Klatzman *et al.*, *ibid.*, p. 767; P. J. Maddon *et al.*, *Cell* **47**, 333 (1986).
4. J. S. McDougal *et al.*, *Science* **231**, 382 (1986).
5. B. E. Bierer, B. P. Sleckman, S. E. Ratnofsky, S. J. Burakoff, *Annu. Rev. Immunol.* **7**, 579 (1989).
6. L. A. Lasky *et al.*, *Cell* **50**, 975 (1987).
7. A. Cordonnier, L. Montagnier, M. Emerman, *Nature* **340**, 571 (1989).
8. J. Arthos *et al.*, *Cell* **57**, 469 (1989); B. H. Chao *et al.*, *J. Biol. Chem.* **264**, 5812 (1989).
9. N. R. Landau, M. Warton, D. R. Littman, *Nature* **334**, 159 (1988); L. K. Clayton *et al.*, *ibid.* **335**, 363 (1988).
10. L. K. Clayton, M. Sieh, D. A. Pious, E. L. Reinherz, *ibid.* **339**, 548 (1989).
11. M. R. Bowman, K. D. MacFerrin, S. L. Schreiber, S. J. Burakoff, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
12. D. Lamarre *et al.*, *Science* **245**, 743 (1989).
13. D. C. Diamond *et al.*, *J. Immunol.* **141**, 3715 (1988).
14. R. J. Gurley, K. Ikeuchi, R. A. Byrn, K. Anderson, J. E. Groopman, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1993 (1989); K. J. Weinhold *et al.*, *J. Immunol.* **142**, 3091 (1989).
15. D. H. Smith *et al.*, *Science* **238**, 1704 (1987); R. A. Fisher *et al.*, *Nature* **331**, 76 (1987); K. C. Deen *et al.*, *ibid.*, p. 82; A. Traunecker, W. Luke, K. Karjalainen, *ibid.*, p. 84.
16. R. E. Hussey *et al.*, *Nature* **331**, 78 (1987).
17. D. J. Capon *et al.*, *ibid.* **337**, 525 (1989); A. Traunecker, J. Schneider, H. Kiefer, K. Karjalainen, *ibid.* **339**, 68 (1989).
18. D. C. Diamond, R. Finberg, S. Chaudhuri, B. P. Sleckman, S. J. Burakoff, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
19. D. C. Diamond *et al.*, unpublished observations.
20. Q. J. Sattentau, A. G. Dalgleish, R. A. Weiss, P. C. L. Beverley, *Science* **234**, 1120 (1986).
21. D. Schols, M. Baba, R. Pauwels, J. Desmyter, E. de Clercq, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3322 (1989).
22. R. W. Finberg *et al.*, unpublished observations.
23. B. A. Larder, G. Darby, D. D. Richman, *Science* **243**, 1731 (1989); B. A. Larder and S. D. Kemp, *ibid.* **246**, 1155 (1989).
24. B. A. Jameson *et al.*, *ibid.* **240**, 1335 (1988).
25. J. D. Lifson *et al.*, *ibid.* **241**, 712 (1988); P. L. Nara, K. M. Hwang, D. M. Rausch, J. D. Lifson, L. E. Eiden, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7139 (1989).
26. M. Baba *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6132 (1988); H. Mitsuya *et al.*, *Science* **240**, 646 (1988).
27. F. A. Miller *et al.*, *Appl. Microbiol.* **16**, 1489 (1968).
28. S. K. Burley and G. A. Petsko, *Science* **229**, 23 (1985).
29. E. Nicolaides *et al.*, *J. Med. Chem.* **11**, 74 (1968).
30. C. D. Richardson, A. Scheid, P. W. Choppin, *Virology* **105**, 205 (1980); C. D. Richardson and P. W. Choppin, *ibid.* **131**, 518 (1983); K. Asano and A. Asano, *Biochemistry* **27**, 1321 (1988).
31. G. Ginther-Luce *et al.*, *Biotechniques* **3**, 270 (1985).
32. A. Haregerwoin *et al.*, *Nature* **340**, 309 (1989).
33. Support of this research was provided by grants from the NCI (R.W.F.), the U.S. Army (R.W.F.), the NIGMS (S.L.S.) and NIAID (S.J.B.). An NIH Predoctoral Fellowship (D.B.M.) is gratefully acknowledged. We thank J. Bergelson for performing the picornarvirus plaque assays, S. Goldman, R. Moreland, and R. F. Standaert for their helpful discussions, and J. Fingeroth for critical review of the manuscript.

