Biochemical Pharmacology, Vol. 20, pp. 2213–2220. Pergamon Press, 1971. Printed in Great Britain

L5178Y ASPARAGINE-DEPENDENT CELLS AND INDEPENDENT CLONAL SUBLINES

TOXICITY OF 5-DIAZO-4-OXO-L-NORVALINE*

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(*Received* 5 *November* 1970; *accepted* 15 *January* 1971)

Abstract—Sublines of the asparagine-dependent L5178Y mouse lymphoblastic leukemia cells have been obtained by single-step clonal selection in asparagine-free medium. These sublines grow equally well with or without this amino acid in the medium. Inhibition of the growth of L5178Y cells in culture by the L-asparagine analog, 5-diazo-4-oxo-L-norvaline (L-DONV), can be attributed to two different mechanisms. Both the L and D-stereoisomers of DONV inhibited the growth of the parent L5178Y line in Fischer's medium (with asparagine) as well as the growth of the asparagine-independent cells in the presence of asparagine. In a medium devoid of asparagine, however, L-DONV caused a more immediate cessation of growth of the asparagine-independent cells than in the medium normally supplemented with this amino acid. The toxicity of D-DONV was not enhanced by omission of asparagine. Evaluation of the effects of the analogs by cell cloning revealed a rapid loss of viability of both dependent and independent cells in the presence or absence of asparagine. These results indicate that L-DONV acts as an L-asparagine analog to inhibit the growth of cells dependent on endogenous asparagine synthesis, but that an additional cytotoxic effect of either stereoisomer of the analog exists.

PREVIOUS reports from this laboratory have indicated that 5-diazo-4-oxo-L-norvaline (L-DONV) is an effective analog of L-asparagine (ASN). Some of the observed effects include the stereospecific inhibition of ASN synthesis by intact mouse lymphoma cells (6C3HED)[1] or by purified ASN synthetase from these cells,[2] catalytic decomposition of L-DONV by L-asparaginase,[3] and irreversible and stereospecific titration of the active site of L-asparaginase.[4] An earlier study in this laboratory also demonstrated that L-DONV reduced cell viability more rapidly in L-ASN-dependent L5178Y lymphoblastic leukemia cells than in the nondependent P815Y mast cell leukemia cell line.[5]

This report presents a further analysis of the cytoxicity of the diazo analog as well as some effects of the D-isomer of DONV (D-DONV). Experiments with sublines of L5178Y cells selected for ASN independence indicate that L-DONV is inhibitory to cell growth and viability by at least two mechanisms.

MATERIALS AND METHODS

L5178Y cells obtained from Dr. G. A. Fischer were propagated as previously described.[6] The requirement for L-ASN[7] was supplied by inclusion of 10 mg/l.

* This work has been supported by the United States Public Health Service (Grants CA5012 and CA10748) as well as by grants from the American Cancer Society (T112 and PR27).

(8×10^{-5} M) in the standard Fischer's medium.[6] The cells were maintained in exponential growth in Fischer's medium supplemented with 10% horse serum. Fischer's medium with ASN omitted was prepared for use in experiments requiring ASN-free medium. All serum and media were obtained from the Grand Island Biological Company.

The method of Chu and Fischer[8] for cloning L5178Y cells in a soft agar gel suspension was employed. The level of ASN synthetase activity of intact cells was measured by brief incubation of concentrated suspensions (10 to 20 $\times$ 10^6/ml) of cells in tissue culture medium and determination of the accumulation of soluble ASN in medium and cells. These methods, including the procedure for the coupled enzymatic assay for ASN, have been described.[9,10] The L- and D-DONV were synthesized by Dr. P. Chang by the method described earlier for L-DONV.[5]

The L-asparaginase (180 U/mg) used for these experiments was prepared from *Escherichia coli* and kindly supplied by Dr. B. Berk at E. R. Squibb.

RESULTS

Selection and properties of ASN-independent L5178Y *cells*

For meaningful comparisons between ASN-independent and dependent cells, it was desirable to have cells identical except for this property. Since most mammalian cells normally synthesize ASN, a selection of an ASN-independent line was attempted from the L5178Y cell line which normally requires this amino acid for growth.

