

Alcohol Inhibits Cell–Cell Adhesion Mediated by Human L1

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Abstract. Mental retardation, hydrocephalus, and agenesis of the corpus callosum are observed both in fetal alcohol syndrome (FAS) and in children with mutations in the gene for the cell adhesion molecule L1. We studied the effects of ethanol on cell–cell adhesion in mouse fibroblasts transfected with human L1. L1-transfected fibroblasts exhibited increased cell–cell adhesion compared with wild-type or vector-transfected controls. Ethanol potently and completely inhibited L1-mediated adhesion both in transfected L cells and NIH/3T3 cells. Half-maximal inhibition was observed at 7 mM ethanol, a concentration achieved in blood and brain after ingesting one alcoholic beverage. In contrast, ethanol did not inhibit the adhesion of fibroblasts transfected with vector alone or with N-CAM-140. L1-mediated

cell–cell adhesion was inhibited with increasing potency by n-propanol and n-butanol, but was not inhibited at all by n-alcohols of 5 to 8 carbons, acetaldehyde, or acetate, suggesting that ethanol interacts directly with a small hydrophobic pocket within L1. Phenylalanine, teratogenic anticonvulsants, and high concentrations of glucose did not inhibit L1-mediated cell–cell adhesion. Ethanol also inhibited potently the heterotypic adhesion of rat cerebellar granule cells to a monolayer of L1-transfected NIH/3T3 cells, but had no effect on their adhesion to N-CAM-140 or vector-transfected NIH/3T3 cells. Because L1 plays a role in both neural development and learning, ethanol inhibition of L1-mediated cell–cell interactions could contribute to FAS and ethanol-associated memory disorders.

FETAL alcohol syndrome (FAS)¹ is one of the most common recognizable syndromes associated with mental retardation in the Western world (1). The full syndrome, characterized by growth retardation, neurological abnormalities, and facial malformations, occurs in ~5% of the offspring of alcoholic women (1, 72). Neuropathological examination and neuroimaging of children with FAS have disclosed a spectrum of neurodevelopmental abnormalities, including hydrocephalus, agenesis of the corpus callosum, neuronal–glial heterotopias, cerebellar dysplasia, and microcephaly (8, 27, 62). Some of these lesions arise from disordered migration of neural cells, which has also been demonstrated experimentally in animal models of FAS (45, 46).

Mental retardation, hydrocephalus, cerebellar dysplasia, and agenesis of the corpus callosum also occur as part of an

X-linked syndrome associated with mutations in the gene encoding L1, an immunoglobulin cell adhesion molecule (IgCAM) (4, 20, 28, 31, 66, 74, 83). Three overlapping syndromes, X-linked hydrocephalus, MASA syndrome (mental retardation, aphasia, shuffling gait, and adducted thumbs), and X-linked complicated spastic paraplegia, were each mapped to the same region (Xq28) of the X chromosome (32, 76, 82). A large number of different missense mutations, short deletions, and defects of alternative splicing within the L1 gene have been identified in individuals with these X-linked disorders (83); similar mutations have never been encountered in large numbers of control subjects. A single point mutation in L1 can cause either X-linked hydrocephalus or MASA syndrome within the same family (20), confirming that these disorders are allelic variants.

L1 is expressed in postmitotic neurons of the central nervous system and in Schwann cells and axons of the peripheral nervous system (60). L1 is localized primarily along the course of axons, and particularly on growth cones at points of cellular contact (60, 68). Calcium-independent binding of L1 molecules in adjacent membranes (homophilic binding) promotes cell–cell adhesion and activates a tyrosine kinase signaling cascade involved in neurite outgrowth (36, 77, 79, 81). L1 also binds heterophili-

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1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; IgCAM, immunoglobulin cell adhesion molecule; FAS, fetal alcohol syndrome; FN3, fibronectin type III.

cally to axonin-1, TAG-1, F3/F11, and phosphacan (34, 71). L1 regulates axon targeting, axon fasciculation, cell migration, and synaptic plasticity (3, 36, 38, 39, 51, 71). Interactions of L1 with the fibroblast growth factor receptor (13, 77) and pp60^{c-src} (25) appear to play an important role in its morphogenetic actions.

Members of the IgCAM superfamily are characterized by extracellular domains comprising variable numbers of C2 Ig-like domains and fibronectin type III (FN3) homology domains (71). These are linked either to a glycosylphosphatidylinositol membrane anchor or to a transmembrane domain and cytoplasmic tail. L1 consists of six Ig domains, five FN3 repeats, a transmembrane domain, and an alternatively spliced cytoplasmic tail (22, 53). In contrast, the neural cell adhesion molecule, N-CAM, consists of five Ig and two FN3 domains anchored to a transmembrane and cytoplasmic region (N-CAM-140 and N-CAM-180) or to a GPI linkage (N-CAM-120). Using recombinant fragments and deletion constructs, the adhesive properties of mouse L1, the chick homologue Ng-CAM, and the *Drosophila* homologue neuroglian have been mapped to portions of the extracellular domain (2, 23, 24, 84).

The presence of various L1 mutations in children with brain dysmorphology and the failure to detect such mutations in unaffected individuals indicates that L1 is essential for normal human brain development (83). In view of the considerable overlap in the syndromes produced by L1 mutations and prenatal ethanol exposure, we asked whether ethanol disrupts cell-cell adhesion mediated by human L1. Here we show that ethanol potently and reversibly inhibits the aggregation of fibroblasts transfected with human L1 and the adhesion of cerebellar granule cells to a monolayer of L1-transfected fibroblasts. In contrast, ethanol has no effect on cell-cell interactions mediated by human N-CAM-140.

Materials and Methods

Cell Transfection

The vector pRc/RSV (Invitrogen, San Diego, CA) was modified by deletion of the fl origin of replication. Full-length human cDNAs for L1 (Dr. John Hemperly, Becton-Dickenson, Research Triangle Park, NC) or N-CAM-140 (Dr. Louis Lanier, DNAX, Palo Alto, CA) were ligated into the BstXI site of pRc/RSV downstream from the RSV promoter and transfected into mouse L cells or NIH/3T3 cells by the Lipofectamine method (Life Technologies, Frederick, MD). The cells were subcultured in 100-mm Petri dishes in medium containing 400 µg/ml of G418, and positive clones were selected by limiting dilution. Clones were screened for L1 expression by Northern blot, indirect immunofluorescence of live cells, and Western blot, as described (6, 56). For Western blots, the reaction products were visualized using the 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate kit (Vector Laboratories, Burlingame, CA). Clones were screened for N-CAM-140 expression using monoclonal antibody 5B8 (Developmental Studies Hybridoma Bank) in an ELISA, as described (57). N-CAM expression was verified by immunoblot using monoclonal antibody 5B8 and [¹²⁵I]-goat anti-mouse IgG (ICN Biomedicals, Costa Mesa, CA) as a secondary antibody (58).

