Permeability Difference as a Source of Resistance to Alkylating Agents in Ehrlich Tumor Cells

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Repeated in vivo exposure of Lettre-Ehrlich ascites tumor cells to high doses of HN2 (nitrogen mustard) (3 to 80mg/kg) permits the selection of resistant sublines. Using two of these lines, L2RA, selected against 3mg/kg HN2 and $L2RA_{80}$, selected against doses up to 80mg/kg, we have observed a decreased permeability to HN2, accompanied by a reduction in the extent of alkylation of the DNA.

Methods

LES ascites cells in Swiss mice were exposed to 5mg/kg HN2 i.p. and transplanted after 14-21 days; 5 to 7 challenges are sufficient to select a stable, resistant cell line. Use of higher doses (6-80mg/kg HN2) to select more resistant lines requires transfer of the washed, exposed cells to a fresh host prior to growth and transplantation.

Results

Lines L2RA and $L2RA_{80}$ show clearcut in vivo resistance to HN2; i.p. doses of 0.4 to 0.8mg/kg administered day 1 to 6

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Table 1

In Vitro Effect of HN2 on Viability of Sensitive and Resistant Ehrlich Cells[1)]

[HN2]	LES	L2RA	$L2RA_{80}$
	Fraction Surviving[2)]		
10^{-6}	~ 1	~ 1	~ 1
10^{-5}	< 0.01	0.1	~ 1
10^{-4}	< 0.01	< 0.01	0.1
10^{-3}	≪ 0.01	≪ 0.01	< 0.1

1) 50×10^6 cells incubated in autologous ascites fluid diluted ~ 1/1 with Krebs-Ringer phosphate buffer, pH 7.0, 37°C in air for 15 or for 60 minutes with indicated [HN2] (Merck).

2) Each point represents 2 groups of ten mice receiving 10^7 cells after in vitro incubation with HN2, centrifugation, washing with saline and resuspension in saline. Viability estimated as fraction surviving by comparison with survival time data for mice receiving serial dilutions of cells (10^7 to 10^1) after 60 minutes incubation without HN2, essentially according to procedure of Skipper et al. (1).

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following tumor implabtation cause no significant extension of host survival time. At these doses the parent LES line shows >200% extension of survival time and 50% survivors at 90 days. The relative in vitro resistance is given in Table 1. As can be seen, a 10 to 50x enhancement of resistance occurs in going from LES to L2RA; $L2RA_{80}$ shows an additional 10x increase in resistance over L2RA.

When DNA samples from 6 day growths of LES and L2RA cells are isolated and purified (2) after 60' in vivo exposure to HN2 (3mg/kg), the resistant cells show a reduction of approximately 50% in the extent of alkylation. This decrease is highly signif-

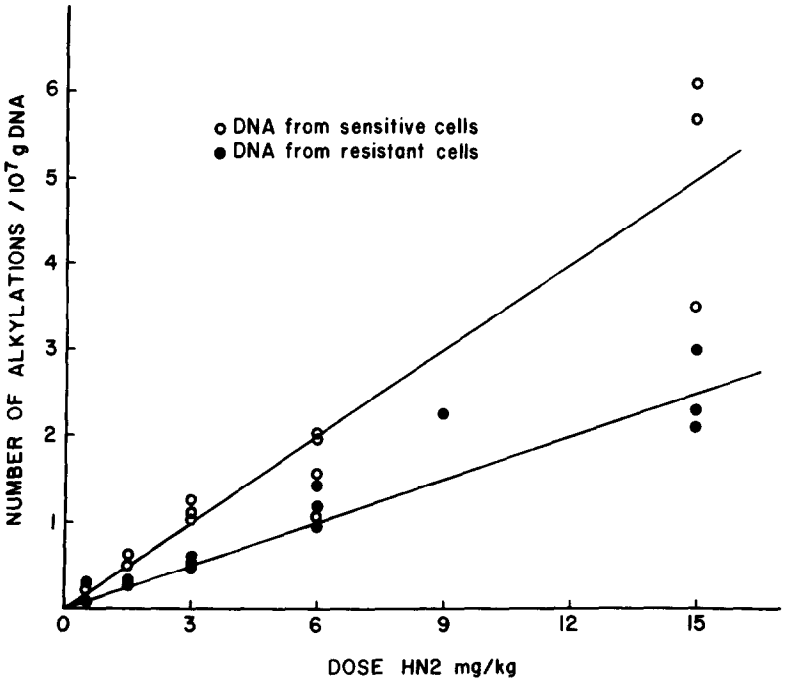

Fig. 1. Mice bearing 6-7 day old Ehrlich cell growths given single i.p. doses of ^{14}C-HN2 as indicated. Cells harvested after 60 minutes in vivo; DNA isolated by procedure of Marmur (2); assayed in Cab-o-sil suspension by scintillation counting of 0.2 - 0.5mg/sample.