In two experiments L5178Y cells were washed with ASN-free medium and cloned at 1 to 2 $\times$ 10^5 cells per ml in ASN^- agar cloning medium with 15% horse serum.[8] Several early clones were isolated and tested for growth in ASN^- medium. One clone from each experiment was established as a permanent ASN-independent (L5178Y-ASN^+) cell line and maintained in ASN^- medium. After a few generations in ASN-free medium, the new sublines achieved the same doubling time (11–12 hr) as the parental line. The cells produced ascites tumors in AKD_2F_1 mice and, after 1 year of continuous weekly passages in mice, were still ASN-independent when tested in culture.

The stability of these ASN-independent cell lines suggests that L5178Y cells may occasionally become independent by a spontaneous mutation. Thus the ASN dependence of L5178Y cells is probably not a result of permanent loss of the genetic information required for ASN synthesis. The possibility that the new cell sublines had become ASN-independent by a marked increase of ASN synthetase activity was tested. Both sublines produced ASN (3 nmole/10^6 cells/hr) when the intact cells were incubated as previously described for other cell lines.[10] There was no detectable accumulation of ASN when cells of the original L5178Y line were incubated under the same conditions ($<0{\cdot}2$ nmole/10^6 cells/hr). Ascites cells from animals with L5178Y-ASN-independent tumors transplanted weekly for 1 year had somewhat lower levels of ASN synthetase activity (1·1–1·3 nmole/10^6 cells/hr), with no measurable synthesis in the parental L5178Y ascites cells. Twelve additional ASN-independent sublines were derived and each had measurable ASN synthetase (0·4–2 nmoles/10^6 cells/hr) when assayed in this manner after passage in mice.

Figure 1 shows a comparison of the effect of ASN-free medium or L-asparaginase on growth of the L5178Y cells and of one of the ASN-independent lines. The amount of

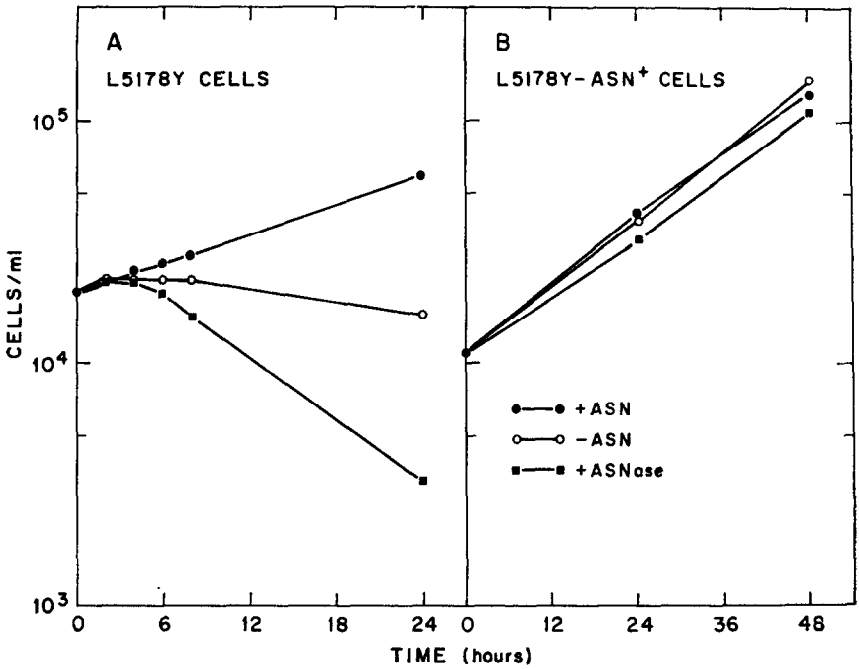

FIG. 1. Growth of L5178Y cells without ASN or with ASNase. (a) L5178Y cells. Cells in exponential growth in Fischer's medium were collected by centrifugation and resuspended in ASN-free Fischer's medium. ASN (8×10^{-5} M) was added immediately to control cultures. *E. coli* ASNase (0·1 U/ml) was added at zero time to some cultures. The cell number was determined with a Coulter counter. (b) L5178Y-ASN-independent subline (L5178Y-ASN$^+$). Cells from stock cultures (already growing in ASN-free Fischer's medium) were diluted with fresh medium at zero time and ASN (8×10^{-5} M) or ASNase (0·05 U/ml) was added at once. (In another part of the same experiment this level of ASNase caused rapid lysis of L5178Y cells as in Fig. 1a.)

enzyme was sufficient to hydrolyze the ASN in the growth medium in less than 1 min. Both methods of ASN deprivation rapidly inhibited cell replication in the L5178Y cells (Fig. 1a). The very rapid cell lysis seen when the L5178Y cells were treated with ASNase was also observed when these cells were washed and put into ASN-free growth medium supplemented with 10% dialyzed horse serum to assure maximal reduction of exogenous ASN.