Homotypic Cell Aggregation Assay

L cells or NIH/3T3 cells were incubated for 30 min at 37°C in calcium/magnesium-free PBS, pH 7.4 containing 1 mM EDTA and 0.1 mg/ml DNAase, detached by gentle agitation, washed, and mechanically dissociated to obtain a single-cell suspension in buffer supplemented with drugs as indicated. 1 ml of the same buffer containing 300,000 single cells was added to 4.5 cm² 12-well tissue culture plates and rotated at 60 rpm for 30 min

at room temperature. Calcium/magnesium-free buffer was employed to reduce the adhesion due to calcium-dependent CAMs. Evaporation of ethanol and other volatile compounds was prevented by wrapping the plates with parafilm. Neither the addition of alcohols nor the use of parafilm reduced cell viability, which remained at >90% throughout the assay. Three random fields, each containing ~200 cells, were viewed at 200 magnification and scored for the presence of single and adherent cells. Two cells were considered adherent if there was an increase in phase brightness at a point of cell-cell contact. The percentage of adherent cells was calculated for each field and averaged. The coefficient of covariance for this assay was less than 10%.

Homotypic and Heterotypic Fibroblast Cell Adhesion Assay

Vector-transfected (7a1a-V) or L1-transfected (7b1i-L1) NIH/3T3 cells were incubated at 37°C for 2 h in DMEM containing 3 µM of 1,1'-dioctadecyl-3,3,3'-tetramethyl-indocarbocyanine perchlorate (DiI), as described (49). Cells were harvested in calcium/magnesium-free PBS, as described above. A suspension of 2,000 single, labeled cells in 100 µl of DMEM was added to a 48-well plastic plate containing a monolayer of vector-transfected or L1-transfected NIH/3T3 cells. After adding an additional 100 µl of DMEM supplemented with or without ethanol, the plate was wrapped in parafilm, swirled three times, and incubated for 15 min at 37°C. Longer incubation did not produce greater adhesion of the target cells. The medium was removed with a Pasteur pipette and the monolayer was gently washed once with 200 µl of DMEM. Cocultures were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. DiI-labeled cells were counted in duplicate wells in six fields viewed at 100 magnification.

DiI Labeling of Cerebellar Granule Cells

Cerebellar cortex from P8 Sprague-Dawley rats was minced and dissociated by incubation with 0.125% trypsin for 20–30 min at 37°C. Trypsin was inactivated by adding DMEM supplemented with 10% FBS. A single-cell suspension was obtained by triturating the cells with a fire-polished Pasteur pipette. After centrifugation, dissociated cerebellar granule cells were plated onto Petri dishes and labeled with DiI, as described above. Labeled cells were washed and resuspended in DMEM. A single-cell suspension was obtained by trituration using a 10-ml serological pipette. At this developmental age, more than 90% of the labeled cells are cerebellar granule cells (21).

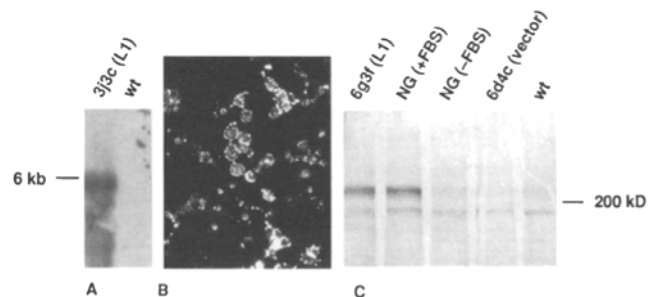


Figure 1. Expression of human L1 in mouse L cells. (A) Northern blot demonstrating L1 mRNA (appearing at ~6 kb) in 3j3c-L1 cells with no detectable message in wild-type cells (wt). (B) Indirect immunofluorescence of live 3j3c-L1 cells using a polyclonal rabbit antiserum against L1 (a gift from Dr. Vance Lemmon). No staining was detected in wild-type cells (not shown). (C) Western blot of 6g3f-L1, 6d4c-V, and wild-type cells using monoclonal antibody 74-5H7 (a gift from Dr. Vance Lemmon) (36, 56). NG108-15 cells (NG) cultured in medium supplemented with fetal bovine serum (FBS) were used as a positive control for L1 expression (57). A predominant band at ~220 kD was detected in 6g3f-L1 and FBS-treated NG108-15 cells, but was only faintly visible in wild-type or vector-transfected L cells or in NG108-15 cells cultured in serum-free medium. The second band at ~190 kD is a presumed proteolytic product.