1 May 1990; accepted 19 June 1990

Genetic Differences in the Ethanol Sensitivity of $GABA_A$ Receptors Expressed in *Xenopus* Oocytes

K. A. WAFFORD, D. M. BURNETT, T. V. DUNWIDDIE, R. A. HARRIS

Animal lines selected for differences in drug sensitivity can be used to help determine the molecular basis of drug action. Long-sleep (LS) and short-sleep (SS) mice differ markedly in their genetic sensitivity to ethanol. To investigate the molecular basis for this difference, mRNA from brains of LS and SS mice was expressed in *Xenopus* oocytes and the ethanol sensitivity of gamma-aminobutyric acid A ($GABA_A$)– and *N*-methyl D-aspartate (NMDA)–activated ion channels was tested. Ethanol facilitated GABA responses in oocytes injected with mRNA from LS mice but antagonized responses in oocytes injected with mRNA from SS animals. Ethanol inhibited NMDA responses equally in the two lines. Thus, genes coding for the $GABA_A$ receptor or associated proteins may be critical determinants of individual differences in ethanol sensitivity.

INDIVIDUAL HUMANS AND RODENTS differ markedly in their behavioral sensitivity to ethanol (*1*). Behavioral sensitivity has also been used as a marker or index for selection of a number of different rodent lines (*2, 3*). The LS and SS mouse lines (*2*) were selected for differential sensitivity to the acute hypnotic effect of ethanol determined by the duration of loss of the righting reflex ("sleep time") after injection of ethanol. The lines differ markedly in this respect. Behavioral and biochemical studies on LS and SS mice indicate that they have similar ethanol metabolism and pharmacokinetics. However, the two lines differ in the response of neuronal GABA receptors to ethanol (*4*) as well as in the sensitivity of the GABA receptor to benzodiazepines (*5*), suggesting that the genetic differences seen in response to ethanol may be the result of modifications of the $GABA_A$ receptor–chloride channel complex. This complex is composed of multiple subunits, many of which have been cloned, sequenced, and expressed in *Xenopus* oocytes or transfected cells (*6, 7*). Thus, there are many subunits, and probably different subtypes, of $GABA_A$ receptors that could underlie genetic differences in drug action.

The similarity between the actions of ethanol and sedative drugs such as benzodiazepines and barbiturates that enhance GABA action, as well as behavioral studies with GABAergic drugs, provides evidence that ethanol exerts at least some of its effects by enhancing the function of the GABA receptor (*8*). More directly, biochemical techniques with radioactive Cl^- show a potentiating effect of intoxicating concentrations of ethanol on functional GABA-activated Cl^- channels in brain membranes and in cultured spinal cord neurons (*4, 9*); electrophysiological studies are less consistent with regard to ethanol–GABA receptor interactions (*10*). $GABA_A$ receptor subtypes or different membrane environments may account for these diverse results.

Our approach to defining differences in the $GABA_A$ receptor–Cl^- channel complex that might underlie the genetic differences in ethanol susceptibility was to prepare mRNA from LS and SS mouse whole brain and express this mRNA in *Xenopus* oocytes (*7, 11*). Receptor function was studied electrophysiologically with voltage-clamp techniques (*11*). We observed inward currents in response to application of GABA when the oocyte membrane was voltage-clamped between −60 and −100 mV, which is consistent with the activation of a Cl^- conductance (the reversal potential for Cl^- is approximately −20 mV in the oocytes) (Fig. 1). Maximum currents observed with 100 μM GABA were 147 ± 30 nA and 163 ± 52 nA (mean ± SEM, n = 30 for each group) for LS and SS GABA receptors, respectively. GABA was used at 10 or 30 μM (20 to 50% of the maximum response) in combination with modulatory drugs. These submaximal concentrations also produced currents that were not statistically different between oocytes injected with LS or SS mRNA (n= 7 to 15). The receptors that were expressed showed pharmacological responses typical of native $GABA_A$ receptors; they were facilitated by pentobarbital and diazepam and inhibited by picrotoxinin (Fig. 1A). Exposure of oocytes to ethanol alone at concentrations less than 100 mM did not elicit any current response.