Figure 1b shows that a low level of ASNase had negligible effect on the growth of the ASN-independent cells for at least 2 days. These cells could be inhibited by higher levels of ASNase or by the low level during longer periods of growth. This delayed inhibition, however, could be entirely prevented by supplementation with additional glutamine ($1{\cdot}4 \times 10^{-2}$ M) and thus was at least partially attributable to the glutaminase activity of the *E. coli* asparaginase.[11]

The two ASN$^+$ sublines were similar in all properties examined and only one of the sublines (L5178Y-ASN$^+$) was used for all the subsequent experiments.

Effect of L- *or* D-*DONV on cell growth*

L5178Y cells grown in Fischer's medium (with 10% horse serum and 8×10^{-5} M ASN) were inhibited equally by either isomer of DONV (Fig. 2) at several concentrations. Increasing the concentration of ASN by 10-fold (to 8×10^{-4} M) did not affect

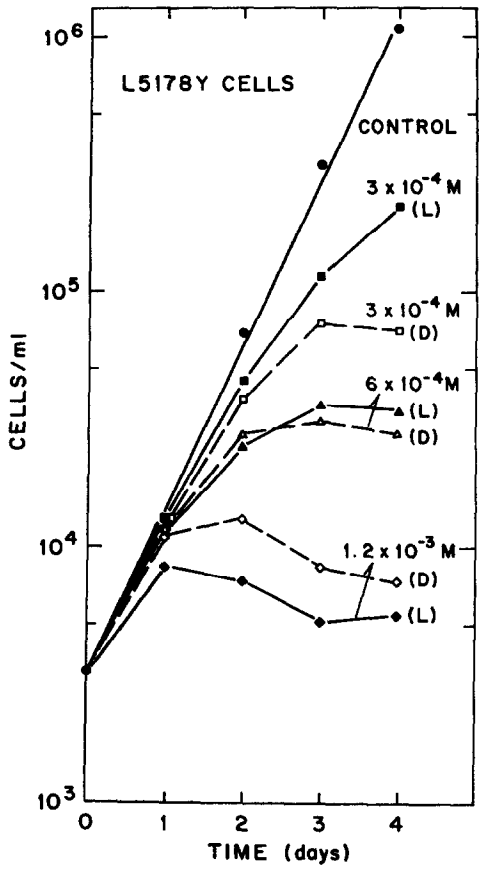

FIG. 2. Effect of various concentrations of L- or D-DONV on the growth of L5178Y (ASN-dependent) cells. Cells in exponential growth were diluted with fresh Fischer's medium containing the standard concentration of ASN (8×10^{-5} M) as well as L- or D-DONV in the concentrations indicated.

growth of control cultures and did not protect against either D- or L-DONV at 6×10^{-4} M.

The L5178Y-ASN$^+$ cells, which grew equally well in the presence or absence of added ASN, provided evidence for the dual toxicity of DONV. With ASN present, L- or D-DONV (6×10^{-4} M) had the same delayed effect on cell growth (Fig. 3). When ASN was omitted, however, cell growth ceased immediately upon addition of L-DONV, while those cultures to which D-DONV was added followed the growth pattern seen in the presence of ASN. This effect of L-DONV was similar to the effect on growth of the parental L5178Y cells when they were transferred to ASN-free medium (Fig. 1). Thus, partial protection against L-DONV is provided by L-ASN. The known capacity of the L-DONV to inhibit ASN synthetase suggests that the observed immediate cessation of growth is caused by inhibition of endogenous synthesis of ASN. The presence of ASN in the medium apparently protects the cells from this ASN deficiency but does not protect against the secondary, delayed DONV effect. Higher levels of ASN, up to 100 times the normal concentration, afforded no further protection of the ASN-independent cells from 6×10^{-4} M DONV. Inhibition