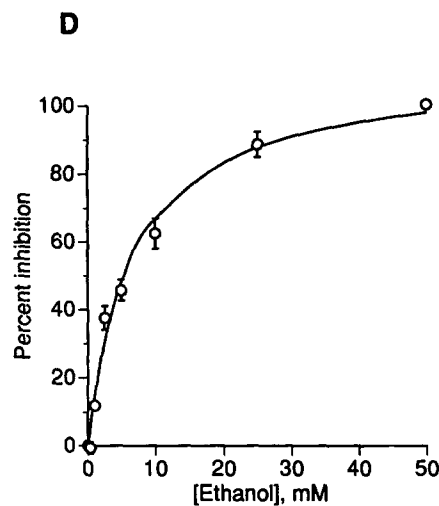
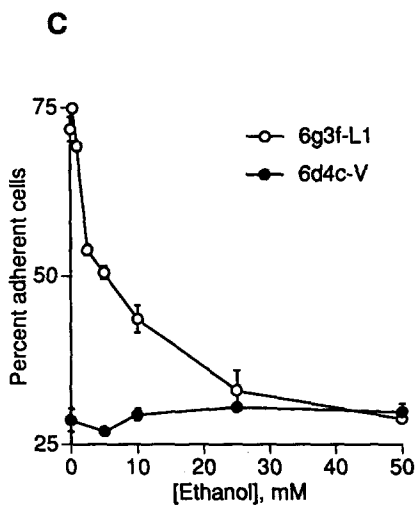
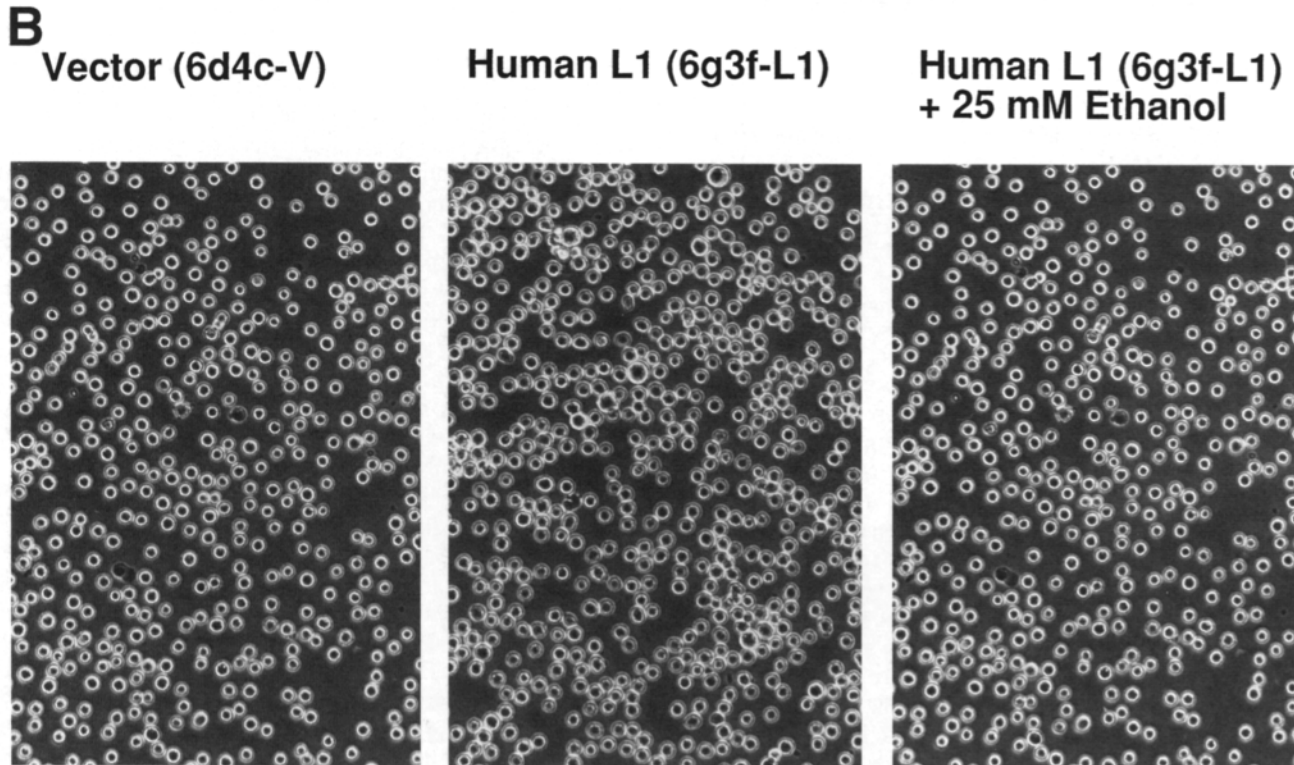
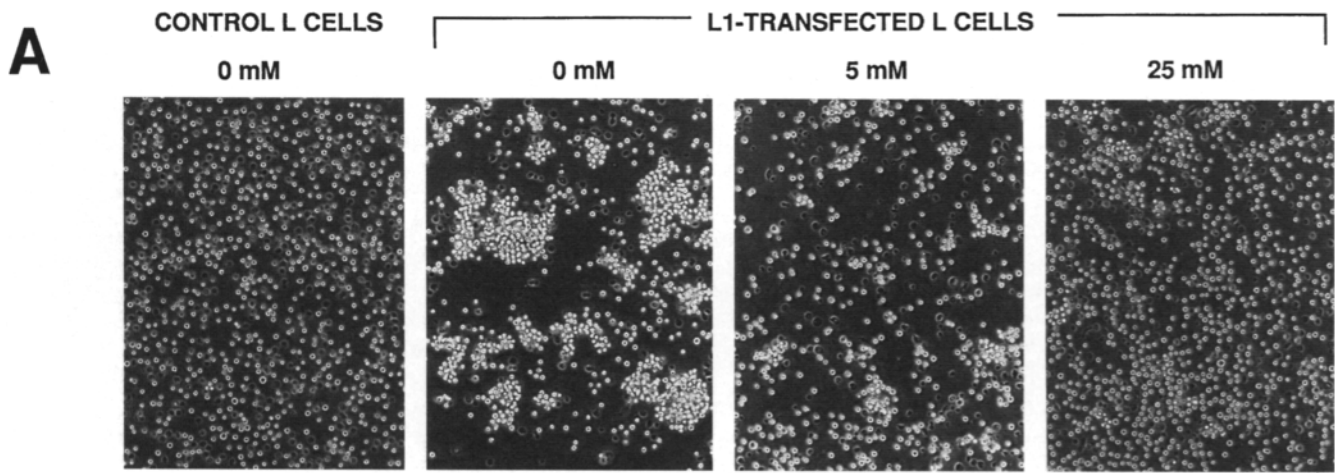


Figure 2. Ethanol inhibition of L1-mediated cell-cell adhesion. Short-term (30 min) aggregation assays were performed using suspensions of single cells. (A) Inhibition by 5 mM or 25 mM ethanol of cell-cell adhesion in L1-transfected mouse L cells (3j3c-L1). The photomicrograph shows suspended cells, which have settled onto the tissue culture plate, viewed under phase contrast microscopy. A minority of wild-type cells (control) were adherent, whereas a majority of 3j3c-L1 cells formed large aggregates. The size and number of these aggregates were visibly reduced when the aggregation assay was carried out in the presence of 5 or 25 mM ethanol. (B) Photomicrograph of a short-term aggregation assay using L cells transfected with vector

alone (6d4c-V) or human L1 (6g3f-L1). Aggregation of 6g3f-L1 cells was performed in the absence and presence of 25 mM ethanol. (C) Dose-dependent reduction by ethanol in the percentage of adherent 6g3f-L1 cells. Data shown are the mean \pm SEM from 4 to 13 independent experiments using 6g3f-L1 cells and 2-3 independent experiments using vector-transfected 6d4c-V cells. (D) Percent inhibition of L1-mediated cell-cell adhesion in 6g3f-L1 cells. L1-mediated cell-cell adhesion was defined as the percent adherent L1-transfected cells minus the percent adherent vector-transfected cells (2 B above). Because ethanol did not inhibit the adhesion of vector-transfected cells, ethanol inhibition was calculated as $100 - \frac{\text{ratio of L1-mediated adhesion in the presence or absence of ethanol}}$.

