Lysosomal Acid Hydrolases in Primary Monolayer Cultures Derived from Neonatal Brains Exposed Transplacentally to Ethylnitrosourea[1] (36834)

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It has been demonstrated that one inoculation of ethylnitrosourea (ENU) will induce neoplasms of the central nervous system transplacentally in the progeny of pregnant rats (1, 2). Koestner, Swenberg and Wechsler (2) refined this oncogenic model, developing a transplacental ENU treatment that resulted in nervous system neoplasms in 100% of the progeny. Further, the neoplasms so induced were derived almost always from glial cells or glial cell precursors of ectodermal origin. The neoplasms that appeared in the rat nervous system subsequent to transplacental exposure to ENU exhibited markedly elevated lysosomal acid hydrolase activities compared to normal cerebrum (3). The consideration that these neoplasms in the adult nervous system were presumably induced by the effect of ENU on fetal glial cell precursors during a single transplacental exposure thus suggests that ENU induces postnatal differentiation of glial cells with altered lysosomal acid hydrolase activities.

The principal aim of the present study was to determine whether transplacental exposure of the fetal brain to ENU alters glial cell lysosomal acid hydrolase activities. Primary monolayer cultures of brain cells derived from the brains of newborn rats were used to provide concentrated samples of glial cells for hydrolase assay. Specifically, hydrolase activities were assayed in four sets of cultures, each set consisting of experimental cultures derived from the brains of newborn litters exposed transplacentally to ENU and controls derived from the brains of unexposed litters.

Materials and Methods. One member of each of 4 pairs of 20-day-pregnant Fischer rats was injected with ENU (iv, 100 mg/kg, 10 mg/ml 0.90% NaCl solution, adjusted to pH 4.5 with ascorbic acid) to effect transplacental exposure of the fetal brains to ENU. The control rat of each pair received buffered saline only. Treated cultures were derived from the pooled brains of 10 newborn littermates from the ENU treated mother while the control cultures were derived from the pooled brains of 10 newborn littermates from the saline injected mother. Brain cell cultures, predominantly glial cells and their precursors, were prepared as previously reported (4) with the following minor modifications. The pooled treated brains and the pooled control brains were collected aseptically in separate volumes of Hanks' balanced salt solution (HBSS) with antibiotics, washed in fresh HBSS and minced. Brain cells were dispersed from these fragments by agitation in 0.25% trypsin for 15 min at 22°. The first cell suspensions, containing meningeal elements and other tissue debris, were discarded and the dispersion procedure was repeated on the same brain fragments. The next 3 cell suspensions from the remaining treated and control pools of brain fragments were collect-

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ed in bovine calf serum and centrifuged (300 RCF, 6 min). The resulting cell pellets were suspended in separate volumes of growth medium (about 200,000 cells/ml) and aliquots (20 ml) were transferred to 100 ml bottles for incubation (37°). Four sets of brain cell cultures were subsequently prepared from the litters of the 4 pairs of rats. Each set consisted of 10–15 treated cultures derived from the brains of newborn transplacentally exposed to ENU and 10–15 control cultures. The growth medium consisted of HBSS, 0.5% lactalbumin hydrolysate, 10% fetal bovine serum (FBS), 2% $NaHCO_3$ and antibiotics (100 units penicillin/ml, 100 μg streptomycin/ml). After 10–14 days and 25 days incubation, the media were replaced with ones containing 7.5% FBS and 2.5% $NaHCO_3$, and 5% FBS and 3% $NaHCO_3$, respectively, with no antibiotics.

The incubation periods for sample cultures from the four culture sets are indicated in Tables I, II, III and IV. For hydrolase assays, monolayers from 2 or 3 bottles of treated and a like number of control cultures were removed with trypsin (0.25%), collected into separate treated and control pools, centrifuged and washed with cold 0.32 *M* sucrose. Treated and control cell pellets were homogenized in 0.32 *M* sucrose containing Triton-X-100 (0.1% final concn) for assay of total hydrolase activities.

β-Glucuronidase (β-G) activities were determined using phenolphthalein β-glucuronide (5) as substrate, acid phosphatase (AP) activities using β-glycerophosphate (6), arylsulfatase A and B (AS) activities using nitrocatechol sulfate (7) and *N*-acetyl-β-D-glucosaminidase (GAD) using *p*-nitrophenylglycoside (8). Activities were expressed as micromoles of substrate hydrolyzed per hour per milligram protein (9).