Application of 10 to 50 mM ethanol together with GABA to oocytes expressing

Department of Pharmacology, University of Colorado Health Sciences Center, and Veterans Administration Medical Center, Denver, CO 80262.

mRNA from LS brain resulted in a dose-dependent potentiating effect of ethanol on the GABA current (Fig. 1, A and B). Concentrations of ethanol above 60 mM inhibited the GABA-induced current, possibly via the release of intracellular Ca^{2+} [which we have observed at ethanol concentrations of 100 mM or greater (*12*)]. However, when ethanol was applied together with GABA at low concentrations in oocytes expressing SS mRNA, a dose-dependent inhibition of the GABA-induced current was observed (Fig. 1, A and B). Thus, ethanol exerted opposing effects on GABA responses in oocytes expressing mRNA from LS or SS mice. In contrast, pentobarbital or diazepam potentiation of GABA responses was not significantly different for the two lines (Fig. 1A). These actions of pentobarbital are consistent with the similar sensitivity of the two lines of mice to pentobarbital anesthesia (*13*), but the lines have been shown to differ in some actions of benzodiazepines (*5*), and the failure of diazepam to discriminate between LS and SS channels was unexpected. Possible differences in benzodiazepine receptor subtypes have not been studied with these mice.

To determine if the differences in susceptibility to ethanol found with the GABA-activated Cl^- channel were also seen with another channel, we tested the effects of ethanol on NMDA responses in oocytes expressing mRNA of LS and SS mice. NMDA receptors are inhibited by low (10 to 50 mM) concentrations of ethanol (*14*).

Fig. 1. (**A**) Examples of expression of mRNA from LS or SS mouse brain in defolliculated oocytes (*11*). GABA (30 μM), ethanol (EtOH) (20 mM), diazepam (DZ) (0.01 or 0.1 μM), picrotoxinin (Picro) (10 μM), and pentobarbital (Pento) (100 μM) applications are indicated by bars above the traces. The horizontal line illustrates the response to GABA alone (control response); note the scale difference for pentobarbital responses. Currents were recorded from single oocytes injected with LS or SS mRNA; at least five oocytes from LS and SS groups were tested with each drug, and responses similar to those shown here were observed in all experiments. Oocytes were voltage-clamped at resting potential (range, −60 to −100 mV), and drugs were applied by perfusion for 1 min. No drug effects were seen in uninjected oocytes. Pentobarbital evoked small responses (10 to 20 nA) when applied alone. (**B**) Changes elicited by increasing ethanol concentrations on GABA-induced Cl^- currents evoked by 10 μM GABA in oocytes injected with mouse brain mRNA from LS brain (circles) and oocytes injected with mRNA from SS brain (squares). Each point represents the mean ± SEM from at least six separate oocytes. Oocytes were continuously perfused with MBS, and GABA and ethanol were applied either separately or together for 1 min before the oocytes were returned to MBS (*12*). At least 5 min were allowed between each pair of applications to prevent desensitization.

We observed responses to NMDA in oocytes also expressing GABA receptors and found that ethanol inhibited NMDA-induced currents in a dose-dependent manner at concentrations up to 75 mM with 50% maximum inhibition (IC_{50}) occurring at approximately 25 mM. The maximum inhibition observed was approximately 50%, and there was no statistical difference in the efficacy of ethanol inhibition between NMDA receptors from LS and SS mouse brain. The NMDA response was inhibited by 22 ± 3% and 15 ± 4% at 20 mM ethanol and by 36 ± 5% and 40 ± 8% at 50 mM ethanol in oocytes expressing RNA from LS and SS mice, respectively (mean ± SEM, $n = 3$).