Adhesion of Cerebellar Granule Cells to Transfected NIH/3T3 Fibroblasts

Monolayers of NIH/3T3 cells were cultured in DMEM supplemented with 10% FBS on poly-L-lysine-coated 24-well tissue culture plates (Corning, Corning, NY). The medium was aspirated and 10,000 DII-labeled cerebellar granule cells were added to confluent monolayers of vector-transfected (7a1a-V), NCAM-140-transfected (7b1a-N-CAM-140), or L1-transfected (7b1i-L1) NIH/3T3 cells in a volume of 500 μ l of DMEM containing 0, 5, 10, 25, or 50 mM ethanol. Evaporation of ethanol was prevented by wrapping the plates with parafilm. Cocultures were incubated for 90 min at 37°C and the wells were washed twice gently with DMEM to remove non-adherent cells (50). The adherent cells were fixed with 500 μ l of 4% paraformaldehyde for 15 min at room temperature and stored at 4°C. Adherent DII-labeled cells were visualized by fluorescence microscopy and counted in 12 high power fields (200 \times) along the central portion of the horizontal and vertical meridians. The number of adherent cells in duplicate wells was averaged and varied by \sim 10%. IgCAM-mediated adhesion was defined as the difference in the adhesion of cerebellar granule cells to IgCAM-transfected and vector-transfected NIH/3T3 cells.

Results

Homotypic Adhesion of Mouse L Cells Transfected with Human L1 cDNA

Wild-type L cells expressed very low levels of mouse L1 (Fig. 1 C) and no N-CAM (see below), as reported by others (51). Mouse L cells (Amer. Type Culture Collection, Rockville, MD) were transfected with a modified pRc/RSV vector (Invitrogen) containing a full-length cDNA for human L1 (61), selected by growth in G418 medium, and subcloned from single colonies. Stable expression of high levels of human L1 was demonstrated by Northern blot, Western blot, and indirect immunofluorescence of live cells (Fig. 1). Cell-cell adhesion was characterized in two clones of L1-transfected cells (3j3c-L1 and 6g3f-L1) and one clone of vector-transfected cells (6d4c-V) using a 30-min aggregation assay (6). Clone 3j3c-L1 formed large cellular aggregates (Fig. 2 A) that were difficult to count. Clone 6g3f-L1 also expressed L1 mRNA (not shown) and protein (Fig. 1 C), but tended to form smaller aggregates of 2–10 cells (Fig. 2 B), which were more suitable for quantitative assessment of cell-cell adhesion.

Transfection of L cells with the pRc/RSV vector alone (clone 6d4c-V) did not increase cell-cell adhesion above levels observed in nontransfected, wild-type cells (wild-type, 33.6 \pm 2.9% adherent, n = 6; 6d4c-V, 28.7 \pm 1.7% adherent, n = 13). In contrast, L1-transfected cells exhibited significantly greater cell-cell adhesion (6g3f-L1, 71.8 \pm 1.8% adherent, n = 13) than vector-transfected or wild-type cells. Based on these findings, cell-cell adhesion mediated by human L1 was defined as the difference between the adhesion of L1-transfected (6g3f-L1) and vector-transfected cells (6d4c-V).

Ethanol Inhibition of L1-mediated Cell-Cell Adhesion in Mouse L Cells

To determine whether ethanol alters L1-mediated cell-cell adhesion, short-term aggregation assays (30 min) were performed in the absence or presence of 0.3–50 mM ethanol. Ethanol caused a dose-dependent inhibition of L1-mediated cell-cell adhesion (Fig. 2 A), but had no effect on the lower level of adhesion exhibited by control (not shown) and vector-transfected L cells (Fig. 2 C). Treat-

ment of clone 3j3c-L1 with 25 mM ethanol reduced cell-cell adhesion to levels observed in wild-type L cells (Fig. 2 A). These effects were quantitated using clone 6g3f-L1 (Fig. 2, C and D). Significant inhibition of cell-cell adhesion in 6g3f-L1 cells was evident at 1 mM ethanol (12.0 \pm 1.1%, t = 10.9, P < 0.01), and half-maximal inhibition was observed at 7 mM ethanol, a concentration achieved in the blood after ingesting one alcoholic beverage (42, 52). At a concentration of 50 mM, ethanol completely inhibited cell-cell adhesion mediated by human L1. The ethanol inhibition curve was a rectangular hyperbola (Fig. 2 D) with a pseudo Hill number of 1.2, suggesting a noncooperative interaction between ethanol and L1-transfected cells.

Cut-Off Effect for *n*-Alcohol Inhibition of L1-mediated Cell-Cell Adhesion

Ethanol is believed to disrupt membrane proteins either directly or secondarily by increasing membrane lipid disorder (11, 17, 19, 75). If ethanol inhibits L1-mediated cell-cell adhesion by disordering membrane lipids, then the potency of higher alcohols should increase as a linear function of chain length and lipid solubility. In contrast, if ethanol interacts with specific protein binding sites within L1, then a cutoff should be demonstrable above which higher chain-length alcohols no longer interact with L1, despite increasing membrane disorder (19, 37, 55). To explore these two possibilities, we performed aggregation assays of clone 6g3f-L1 in the absence or presence of *n*-alcohols of one to eight carbon atoms. We selected aqueous concentrations of *n*-alcohols that would produce the same membrane concentration and the same degree of membrane disorder as 25 mM ethanol (40, 43). At these concentrations, methanol, ethanol, *n*-propanol, and *n*-butanol reduced cell-cell adhesion in clone 6g3f-L1 to levels observed in nontransfected- and vector-transfected cells (Fig. 3). The potency for inhibition increased as a function of alcohol hydrophobicity, in that 7.5 mM propanol and 2 mM

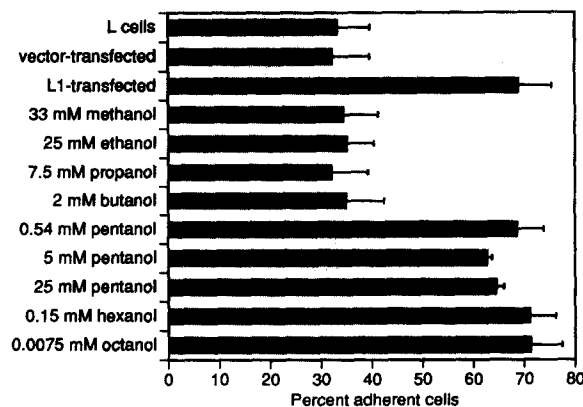


Figure 3. Inhibition of L1-mediated aggregation of L cells by a series of *n*-alcohols. Aggregation assays were performed using nontransfected (wild-type), vector-transfected (6d4c-V) and L1-transfected (6g3f-L1) mouse L cells. Parallel experiments were performed with 6g3f-L1 cells using aqueous concentrations of *n*-alcohols that were calculated to produce equal membrane concentrations (43). All bars labeled with an alcohol represent data from 6g3f-L1 cells. Data shown are the mean \pm SEM from 3 to 4 independent experiments. None of the alcohols reduced cell viability.

butanol were much more effective than the equivalent concentrations of ethanol. By contrast, n-pentanol, n-hexanol, and n-octanol were completely ineffective. A dose-response curve for pentanol did not disclose any inhibition of L1-mediated cell-cell adhesion at aqueous concentrations up to 25 mM. These data suggest that ethanol inhibits L1-mediated cell-cell adhesion by interacting with a hydrophobic site on the L1 molecule, the volume of which is between that of n-butanol and n-pentanol.