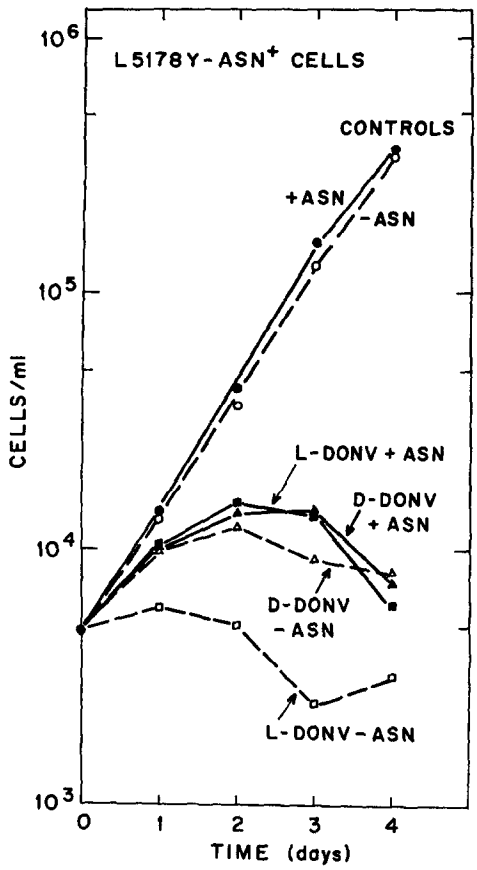

FIG. 3. Effect of L- or D-DONV (6×10^{-4} M) on the growth of ASN-independent (L5178Y-ASN$^+$) cells in medium with or without ASN. Cells from stock cultures (growing in ASN-free Fischer's medium) were diluted to the starting concentration with fresh ASN-free medium. At zero time L- or D-DONV was added (6×10^{-4} M) and the indicated cultures were supplemented with ASN (8×10^{-5} M).

of cell growth by D-DONV was not affected by addition of D-ASN (8×10^{-5} to 8×10^{-3} M) or L-glutamine (to $1{\cdot}4 \times 10^{-2}$ M, 10 times the normal concentration).

Effect of L- *or* D-*DONV on cloning*

The cloning of cells after brief treatment with drugs permits the detection of early effects which may prevent the cells from continuing to divide for the number of generations (at least eight) required to form a visible colony from a single cell. Thus rapidly lethal effects are often more apparent when tested by this method than by measuring cell growth in liquid cultures for a short time.

Table 1 summarizes the results of experiments to determine the effects of 24-hr treatment with L- or D-DONV on the viability of the ASN-dependent and independent cells. The relative numbers of total viable cells after 24 hr are a product of the combined effects of inhibition of cell division and reduction of cell viability by the treatments employed. Although DONV had little effect on cell growth as determined by the Coulter counter after 24 hr (cf. Figs. 2 and 3), these studies indicate that both

TABLE 1. EFFECT OF 24-hr TREATMENT WITH L-DONV OR D-DONV ON TOTAL VIABLE CELLS PER CULTURE*

Cells and treatment	Viable cells per culture (% of control)
L5178Y (ASN-dependent)	
+ ASN (controls)	100
− ASN	10 ± 3 (S.D.)
+ ASN + L-DONV	30 ± 10
− ASN + L-DONV	2 ± 1
+ ASN + D-DONV	17 ± 7
− ASN + D-DONV	2 ± 1
L5178Y-ASN$^+$ (ASN-independent)	
+ ASN (controls)	100
− ASN	114 ± 13
+ ASN + L-DONV	22 ± 6
− ASN + L-DONV	4 ± 2
+ ASN + D-DONV	4 ± 4
− ASN + D-DONV	6 ± 5

* In three separate experiments with each cell line, cells in exponential growth were collected by centrifugation and resuspended at 2–3 × 10^4 cells/ml in ASN-free medium. ASN (8 × 10^{-5} M) and L- or D-DONV (6 × 10^{-4} M) were added at once to the indicated cultures. The growth in 24 hr was similar to that for the corresponding cultures in Figs. 2 and 3. At 24 hr the cells were washed and cloned in Fischer's medium (with ASN) in soft-agar cultures. At this time the number of viable cells in control cultures had increased to four to five times the initial cell concentration. A total of 400–500 cells were cloned in five tubes for each treatment. The data within each experiment were normalized to controls (with ASN) = 100% viability. (Absolute cloning efficiency of controls was 60–80%.) The above data compare total viable cells per culture after 24 hr. The calculation was made on the basis of per cent viability in each cloned culture as follows:

$$\frac{\text{test cells/ml} \times \%\ \text{clonable cells} \times 100}{\text{control cells/ml} \times \%\ \text{clonable cells}}.$$