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icant ($P < 0.001$) and is shown in Fig. 1. $L2RA_{80}$ cells show the same reduction in alkylation as do L2RA.

Reduction of alkylation in the resistant cells is accompanied by a proportional decrease in the amount of cross-linked DNA, as estimated by means of CsCl density gradient centrifugation of samples thermally denatured at 65°C in 7.2 M $NaClO_4$ (3). This procedure was used because it provides direct physical evidence of the persistence of "native" density DNA in denatured samples, the presence of which in alkylated samples can be attributed to covalent cross-links between the complementary strands of the DNA (4,5). (We have shown that these cross-links are stable during

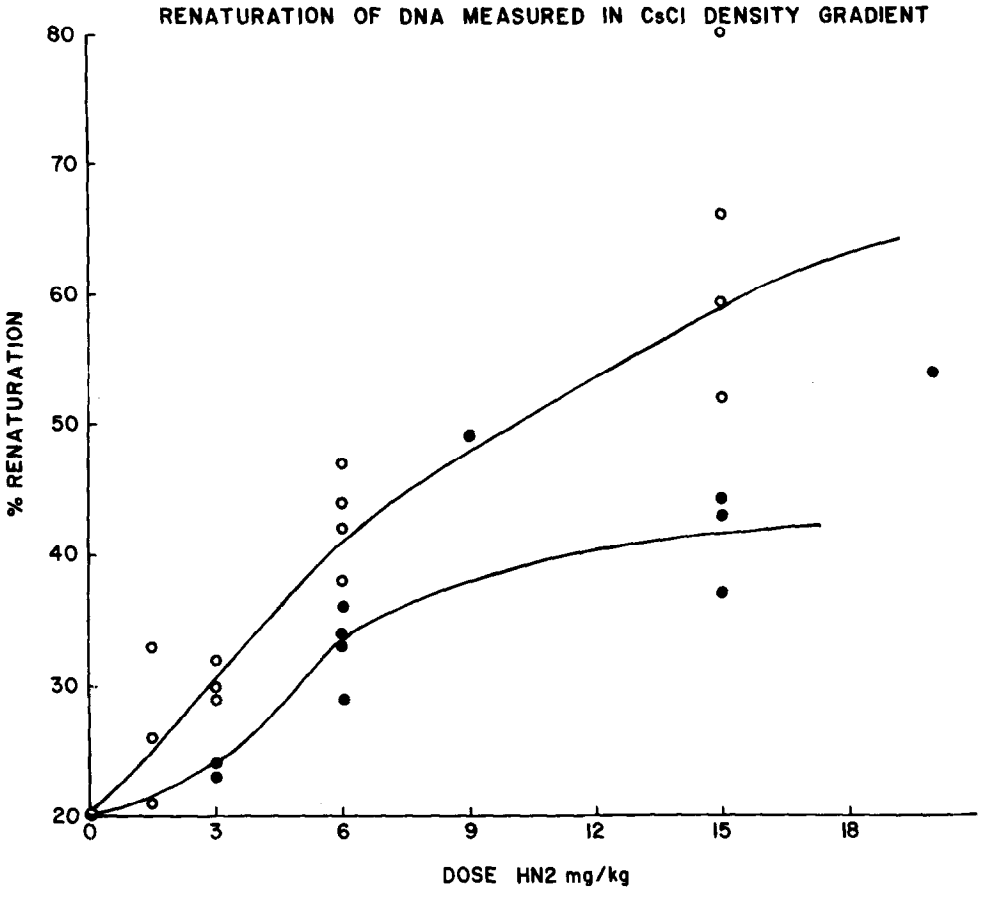

Fig. 2. Crosslinking of DNA by HN2 *in vivo*. DNA samples from LES (o) and L2RA (●) cells denatured in 7.2 M $NaClO_4$ at 65°C, quenched to 0°, equilibrated at 25°C and then dialyzed against 0.01 phosphate buffer, pH 7.0. Denatured samples centrifuged in a 5.7 M CsCl gradient 20 hrs. at 44,770 RPM. % renaturation determined from densitometer tracing comparisons of the native (ρ = 1.704) and denatured (ρ = 1.716) bands. (Results corrected for ~10% spontaneously renaturable DNA present in mouse cells.)