Reversibility of Ethanol's Inhibition of Adhesion

Ethanol inhibition of L1-mediated cell-cell adhesion was fully reversible. The percentage of adherent 6g3f-L1 cells was not reduced if cells were pretreated for 30 min with calcium/magnesium-free PBS containing 25 mM ethanol, washed, and resuspended in ethanol-free buffer before the aggregation assay (Fig. 4). Likewise, ethanol pretreatment did not alter its subsequent inhibition of L1-mediated adhesion. Hence, ethanol does not inhibit cell-cell adhesion through nonspecific and irreversible effects on L1, cellular integrity, or cell survival.

Effects of Ethanol Metabolites or Other Teratogens

Ethanol appears to inhibit L1-mediated cell-cell adhesion directly, rather than through the production of its primary metabolites (Table I). Neither acetaldehyde nor acetate inhibited 6g3f-L1 cell-cell adhesion at concentrations higher than those typically measured in blood after heavy alcohol ingestion (15, 26). Ethanol inhibition of cell-cell adhesion likewise does not arise through indirect osmolar actions because 2 mM butanol was as effective as 25 mM ethanol, and neither 25 mM pentanol nor 22 mM glucose had any effect. Prenatal exposure to anticonvulsants and high concentrations of phenylalanine and glucose have all been associated with central nervous system malformations (33, 41, 48); however, among these agents only alcohol inhibited L1-mediated cell-cell adhesion (Table I). These data

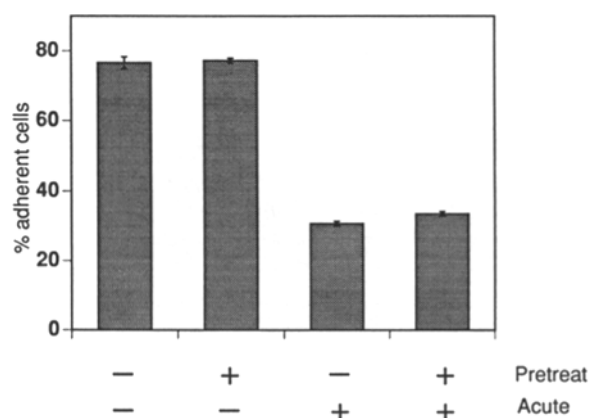


Figure 4. Ethanol's actions are reversible. Monolayers of L1-transfected (6g3f-L1) mouse L cells were incubated for 30 min at 37°C in the absence (pretreat -) and presence (pretreat +) of 25 mM ethanol, washed three times to remove the ethanol, and harvested for aggregation assays. Aggregation assays were performed in the absence (acute -) and presence (acute +) of 25 mM ethanol. Shown are the mean ± SEM percent adherent cells from three independent experiments.

Table I. Effect of Ethanol Metabolites and Teratogens on Cell-Cell Adhesion Mediated by Human L1

Cells	Treatment	Adherent % ± SEM	n
Nontransfected	none	34 ± 5	4
Vector-transfected	none	32 ± 5	4
L1-transfected	none	69 ± 5	4
L1-transfected	25 mM ethanol	35 ± 5	3
L1-transfected	50 μM acetaldehyde	64 ± 2	3
L1-transfected	7 mM acetate	65 ± 1	3
L1-transfected	2 mM phenylalanine	68 ± 2	3
L1-transfected	5.6 mM glucose	62 ± 3	3
L1-transfected	22.2 mM glucose	62 ± 1	3
L1-transfected	70 μM phenytoin	64 ± 1	3
L1-transfected	600 μM sodium valproate	70 ± 1	3
L1-transfected	42 μM carbamazepine	70 ± 1	3

Adhesion assays were performed in the absence or presence of the indicated drugs (59). The drug concentrations represent high therapeutic and teratogenic levels of anticonvulsants, teratogenic levels of phenylalanine in phenylketonuria, and levels of glucose observed in normal subjects (5.6 mM) and in diabetics with poor glucose control (22.2 mM).

suggest that the mechanism by which alcohol perturbs L1-mediated effects on brain development may be unique.

Effects of Ethanol on N-CAM-mediated Cell-Cell Adhesion

To learn whether ethanol sensitivity is a general attribute of IgCAMs, we tested the effects of ethanol on cell-cell adhesion in NIH/3T3 cells transfected with human N-CAM. We used NIH/3T3 cells rather than mouse L cells because they formed a more uniform monolayer for the heterotypic cell adhesion assays described below. NIH/3T3 cells were transfected with the pRc/RSV vector containing human L1 or human N-CAM-140. Cells were selected by Western blot analysis for stable expression of L1, N-CAM-140, or vector alone (Fig. 5 A). Wild-type NIH/3T3 cells expressed low levels of mouse N-CAM-140 and no L1. Cell aggregation was significantly increased in NIH/3T3 cells transfected with human L1 (7b1i-L1) or human N-CAM-140 (7b1a-N-CAM) as compared with cells transfected with vector alone (7b1i-V). Ethanol had no effect on the aggregation of vector-transfected or N-CAM-140-transfected NIH/3T3 cells (Fig. 5, B and C). In contrast, cell aggregation mediated by human L1 was inhibited almost completely by 25 mM ethanol. These data indicate that ethanol sensitivity is not a property of all IgCAMs.

Ethanol Inhibition of Homophilic Interactions Mediated by L1

Ethanol could inhibit L1-mediated cell-cell adhesion by blocking homophilic binding, heterophilic binding, or both. To examine these possibilities, we labeled vector or L1-transfected NIH/3T3 cells with the fluorescent lipophilic tracer DiI (vector or L1 probe cells) and studied their adhesion to monolayers of unlabeled vector- or L1-transfected NIH/3T3 cells (vector or L1 target cells). DiI-labeled probe cells were incubated with target cells for 15 min at 37°C. Non-adherent cells were removed by gentle washing, and the number of attached DiI-labeled probe cells was counted under fluorescence microscopy.