Ro15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5α][1,4]benzodiazepine-3-carboxylate) is a benzodiazepine inverse agonist that antagonizes some of the behavioral, biochemical, and electrophysiological actions of ethanol (*15*). In order to directly assess the effects of this compound on ethanol–GABA receptor interactions, we applied Ro15-4513 together with GABA and together with GABA and ethanol. At a concentration of 1 μM, this compound had no effects by itself and no direct antagonistic effects on GABA currents alone, but it inhibited the potentiating effect of ethanol on LS GABA receptors (Fig. 2). Ro15-4513 had no significant effect on the inhibition of SS GABA receptors by ethanol.

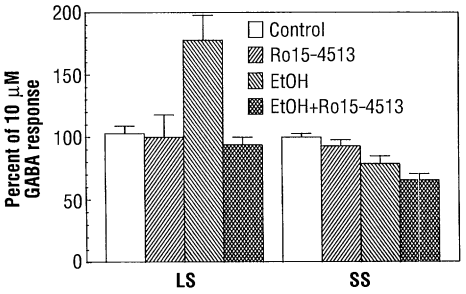

Fig. 2. Effect of Ro15-4513 on Cl^- currents elicited by 10 μM GABA in *Xenopus* oocytes and on GABA currents in the presence of 20 mM ethanol in oocytes injected with mRNA from LS or SS mice. Ro15-4513 (1 μM) was applied by perfusion together with GABA or GABA plus ethanol for 1 min as in Fig. 1. Each bar represents the mean ± SEM for five different oocytes.

Our results suggest that the genetic differences in ethanol sensitivity between SS and LS mice could be related at the cellular level to marked, but selective, changes in the function of GABA-activated Cl^- channels. The fact that ethanol has opposite effects on GABA responses in the two lines without changes in GABA or pentobarbital sensitivity points to identification and comparison of the GABA receptor subunits that are expressed in LS and SS mice as the next step in elucidating the site of action of ethanol at a molecular level. This site could be a subunit of the GABA receptor–Cl^- channel complex or a related protein that modulates channel function. The LS and SS lines do not differ in the nucleotide sequence of the α1 subunit or the brain regional levels of mRNA for the α1 or γ2 subunit (*16*), but differences in other subunits have not been evaluated. How these differences in GABA receptor function translate into altered patterns of neural activity in the intact animal remains to be determined. Nevertheless, given the important role of these receptors in regulating the activity of most brain regions, our results suggest that the differential effect of ethanol on GABA receptors will influence networks in many parts of the nervous system. In contrast to the GABA response, genetic selection did not alter the ethanol sensitivity of the NMDA receptor. This result suggests that actions of ethanol at the NMDA receptor are not important for the particular action of ethanol that was the

basis for the genetic selection (duration of the loss of the righting reflex).

Our results indicate that a gene expression system can be used to define genetic differences in brain function. Expression studies in coordination with pharmacological genetic selection offer a promising strategy for the study of drug-receptor interactions.

