immunol. 4, 593-619.
6. Imboden, J.B., and Stobo, J.D. (1985) J. Exp. Med. 161, 446-456.
7. Klausner, R.D., Patel, M.D., O'Shea, J.J., and Samuelson, L.E. (1987) J. Cell. Phys. Supp. 5, 49-51.
8. Nel, A.E., Bouic, P., Lattanze, G.R., Stevenson, H.C., Miller, P., Dirienzo, W., Stefanini, G.F., and Galbraith, R.M. (1987) J. Immunol. 138, 3519-3524.
9. Ledbetter, J.A., Gentry, L.E., June, C.H., Rabinovitch, P.S., and Purchio, A.F. (1987) Mol. Cell. Biol. 7, 650-656.
10. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) Biol. Chem. 257, 7847-7851.
11. Costa-Casnellie, M.R., Segel, G.B., and Lichtman, M.A. (1985) Biochem. Biophys. Res. Commun. 133, 1139-1144.
12. Mire, A.R., Wickremasinghe, R.G., and Hoffbrand, A.V. (1986) C. Biochem. Biophys. Res. Commun. 137, 128-134.
13. Ashwell, J.D., Cunningham, R.E., Niguchi, P.D., and Hernandez, D. (1987) J. Exp. Med. 165, 173-194.
14. Ashwell, J.D., Longo, D.L., and Bridges, S.H. (1987) Science. 237, 61-64.
15. Clark, R.B., Love, J.T. Jr., Sgroi, D., Lingenheld, E.G., and Sha'afi, I. (1987) Biochem. Biophys. Res. Commun. 145, 666-672.
16. Fields, A.P., Bednarik, D.P., Hess, A., and May, W.S. (1988) Nature. 333, 278-280.
17. Nel, A.E., Schabort, I., Rheeder, A., Bouic, P., and Wooten, M.W. (1987) J. Immunol. 139, 2230-2236.
18. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) C. Biochem. 23, 5036-5041.
19. Mizuguchi, J., Yong-Yong, J., Nakabayaschi, H., Huang, K., Beaven, M.A., Chused, T., and Paul, W.E. (1987) J. Immunol. 139, 1054-1059.
20. Young, S., Parker, P.J., Ullrich, A., and Stabel, S. (1987) Biochem J. 244, 775-779.
21. Borner, C., Eppenberger, U., Weiss, R., and Fabbro, D. (2988) Proc. Natl. Acad. Sci. (USA) 85, 2110-2114.
22. Larsen, C.S., Christiansen, N.O., and Esmann V. (1988) Biochim. et Biophys. Acta 969, 281-288.
23. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397-402.
24. Nakano, H., Kobayashi, E., Takahashi, I., Tamaoki, T., Kuzuu, Y., and Iba, H. (1987) J. Antibio. 40, 706-708.