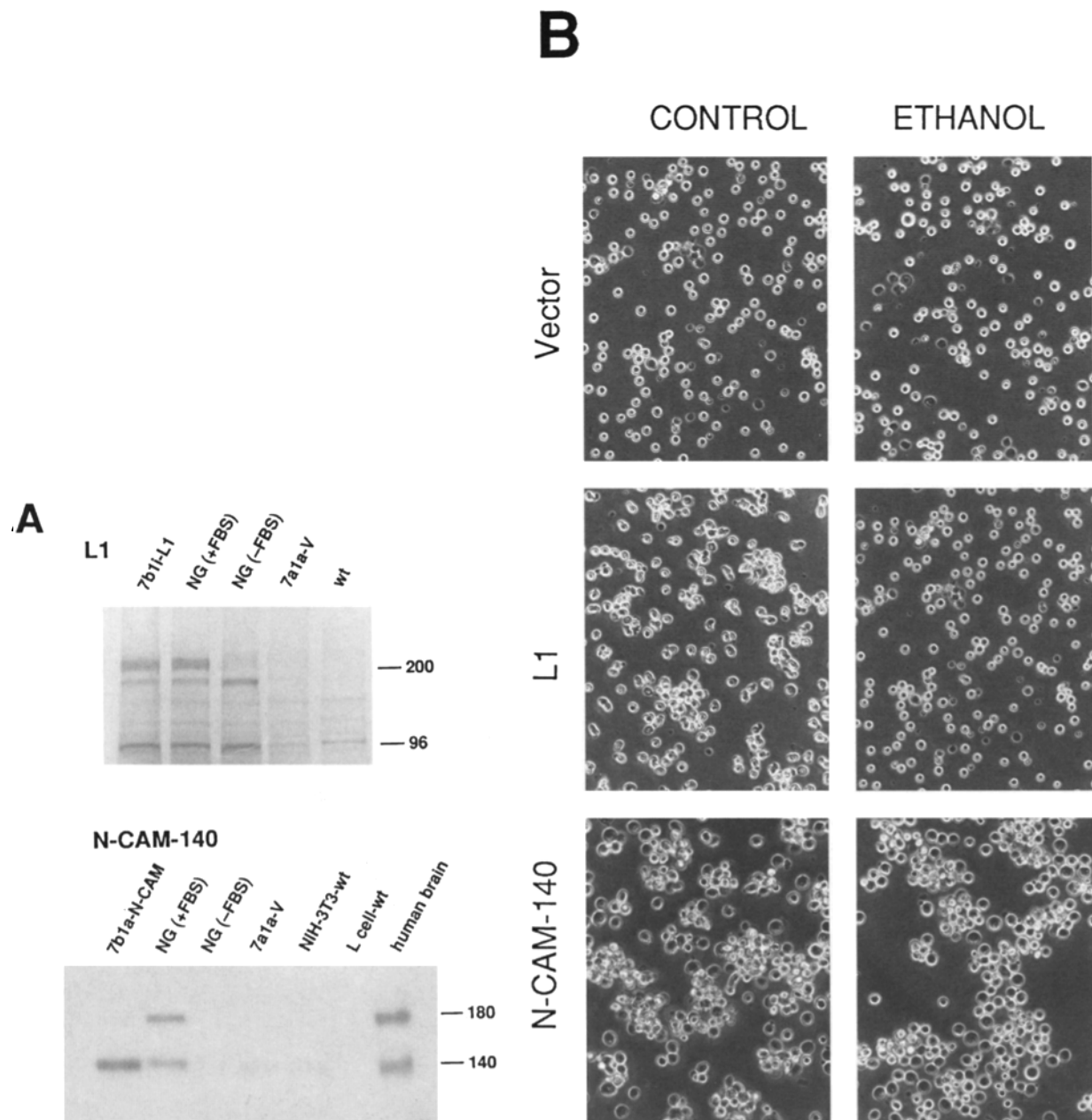
Low numbers of vector probe cells adhered reproducibly to monolayers of vector target cells (Fig. 6). An increase over this number in the adhesion of L1 probe cells to vector target cells or vector probe cells to L1 target cells provides an indication of the heterophilic binding of L1 to other molecules expressed by NIH/3T3 cells, such as $\beta 1$ integrins (16, 81). The adhesion of vector probe cells to L1 target cells was only slightly greater than that of vector probe cells to vector target cells. In contrast, the adhesion of L1 probe cells to L1 target cells was nearly twice that of L1 probe cells to vector target cells. Thus, most of the adhesion of L1 probe cells to L1 target cells can be attributed to homophilic interactions between L1 molecules on adjacent probe and target cells.

Ethanol had no effect on the adhesion of vector probe to vector target cells. Likewise, ethanol did not inhibit the adhesion of vector probe to L1 target cells or L1 probe to

vector target cells. In contrast, the adhesion of L1 probe cells to L1 target cells was reduced by ethanol to levels observed between vector-transfected and L1-transfected cells (Fig. 6). This finding suggests that ethanol inhibits the homophilic binding of L1. Ethanol does not appear to perturb the low level of heterophilic binding mediated by L1 in these cells. However, additional experiments are required to exclude an effect of ethanol on L1 heterophilic binding.

Ethanol Effects on Adhesion of Cerebellar Granule Cells to L1-transfected NIH3T3 Fibroblasts

Our data thus far indicate that ethanol selectively inhibits L1-mediated cell-cell adhesion in transfected fibroblasts. To learn whether ethanol inhibits L1- or N-CAM-140-mediated adhesion in neurons of the central nervous system, we



examined its effects in cerebellar granule cells, which express both IgCAMs (35, 60). In these experiments, we studied the heterotypic binding of DiI-labeled rat cerebellar granule cells to a monolayer of L1-transfected (7b1i-L1), N-CAM-transfected (7b1a-N-CAM-140), or vector-transfected (7a1a-V) NIH/3T3 cells. DiI-labeled cerebellar granule cells obtained from postnatal day 8 (P8) rats were allowed to adhere to fibroblast monolayers. Nonadherent cells were removed by gentle washing and the number of attached DiI-labeled cells was counted under fluorescence microscopy.

A significantly larger number of P8 cerebellar granule cells adhered to the L1 or N-CAM target cells than to vector target cells (Fig. 7). L1- and N-CAM-mediated adhesion were defined as the difference in the number of DiI-labeled cerebellar granule cells adherent to the IgCAM target cells and to the vector target cells. Ethanol caused a dose-dependent reduction in the adhesion of cerebellar granule cells to L1 target cells, but had no effect on their

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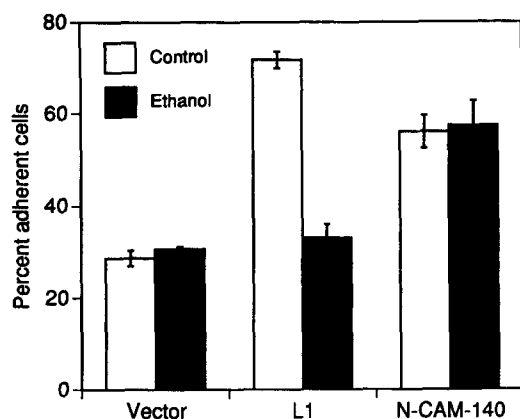