Results. Observations of the brain cell cultures during incubation and in stained preparations revealed no morphological differences between cultures derived from newborn brains exposed transplacentally to ENU and control cultures. Almost all cells resembled the glial cells *in vitro* described previously (4, 9).

There were no clear differences between hydrolase activities of the brain cell cultures derived from brains of newborn exposed transplacentally to ENU and those of controls. Since each of the 4 sets of treated and control cultures developed its own level of activity for each hydrolase, and these activities changed with time in culture the ratios between treated and control culture activities for each set were used for comparison. Mean ratios for each hydrolase at each stage of incubation are recorded in Tables I–IV. Kendall's coefficient of rank correlation was calculated to determine whether the ratios between treated activities and control activities were correlated with time in culture. There was no significant correlation at 5.0% level for β-G, AP, GAD or AS.

Culture sets 3 and 4 were serially assayed for changes in hydrolase activities with time in incubation. GAD activities increased steadily throughout the 42–49 day incubation period to almost three times the level of the activity observed after 14 days and four times that after 7 days incubation (Table I). The β-G activities increased by about 25 to 50% through 35 days incubation, then decreased by 42 to 49 days (Table II). The AS activities reached their maximum between 21 to 35 days incubation, then decreased or remained little changed thereafter (Table III). The AP activities were highest after 14 to 21 days incubation, then generally declined through 42 and 49 days incubation (Table IV).

Discussion. Several investigations have correlated increased lysosomal hydrolase activities with neoplasia. For example, in rat gliomas and neurinomas induced transplacentally with ENU, Allen *et al.* (3) noted marked increases in β-G, GAD, AP and AS activities compared to normal brain. Kordac, Braun and Schön (10) and Hoch-Ligeti, Lobl and Arvin (11) reported increases in serum and liver β-G activities preceding the onset of identifiable liver tumors in mice after administration of diethylnitrosoamine. Further, Allison (12) has observed the appearance of cytoplasmic hydrolase activity after exposure of cells to carcinogens. However, the results of the present *in vitro* experiments suggest that general increases in hydrolase activities,

TABLE I. *N*-Acetyl-β-D-glucosaminidase Activities in Primary Monolayer Cultures Derived from the Whole Brains of Newborn Rats Exposed Transplacentally or Unexposed to Ethylnitrosourea.

Culture sets		Days in culture					
		7	14	21	35	42	49
1	Treated[a]				12.26		
	Control				11.90		
2	Treated		13.25				
	Control		9.23				
3	Treated		13.31	23.37	38.08	36.98	
	Control		13.52	19.33	39.17	35.54	
4	Treated	6.76	10.06	16.17	20.57	21.87	23.04
	Control	5.71	10.42	17.00	24.46	25.04	28.06
Treated/control		1.18	1.32[b]	1.08[b]	0.95[b]	0.96[b]	0.82
Correlation		—0.42[c] (not significant)					

[a] All data recorded as micromoles of substrate hydrolyzed per hour per milligram of protein.
[b] Mean ratio.
[c] Kendall's coefficient of rank correlation.

least for those hydrolases studied, are not rectly induced by the carcinogen ENU. he data show that transplacental exposure ENU had no effect on hydrolase activities subsequent brain cell cultures through 49 ys of incubation. On the other hand, they ay suggest that transplacental exposure to NU induces neoplastic changes in only a few cells rather than most. Thus the detection, by chemical analysis, of increased hydrolase activities in relatively few affected cells could be masked by the large majority of unaffected cells. Further the induction of neoplastic changes by ENU may require some other factor, present in the *in vivo* environment, for manifestation. Finally, the

TABLE II. β-Glucuronidase Activities in Primary Monolayer Cultures Derived from the Whole Brains of Newborn Rats Exposed Transplacentally or Unexposed to Ethylnitrosourea.

Culture sets		Days in culture					
		7	14	21	35	42	49
1	Treated[a]				0.375		
	Control				0.362		
2	Treated		0.425				
	Control		0.585				
3	Treated		0.362	0.699	0.895	0.871	
	Control		0.432	0.521	0.758	0.658	
4	Treated	0.267	0.362	0.237	0.245	0.215	0.230
	Control	0.127	0.268	0.268	0.315	0.157	0.287
Treated/control		2.10	0.97[b]	1.11[b]	1.00[b]	1.35[b]	0.80
Correlation		—0.08[c] (not significant)					

[a] All data recorded as micromoles of substrate hydrolyzed per hour per milligram of protein.
[b] Mean ratio.
[c] Kendall's coefficient of rank correlation.