$NaClO_4$ denaturation; higher temperatures (>70°C in $NaClO_4$) can result in losses of renaturability (6).) When the data for sensitive and resistant cells is compared (Fig. 2), two points of overlap are observed in a total of 24 analyses; the lines are however

Table 2

Uptake and Distribution of ^{14}C-HN2 in Sensitive and Resistant Ehrlich Cells[1)]

	LES	L2RA	$L2RA_{80}$
		Distribution of ^{14}C[2)]	
% uptake/10^9 cells	24 ± 8	6.6 ± 2	9.0 ± 2
% uptake/10^9 nucleii	6.6 ± 2	1.7 ± 0.5	2.5 ± 0.5
% uptake by DNA/ 10^9 nucleii	0.46 ± .1	0.20 ± 0.05[3)]	(0.25)[4)]
μμm HN2/cell x 10^5	9.1 ± 3.0	2.6 ± 0.6	3.6 ± 0.5
μμm/nucleus x 10^5	2.5 ± 0.9	0.62 ± 0.2	1.0 ± 0.3
μμm on DNA/nucleus x 10^5	0.17 ± 0.02	0.07 ± 0.01	(0.1)[4)]

1) Mice bearing 6-7 day old i.p. growths of tumor cells given single i.p. dose of 3.0 mg/kg ^{14}C-HN2 and sacrificed after 60 minutes.

2) Cells freed of ascites fluid and washed 2x with saline in cold. Aliquots assayed for total radioactivity. Cells shocked by exposure to hypotonic saline (~0.01 M) 10 minutes in cold, homogenized, nucleii sedimented, resuspended, rehomogenized and sedimented. Cells show less than 5% cytoplasmic tags. Aliquot of nucleii assayed for ^{14}C.

3) DNA isolated and purified by procedure of Marmur (2).

4) Estimated on the basis of measurements of cross-linking of the DNA, using the relationship, % crosslink = 0.07 x alkylations (m HN2/10^7 daltons DNA).

significantly separated ($P < 0.001$) and the resistant L2RA cells undergo approximately 50% of the cross-linking shown by the sensitive cells. As was the case with total alkylation, the same reduction in cross-linking is observed in $L2RA_{80}$ cells.

In seeking for a source of the observed differences in alkylation we compared the uptake and distribution of HN2 in cells exposed to 3mg/kg for 60' *in vivo*. In a series of replicate com-

parisons, LES cells took up significantly greater quantities of HN2 than did the L2RA cells and exhibited a greater absolute level of HN2 in the nucleus. This effect can be seen in Table 2; the LES cells take up 3-4x as much HN2 as do L2RA cells and there is no significant difference between L2RA and $L2RA_{80}$. The relative partition of the HN2 between cytoplasm, nucleus and DNA is, however, constant for all three cell lines.

Discussion

It is possible, by means of standard selection techniques, to produce stable ascites tumor cell lines increasingly resistant to HN2. While our studies have produced no direct evidence that the changes in resistance are stepwise (7), the fact that increasing the exposure to HN2 doses result in progressive enhancement of resistance supports such a conclusion (see below).

The observed permeability difference qualitatively accounts for the reduced alkylation of the DNA observed in the resistant cells. However, even in the L2RA cells, permeability changes are insufficient to account for the greater than 10-fold increase in resistance to HN2, since doubling the dose to these cells should merely equalize the cytotoxicity-this is not the case. Furthermore, our conclusion that selection is a complex sequential change is supported by the enhancement of resistance without further changes in permeability. It appears likely that HN2 selects for a succession of cellular changes with permeability changes appearing early in the process.

Wolpert and Ruddon (8) and Klatt, et al. (9) have now confirmed our observations on the Ehrlich cell lines and similar effects were observed by Reid and Walker (10) using L cells and sulfur mustard. Yamada et al. (11) have provided indirect evidence that permeability limits alkylation damage. These

findings suggest some generality to permeability regulation as a resistance mechanism.

The ability of $L2RA_{80}$ cells to resist single doses of 80mg/kg HN2 indicates that these cells are equipped with extremely active repair mechanisms. A dose of 80mg/kg HN2 can be expected to produce $\sim 1x10^7$ alkylations per $L2RA_{80}$ cell or 1 per 2500 nucleotides. At this frequency, probably every gene in the cell would have undergone chemical modification of its DNA (6). Our studies have also shown that cross-link frequency is $\sim$ 10% of the total alkylations, so that each cell would contain $1x10^6$ cross-links, involving, probably, every cistron. The survival of these cells does not seem possible without extensive, if not complete, excision of the alkylated bases and their non-conservative replacement by normal bases. The existence of such repair mechanisms in mammalian tissue culture cell lines is strongly indicated by Roberts et al. (12); in conjunction with our findings, these observations support a critical role for cross-linking (13, 14).

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