REFERENCES AND NOTES

1. J. R. Wilson and R. Plomin, *Soc. Biol.* **32**, 162 (1986).
2. G. E. McClearn and R. Kakihana, *Behav. Genet.* **3**, 409 (1973).
3. E. J. Gallaher, L. E. Hollister, S. E. Gionet, J. C. Crabbe, *Psychopharmacology* **93**, 25 (1987); J. K. Belknap, N. R. Halti, D. M. Goebel, M. Lame, *Behav. Genet.* **13**, 383 (1983).
4. A. M. Allan and R. A. Harris, *Life Sci.* **39**, 2005 (1986).
5. R. A. Harris and A. M. Allan, *Brain Res.* **490**, 26 (1989); *FASEB J.* **3**, 1689 (1989).
6. P. R. Schofield *et al.*, *Nature* **328**, 221 (1987); E. S. Levitan *et al.*, *ibid.* **335**, 76 (1988); D. B. Pritchett *et al.*, *ibid.* **338**, 582 (1989).
7. K. M. Houamed *et al.*, *ibid.* **310**, 318 (1984).
8. P. D. Suzdak, S. M. Paul, J. N. Crawley, *J. Pharmacol. Exp. Ther.* **245**, 880 (1988); A. M. Allan and R. A. Harris, in *Recent Developments in Alcoholism*, M. Galanter, Ed. (Plenum, New York, 1987), vol. 5, pp. 313–325.
9. A. M. Allan and R. A. Harris, *Pharmacol. Biochem. Behav.* **27**, 665 (1987); S. J. McQuilkin and R. A. Harris, *Life Sci.* **46**, 527 (1990); P. D. Suzdak, R. D. Schwartz, S. M. Paul, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4071 (1986); A. K. Mehta and M. K. Ticku, *J. Pharmacol. Exp. Ther.* **246**, 558 (1988).
10. D. P. Harris and J. G. Sinclair, *Gen. Pharmacol.* **15**, 449 (1984); J. R. Mancillas, G. R. Siggins, F. E. Bloom, *Science* **231**, 161 (1986); J. N. Nestoros, *ibid.* **209**, 708 (1980); J. J. Celentano, T. T. Gibbs, D. H. Farb, *Brain Res.* **455**, 337 (1989); R. Takada *et al.*, *Alcohol* **6**, 115 (1989); P. W. Gage and B. Robertson, *Br. J. Pharmacol.* **85**, 675 (1985).
11. LS and SS mice were obtained from the Institute for Behavioral Genetics (Boulder, CO). Polyadenylated RNA was prepared from whole brain pooled from four mice with a Fast Track isolation kit (Invitrogen, San Diego, CA). Polyadenylated RNA was isolated by oligo(dT) binding, further purified by phenol/chloroform extraction, and stored at −20°C. A total of six different mRNA preparations were made from LS and SS mice, with brains pooled from four randomly selected individuals for each preparation. Similar results were obtained with all preparations from each line of mice. Oocytes were prepared from adult female *Xenopus laevis* (Xenopus I, Ann Arbor, MI) as described (*7*, *12*). The follicular cell layer was removed by a 10-min treatment in Sigma type 1A collagenase (0.5 mg/ml) dissolved in modified Barth's saline (MBS). Oocytes were injected with 50 nl of a solution of mRNA (2 to 4 mg/ml) with a 10-μl micropipette (Drummond Scientific, Broomall, PA) that had an internal diameter of 20 μm. Oocytes were then incubated for 2 to 3 days in MBS supplemented with 2 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 mg/ml), gentamycin (50 mg/ml), and 0.5 mM theophylline. For electrophysiological recording, oocytes were placed on filter paper in a 200-μl bath and continually perfused with MBS, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, pH 7.3 to 7.5, at a rate of 1.7 ml/min. Cells were impaled with two microelectrodes (0.5 to 2 megohms) containing 3 M KCl and voltage-clamped with an Axoclamp IIA amplifier. We applied the perfused drugs by changing the saline solution to saline containing appropriate concentrations of drug. Wash-through time was ~5 s for total changeover of solution. We calculated drug concentrations by assuming complete equilibration; measured concentrations of ethanol at the time of peak response were about 40% less than the indicated concentrations.
12. K. A. Wafford, T. V. Dunwiddie, R. A. Harris, *Brain Res.* **505**, 215 (1989).
13. T. C. Howerton, M. F. O'Connor, A. C. Collins, *J. Pharmacol. Exp. Ther.* **227**, 389 (1983).
14. D. M. Lovinger, G. White, F. F. Weight, *Science* **243**, 1721 (1989); P. L. Hoffman, C. S. Rabe, F. Moses, B. J. Tabakoff, *J. Neurochem.* **52**, 1937 (1989). NMDA responses were studied in the absence of Mg^{2+} and in the presence of 3 μM glycine. A 100-μM concentration of NMDA was used to study ethanol action, and a 300-μM concentration was used to assess the maximal effect of NMDA.
15. M. K. Ticku and S. K. Kulkarni, *Pharmacol. Biochem. Behav.* **30**, 501 (1988); M. R. Palmer, C. van Horne, J. T. Harlan, R. A. Moore, *J. Pharmacol. Exp. Ther.* **247**, 1018 (1988); P. D. Suzdak *et al.*, *Science* **234**, 1243 (1986); R. A. Harris, A. M. Allan, L. C. Daniell, C. Nixon, *J. Pharmacol. Exp. Ther.* **247**, 1012 (1988).
16. W. J. Keir, R. A. Deitrich, J. M. Sikela, *Ann. N.Y. Acad. Sci.*, in press; N. R. Zahniser *et al.*, *Soc. Neurosci. Abstr.*, in press.