TABLE III. Arylsulfatase (A and B) Activities in Primary Monolayer Cultures Derived from the Whole Brains of Newborn Rats Exposed Transplacentally or Unexposed to Ethylnitrosourea.

Culture sets		Days in culture					
		7	14	21	35	42	49
1	Treated[a]				4.69		
	Control				4.90		
2	Treated		8.45				
	Control		10.60				
3	Treated		8.78	13.40	14.86	14.82	
	Control		9.28	9.46	12.30	11.06	
4	Treated	4.24	7.36	7.78	6.88	6.09	6.61
	Control	4.13	6.99	7.91	7.32	6.88	6.22
Treated/control		1.02	0.93[b]	1.19[b]	1.03[b]	1.10[b]	1.06
Correlation		—0.10[c] (not significant)					

[a] All data recorded as micromoles of substrate hydrolyzed per hour per milligram of protein.
[b] Mean ratio.
[c] Kendall's coefficient of rank correlation.

results of the present *in vitro* experiments may mean that increases in lysosomal hydrolase activities reported in induced gliomas (3) follow, rather than precede, neoplastic transformation.

The hydrolase activities in treated and control brain cell cultures were strikingly higher than the maximal activities observed in normal neonatal rat brain (13). However, this does not suggest that glial cells *in vivo* contain greater hydrolase activities relative to neuronal components. Rather, the high hydrolase activities in the brain cell cultures may reflect the reaction of these cells to the *in vitro* environment. This explanation is supported by data from similar studies reported

TABLE IV. Acid Phosphatase Activities in Primary Monolayer Cultures Derived from the Whole Brains of Newborn Rats Exposed Transplacentally or Unexposed to Ethylnitrosourea.

Culture sets		Days in culture					
		7	14	21	35	42	49
1	Treated[a]				6.11		
	Control				7.18		
2	Treated		5.79				
	Control		6.46				
3	Treated		7.52	8.17	5.88	7.19	
	Control		6.67	6.51	6.55	5.48	
4	Treated	2.71	4.36	4.06	2.99	3.27	3.29
	Control	3.73	4.71	4.28	3.25	3.60	4.03
Treated/control		0.72	0.98[b]	1.09[b]	0.89[b]	1.10[b]	0.81
Correlation		—0.02[c] (not significant)					

[a] All data recorded as micromoles of substrate hydrolyzed per hour per milligram of protein.
[b] Mean ratio.
[c] Kendall's coefficient of rank correlation.

by Cotman, Herschman and Taylor (14).

The possibility that the high hydrolase activities of the brain cell cultures reported here were due to the presence of macrophages is not consistent with morphological observations. The cell morphology was comparable to cells in similar cultures, characterized as predominantly glial cells, by light and electron microscopy (15, 4) and to the glial cell cultures described by Lumsden and Pomerat (16) and Wolfgram and Rose (9).

The increased hydrolase activities in the brain cell cultures reported here may reflect an increased rate of cell proliferation in this system. This suggestion is supported by a report (17) demonstrating linear increases in several glycosidases, especially GAD, AP and β-G, during the cell cycle in synchronized cultures of mouse lymphoma. The lowest activities occurred late in mitosis and during G_1, then increased linearly during the DNA synthesis phase to maximum activities during G_2 and early mitosis.

Summary. The effect of transplacental exposure of the fetal rat brain to the carcinogen ethylnitrosourea (ENU) on lysosomal acid hydrolase activities in glial cells has been reported. Primary monolayer cultures of brain cells derived from the brains of newborn rats exposed transplacentally to ENU and from unexposed newborn brains were used to provide concentrated samples of glial cells for hydrolase assay. The hydrolases assayed were β-glucuronidase, *N*-acetyl-β-D-glucosaminidase, arylsulfatase A and B and acid phosphatase and data were expressed as micromoles of substrate hydrolyzed per hour per milligram of protein. Experimental and control cultures were prepared, incubated and assayed contemporaneously and the ratios between treated and control hydrolase activities were used to evaluate the effect of transplacental ENU exposure.

Results showed that there was no effect on the activities of the lysosomal acid hydrolases measured in primary monolayer cultures of brain cells derived from newborn rat brains exposed transplacentally to ENU. Glucosaminidase activities increased through the 49 day incubation period, while maximum activities of β-glucuronidase, arylsulfatase and acid phosphatase were observed after 21 to 35 days incubation. Hydrolase activities in the brain cell cultures were markedly higher than activities previously reported in neonatal whole brain.

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