Each figure in the table is the average of 12–15 separate tubes in three experiments.

forms of DONV greatly reduced cell viability within 24 hr. In the parental L5178Y line, less than half of the cells remained viable after 24 hr with either DONV isomer in the presence of ASN. The extensive loss of viability of these cells due to ASN deprivation alone was further enhanced by either L- or D-DONV, indicating additional toxicity created by the analog. In the ASN-independent cells, omission of ASN had no effect on viability. This permitted a comparison of effects of the two DONV isomers on viability under conditions where ASN deprivation alone was not inhibitory. Both isomers of DONV were toxic to these cells; however, when the cells were exposed to L-DONV there was significant protection in the presence of ASN. With D-DONV there was little difference in the total number of viable cells with or without ASN present during exposure to the drug.

It should be noted that in each cloning experiment 6 × 10^{-4} M D-DONV was consistently more toxic than 6 × 10^{-4} M L-DONV, although the solutions used were of equal concentrations ± 5% as determined by the absorption at 274 nm. Thus, these

experiments indicate that D-DONV was at least as toxic with respect to its effect on cell viability as the L-isomer, but that a portion of the toxicity of L-DONV can be prevented by the inclusion of asparagine in the medium.

DISCUSSION

These experiments have helped to clarify earlier, somewhat paradoxical observations on the toxicity of L-DONV: an early loss of cell viability even though the onset of inhibition of cell growth was less rapid and was dependent on the dose of DONV used.[5] The present results suggest that the decline in cell viability begins immediately whether or not ASN is present and is due to a common property of L- and D-DONV. In the presence of ASN the effects on cell growth are also non-stereospecific and could be a consequence of the same lethal mechanism. Only when cell growth is dependent on endogenous ASN (as in the case of the ASN-independent cells in ASN-free medium) is L-DONV a more potent inhibitor than D-DONV. Under these conditions the pattern of growth resembles that of ASN-dependent cells when they are deprived of exogenous ASN. Protection with ASN is only partial, and the cells are still subject to the nonspecific toxicity of this diazo-ketone derivative. Thus, while L-DONV has proven to be an invaluable analog of L-asparagine for the investigation of certain aspects of ASN metabolism (ASN synthetase or L-asparaginase), studies involving use of DONV in intact biological systems must also take into account the toxicity not overcome by supplemental ASN.

ASN-independent sublines of ASN-dependent tumors have been reported in several earlier studies. Variants of the Jensen sarcoma[12] or of the 6C3HED lymphoma[13] were obtained by incubation of cells *in vitro* in medium without ASN; ASNase resistant sublines (6C3HED,[14–16] Jensen sarcoma,[17] Walker 256 carcinosarcoma,[18] or L5178Y leukemia[19,20]) have also been derived from surviving cell populations *in vitro* or tumors *in vivo* after prolonged treatment with *E. coli* or guinea pig serum asparaginase.

The observation in this report that ASN-independent cells can be selected by single-step cloning from populations of ASN-dependent L5178Y cells indicates that a low frequency of ASN-independent cells occurs even when ASN is continuously available in the medium. The properties of the ASN-independent cell lines suggest that they were derived from cells converted by spontaneous single-step mutation to ASN independence. These results lend strong support to previous suggestions[13,21] that development of ASN-independent sublines of ASN-dependent tumors is the result of a selection of variants in the cell population. These results also support the suggestion that resistance of neoplastic cells to asparaginase therapy is at least partially a result of selective destruction of the ASN-dependent cells and enrichment of the culture or tumor with ASN-independent cells.

Since ASN-independent cells have the same growth characteristics in culture and produce tumors as well as the parental line, dependence on ASN is not a requisite for tumor growth. The ease of selection of ASN-independent cells in this system offers a promising approach to the study of the genetic locus which must have been altered when ASN independence was lost. This may help to elucidate the unexplained concordance of ASN dependence and neoplasia in selected lines of neoplastic cells. Furthermore, it may be possible to use these and other ASN-dependent cell cultures to explore the genetic factors which determine this nutritional requirement.

Acknowledgements—The authors wish to thank Dr. G. A. Fischer and Dr. M. Y. Chu for their valuable assistance and generous provision of cell culture facilities. We are also indebted to Dr. P. Chang for her synthesis of DONV. The technical assistance of Mrs. Barbara Stanley and Miss Celeste Gaumond has been most important in this work.

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