Figure 5. Ethanol inhibits L1- but not N-CAM-mediated cell-cell adhesion in NIH/3T3 cells. (A) Western blots of NIH/3T3 cells transfected with human L1 or human N-CAM-140. mAb 74-5H7 against L1 detected a doublet of ~220 and 190 kD in NIH-3T3 cells transfected with human L1 (7b1i-L1) and in NG108-15 cells (NG) cultured in defined medium supplemented with 10% fetal bovine serum (NG(+FBS)). Only faint bands were detected in NG108-15 cells cultured in serum-free medium (NG(-FBS)), and no bands were detected in vector-transfected (7a1a-V) and wild-type (wt) NIH/3T3 cells. mAb 5B8, directed against the cytoplasmic domains of N-CAM-140 and N-CAM-180, recognized bands of 180 and 140 kD in adult human cerebral cortex and in serum-treated NG108-15 cells, a strong band at 140 kD in N-CAM-140-transfected NIH/3T3 cells (7b1a-N-CAM-140), and weak bands of 140 kD in vector-transfected (7a1a-V) and wild-type (NIH-3T3-wt) NIH/3T3 cells. There was no detectable N-CAM in wild-type L cells. (B) Aggregation assays were performed in the absence and presence of 25 mM ethanol using 7a1a-V, 7b1i-L1, and 7b1a-N-CAM-140 NIH/3T3 cells. Ethanol reduced the aggregation of the 7b1i-L1 cells and changed cell shape from slightly polygonal to round. (C) Quantitative assessment of cell aggregation in vector-, L1-, and N-CAM-140-transfected NIH/3T3 cells. Assays were performed in the absence and presence of 25 mM ethanol. Shown are the mean \pm SEM percent adherent cells from four independent experiments. N-CAM-140-mediated adhesion was not inhibited even in the presence of 100 mM ethanol.

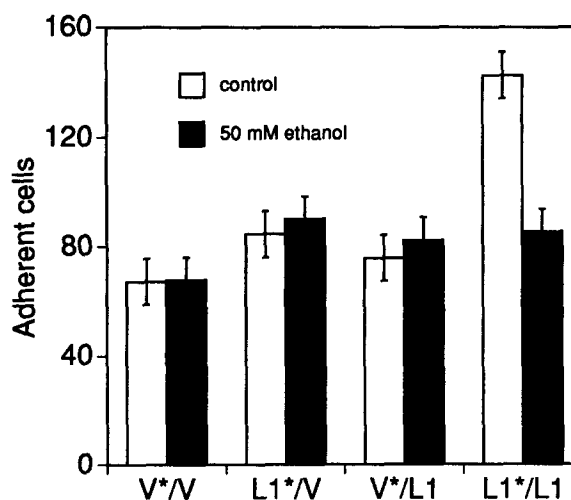


Figure 6. Ethanol inhibits homophilic L1 interactions in NIH/3T3 cells. DiI-labeled L1-transfected (L*) or vector-transfected (V*) NIH/3T3 cells (probe cells) were incubated for 15 min at 37°C with monolayers of L1-transfected (L1) or vector-transfected (V) NIH/3T3 cells (target cells) in the presence or absence of 50 mM ethanol. The number of labeled probe cells adherent to unlabeled target cell monolayers (e.g., V*/L1 represents the adhesion of labeled vector probe cells to a monolayer of unlabeled L1 target cells) was counted in six fields viewed at 100 magnification in duplicate wells. The graph depicts the average \pm SEM number of adherent probe cells per six fields in three independent experiments.

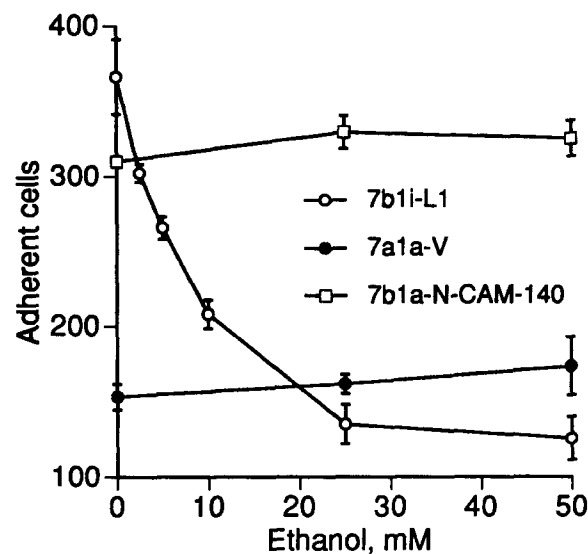


Figure 7. Ethanol inhibits L1-mediated adhesion of cerebellar granule cells. DiI-labeled P8 cerebellar granule cells were incubated for 90 min at 37°C with a monolayer of L1-transfected (7b1i-L1), N-CAM-140-transfected (7b1a-N-CAM-140) or vector-transfected (7a1a-V) NIH/3T3 cells in DMEM supplemented with the indicated concentrations of ethanol. The number of cerebellar granule cells adherent to the monolayer in 12 fields viewed at 200 magnification was counted in duplicate or triplicate wells. The graph shows the average \pm SEM number of adherent cells from four independent experiments.

adhesion to N-CAM or vector target cells. Ethanol's potency in inhibiting the heterotypic adhesion of cerebellar granule cells to L1-transfected NIH/3T3 cells was similar to that observed for inhibiting the homotypic aggregation of L1-transfected mouse L cells (Fig. 2). In both systems, clinically relevant concentrations of ethanol inhibited completely the adhesion attributable to the expression of human L1.

Discussion

The present study establishes that human L1 is a sensitive and specific target of ethanol. Ethanol inhibited L1-mediated cell-cell adhesion in two different assays of homotypic fibroblast cell adhesion and in an assay of heterotypic adhesion of cerebellar granule cells and transfected mouse fibroblasts. Thus, ethanol sensitivity appears to be a property of both rodent and human L1, whether expressed in mesenchymal cells, neural cell lines (6), or neurons. At least in fibroblasts, ethanol appears to inhibit homophilic interactions of L1.

The effects of ethanol on L1 occur at pharmacologically relevant concentrations. Ethanol significantly inhibited L1-mediated cell-cell adhesion at a concentration of 1 mM, which is less than the blood level obtained after ingesting one alcoholic beverage (7). The concentration of ethanol producing half-maximal inhibition, 7 mM, is achieved in both the maternal and fetal circulations after ingesting one or two alcoholic beverages (42, 52). Nearly complete inhibition was apparent at the legal limit of intoxication. Because ethanol equilibrates rapidly between blood and brain (7), these concentrations would also be present in the developing and mature nervous systems.