17. We thank J. Sikela for helpful discussions and assistance with mRNA techniques. Supported by the Veterans Administration and the National Institute of Alcoholism and Alcohol Abuse (AA06399 and AA03527).

27 December 1989; accepted 18 May 1990

MHC-Linked Protection from Diabetes Dissociated from Clonal Deletion of T Cells

JAN BÖHME, BRIGITTE SCHUHBAUR, OSAMI KANAGAWA, CHRISTOPHE BENOIST, DIANE MATHIS

The I-E molecule of the major histocompatibility complex (MHC) can prevent the spontaneous development of diabetes in nonobese diabetic (NOD) mice. The mechanism of this protection has been investigated by breeding wild-type and promoter-mutated E^k_α transgenes onto the NOD genetic background. Animals carrying the various mutated transgenes expressed I-E on different subsets of immunocompetent cells, and thus cells important for the I-E protective effect could be identified. Although the wild-type transgene prevented the infiltration of lymphocytes into pancreatic islets, none of the mutants did. However, all of the transgenes could mediate the intrathymic elimination of T cells bearing antigen receptors with variable regions that recognize I-E. Thus, the I-E molecule does not protect NOD mice from diabetes simply by inducing the deletion of self-reactive T cells.

THE NOD MOUSE PROVIDES A MODEL for studying the immunology of insulin-dependent diabetes mellitus (IDDM) in humans (*1*). The murine disease is similar to the human condition in several ways: (i) it is caused by specific T lymphocytes that invade and destroy the pancreatic islets (*2–4*); (ii) this can be alleviated or prevented by immunosuppressive reagents such as cyclosporin (*5*); and (iii) the disease, although under complex polygenic control, is influenced by a gene (or genes) in the major histocompatibility complex (MHC) (*6–9*).

The class II genes of the NOD MHC constitute a distinct haplotype: the I-A complex has a β chain of unusual sequence (*10*), and the I-E complex is absent because of a deletion in the α chain gene (*7*, *11*). The importance of MHC class II genes, in particular of the defective E_α locus, has been demonstrated by introducing an E^d_α transgene onto the NOD genetic background: this gene prevented lymphocyte infiltration into the pancreatic islets of 10-week-old mice (*12*). However, this result has not been accepted without question [see discussion in (*8*), for example]. Because only one transgenic line was used, the protection from insulitis could have resulted from the chromosomal integration site of the transgene, from special features of the transgene construct, or from peculiarities of the E_α allele employed. Several hypotheses have been suggested to explain the protection phenomenon (*4*, *12*). In one, diabetes in NOD mice was postulated to result from an autoimmune attack by T cells that carried the $V_\beta5$ variable region on their T cell antigen receptors (TCRs). Since T cells displaying $V_\beta5^+$ TCRs are negatively selected in the thymus of mice expressing the I-E molecule (*13*), the diabetogenic anti-islet clones would be deleted intrathymically when a wild-type E_α gene is introduced into NOD mice (*4*).

To verify the original observation and to assess where E_α must be expressed to protect from insulitis, we have crossed the NOD strain with a number of E^k_α transgenic lines. One of them, $E_\alpha16$, expresses the I-E

J. Böhme, B. Schuhbaur, C. Benoist, D. Mathis, Laboratoire de Genetique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Genetique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 67000 Strasbourg, France.
O. Kanagawa, Department of Pathology, Washington University, School of Medicine, St. Louis, MO 63110.