L1 is as sensitive to ethanol as any known membrane protein (11, 75). Understanding the structural requirements for ethanol sensitivity may provide important insights into how ethanol produces its diverse cellular actions. Propanol and butanol were more potent than ethanol in producing inhibition, suggesting that these alcohols interact with a hydrophobic site to disrupt L1. The fact that pentanol and higher alcohols had no effect on L1-mediated cell-cell adhesion implies that ethanol does not disrupt L1 by increasing membrane disorder, since pentanol is far more potent than ethanol in disordering cell membranes (40, 43). The mapping of adhesive domains to the extracellular portion of L1 raises the possibility that an alcohol binding pocket smaller than pentanol is located within the extracellular domain of L1. Experiments in progress involving systematic mutation of L1 should enable us to identify these ethanol-sensitive domains.

The possibility that alcohols and anesthetics interact directly with a transmembrane protein was first suggested from studies on purified firefly luciferase (18, 19). Different n-alcohol cut-offs have been reported recently for several ligand-gated ion channels, including the NMDA receptor (>6–8 carbon atoms) and the ATP-gated channel (>3 carbons) (37, 55). Evidence has also been presented for a direct effect of alcohols and anesthetics on lipid-free protein kinase C (70) and the Shaw2 potassium channel (9). To date, there is no direct evidence that ethanol interacts with the extracellular domain of any transmembrane

protein. Again, mutational analysis of L1 and experiments with purified recombinant fragments may provide a means for demonstrating such an interaction.

We showed previously that ethanol potently, but incompletely, inhibits cell-cell adhesion in NG108-15 neuroblastoma × glioma hybrid cells treated with osteogenic protein-1 (OP-1), a powerful inducer of the genes for N-CAM and L1 (6, 56). In these studies, it was not possible to demonstrate a specific effect of ethanol on L1, since OP-1-treated cells express high levels of both N-CAM and L1. The observation that ethanol inhibits adhesion mediated by L1, but not by N-CAM-140, explains why ethanol inhibition in NG108-15 cells was incomplete. Ethanol did not appear to inhibit cell-cell adhesion by promoting the degradation or endocytosis of L1 in OP-1-treated NG108-15 cells because even prolonged (3 d) exposure to 100 mM ethanol did not alter the induction, cell surface expression, or glycosylation of N-CAM and L1. In L1-transfected L cells, a 30-min pretreatment with ethanol followed by washing did not reduce L1-mediated cell aggregation, providing additional, indirect evidence that ethanol does not alter the synthesis, degradation, or endocytosis of L1.

The ethanol sensitivity of L1 is not a general property of IgCAMs. N-CAM-140 proved insensitive to ethanol, both in homotypic fibroblast aggregation assays and in the adhesion of cerebellar neurons to N-CAM-transfected fibroblasts. Ethanol likewise does not appear to inhibit L1-mediated adhesion by perturbing N-CAM's potentiation of L1 homophilic binding (29, 30). Although low levels of mouse N-CAM could potentially enhance interactions of human L1 in NIH/3T3 cells, this could not occur in mouse L cells, which lack N-CAM. Moreover, ethanol completely inhibited L1-mediated cell-cell adhesion both in NIH/3T3 cells and L cells. The selective ethanol sensitivity of L1 may reside within specific sequences of individual Ig and FN3 domains or may be dependent on the greater length of the L1 extracellular domain compared with that of N-CAM-140. It also remains possible that ethanol inhibits L1-mediated adhesion by disrupting the transmembrane or cytoplasmic domains of the molecule.

Because mutations in the gene for L1 produce mental retardation and a spectrum of dysmorphic brain lesions, these data suggest that FAS may arise partly through ethanol inhibition of L1-mediated cell-cell interactions. It is unknown whether ethanol also inhibits L1-mediated neurite outgrowth or neuronal migration. In general, ethanol has variable effects on neurite outgrowth, causing inhibition in nerve growth factor (NGF)-treated chick dorsal root ganglion cells (14) and potentiation in cerebellar granule cells (85) and NGF- or FGF-treated PC12 cells (44). In contrast to cell adhesion, neurite outgrowth involves a large number of sequential downstream signaling events (12, 78, 80, 81) that are differentially susceptible to ethanol perturbation. For example, ethanol potentiates receptor-stimulated adenylyl cyclase activity, inhibits voltage-dependent calcium channels, and enhances neurite outgrowth by potentiating growth factor-stimulated MAP kinase activity (5, 63). Therefore, the combined effects of ethanol on L1-mediated adhesion and signaling may prove to be counterbalancing or synergistic.

The strikingly high sensitivity of L1 to ethanol was unexpected because the full FAS occurs only in the offspring of

mothers who drink heavily during pregnancy (1, 72). It may be that neural development can proceed relatively normally if L1-mediated adhesion is inhibited only briefly, as occurs following the ingestion of one or two drinks, but is disrupted when L1-mediated adhesion is inhibited for the many hours required to metabolize high blood ethanol concentrations. Recent epidemiological studies suggest that even low levels of prenatal ethanol exposure produce dose-dependent alterations in growth, head circumference, facial morphology, cognitive function, and behavior (10, 73). The present studies indicate a possible mechanism underlying the teratogenicity of low concentrations of ethanol.

Differences in the clinical syndromes produced by prenatal exposure to ethanol and mutations in the gene for L1 should be expected. There is considerable phenotypic diversity in the X-linked syndromes of mental retardation, even among family members bearing the same L1 gene mutation (83). Moreover, ethanol inhibition of L1 is reversible, whereas mutational disruption of L1 is permanent. Finally, ethanol likely produces FAS through a combination of different cellular actions that together perturb the generation, proliferation, survival, migration, and signaling of neural cells (45, 46, 63, 64, 67). Only a small proportion of fetuses exposed to ethanol exhibit the severe developmental abnormalities of FAS. Conceivably, these individuals harbor mutations in L1 or related IgCAMs that render brain development more susceptible to ethanol.

Effects of ethanol on L1 may also have consequences in adults. L1 expression persists in postmitotic cells of the mature nervous system, where it is believed to play a role in neuronal plasticity (39). L1 is expressed in the adult hippocampus (47), a critical structure for learning and memory. Both ethanol and antibodies against L1 prevent the development of long-term potentiation and learning (39, 54, 65, 69). Ethanol inhibition of L1-mediated cell-cell adhesion may therefore contribute to alcoholic blackouts, an amnesic syndrome associated with acute intoxication (7), and may be involved in the pathogenesis of chronic cognitive disorders in alcoholics.

We are grateful to Dr. Vance Lemmon for gifts of polyclonal antisera and hybridoma cells, Dr. John Hemperly for the human L1 cDNA, and Dr. Louis Lanier for the human N-CAM-140 cDNA. Presented in part at the 21st annual meeting of the Society for Neuroscience, San Diego, CA, 1995.

This work is supported in part by USPHS AA09669 (M.E. Charness) and the Medical Research Service, Department of Veterans Affairs (M.E. Charness).

Received for publication 2 January 1996 and in revised form 20 February 1996.

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