

HYPOHAPTOGLOBINEMIA ASSOCIATED WITH FAMILIAL EPILEPSY

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Recurring epileptiform seizures affect more than five million individuals in the United States alone (1). In 5–10% of cases, the development of seizures is associated with prior trauma to the head (1, 2). Epilepsy with no overt cause (i.e., idiopathic) also occurs frequently, and, in some instances, multiple cases of idiopathic epilepsy are present within particular families. In the course of screening sera from a number of kindreds afflicted with this form of epilepsy, we have found a large excess of individuals with very low levels of the hemoglobin-binding protein, haptoglobin (Hp).¹ Experimental results suggest that this hypohaptoglobinemia may be related to the development of epilepsy. Similar hypohaptoglobinemia in mice impairs clearance of intracerebral hemoglobin (Hb). Furthermore, Hb promotes the oxidation of brain lipids. We hypothesize that interstitial Hb acts as a starting point for encephalic inflammation, and that familial or acquired hypohaptoglobinemia predisposes its sufferers to seizure disorders by impeding the clearance of this iron-rich protein from the brain.

Materials and Methods

Subjects. We studied 14 kindreds, in each of which at least two siblings had a history of seizures not associated with precedent central nervous system trauma. Selection criteria for probands were (a) recurrent, idiopathic seizures and (b) seizure onset before age 22. Affected siblings had either recurrent idiopathic or febrile seizures, also with onset before age 22. All members of these families were of northern European extraction, and were participants in the Minnesota Comprehensive Epilepsy Program. Normal control subjects were of similar extraction. Informed consent was obtained from all subjects.

Electrophoresis. Two-dimensional polyacrylamide gel electrophoresis (PAGE) of serum was performed as described by O'Farrell (3) and one-dimensional PAGE as described by Laemmli (4).

Haptoglobin. Serum Hp levels were quantitated using an immunonephelometric technique (5, 6). Human Hp (phenotype 1-1) was purified by affinity chromatography of outdated blood-bank plasma on immobilized turkey Hb (7). The resulting Hp was >90% pure, as judged by determination of total protein and immunoreactive Hp (5, 6).

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¹ *Abbreviations used in this paper:* Hb, hemoglobin; Hp, haptoglobin; MDA, malondialdehyde; PAGE, polyacrylamide gel electrophoresis.

Clearance of Intracerebral Hb. The rate of disappearance of intracerebral Hb was studied in male Swiss-Webster mice, 50–60 g in body weight. Some mice were made hypohaptoglobinemic through intraperitoneal injections of 5 mg of purified Hb (8). Following three daily injections of Hb, serum Hp was undetectable by immunonephelometric assay. Human Hb was radiolabelled by incubating suspensions of washed erythrocytes containing 100 mg Hb with 100 μ Ci 51 Cr (as sodium chromate) in a final volume of 1.0 ml (9). $\sim 8 \mu$ l of 51 Cr-labeled lysate (containing 1 mg of 51 Cr-Hb) was injected into the left cerebral hemisphere of sodium pentobarbital-anesthetized mice (5 mg/kg body weight) via a Hamilton microsyringe inserted to a depth of 4 mm. Some hypohaptoglobinemic animals were injected with a preformed Hb-Hp complex. In these experiments, 1 mg 51 Cr-Hb was complexed with purified Hp (1.3 mg). 24 h after injection, the animals were killed by cervical dislocation, and radioactivity within the liver, spleen, and left and right cerebral hemispheres was measured in a gamma-counter. To correct for variations in the amounts of labeled Hb actually delivered to the injection site, results were calculated as a percentage of total counts recovered in liver (the primary site of deposition of both free Hb and Hb-Hp complex) and brain as follows: (cpm injected hemisphere) \times 100/(cpm liver + cpm brain).

Peroxidation of Brain Lipids. Hb-mediated peroxidation of brain homogenates was assessed by measurements of "malondialdehyde" (MDA) (actually, a mixture of thiobarbituric acid-reactive substances). Fresh mouse brain was homogenized in four volumes of 50 mM Tris buffer, pH 8.0, in an acid-washed Potter-Elvehjem homogenizer. MDA was determined as previously described (10).

Results and Discussion

To identify genetic polymorphisms that might be linked with a hereditary predisposition to epilepsy, we performed two-dimensional PAGE on sera from 67 members of the 14 kindreds under study. In 5 of the 14 families, the index cases showed a decrease, or absence, of two or three major polypeptides of molecular weights $\sim 9,000$, 16,000, and 40,000. These proteins were identified as α type 1, α type 2 and β chains of the Hb-binding protein, Hp. On one-dimensional PAGE, a similar abnormality was observed in $<2\%$ of 612 control subjects, in keeping with earlier reports of the rarity of hypo- and anhapto-globinemia in Caucasian populations (11–14).

Immunonephelometric analyses of serum revealed that most of the patients with epileptiform seizures have hypohaptoglobinemia (Fig. 1). The mean serum Hp values of the 16 patients with seizures are significantly lower than those of 20 relatives without seizures ($t = 2.64$, $P < 0.02$ by Student's " t " test). Values for all but 4 of these 16 individuals fall at least one standard deviation below the normal mean. More importantly, the mean Hp values for these 16 patients are significantly below those of 120 normal controls ($t = 3.99$, $P < 0.001$). This difference remains significant even after exclusion of the five probands ($t = 3.63$, $P < 0.01$). Although the heritable nature of this abnormality is indicated by the high frequency of occurrence within these kindreds, the precise mode of inheritance cannot now be distinguished.

Hp, which is synthesized in the liver, avidly binds free Hb. The complex is then rapidly cleared by the reticuloendothelial system (15, 16). In the event of hepatic disease or increased intravascular destruction of erythrocytes, serum Hp may decrease. However, no evidence of such disease was found in any members of the affected kindreds. Furthermore, the mean serum Hp levels of 17 patients with nonfamilial epilepsy, under heavy medication to prevent frequent seizures

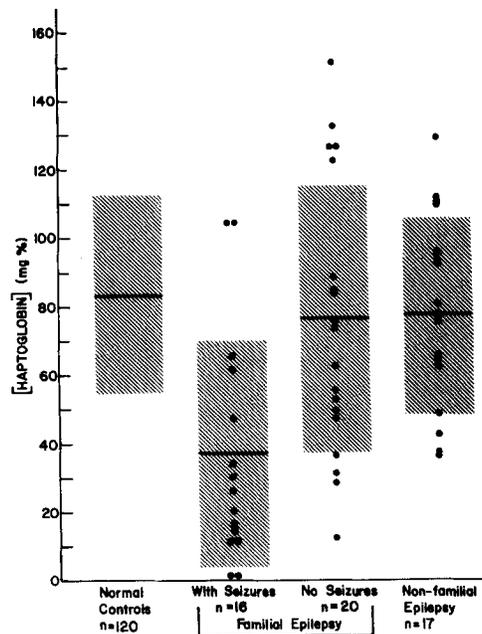


FIGURE 1. Distribution of serum haptoglobin levels in 16 people (from five families) affected by seizures, 20 (presently unaffected) members of the same families, 17 patients with nonfamilial epilepsy, and 120 normal controls (values for the latter derived from Beckman Instruments, Inc., Palo Alto, CA, using precisely the same reagents, instrument and technique). Shaded bars denote one standard deviation; heavy lines indicate the mean for each group. Circles show values for individual subjects.

similar to those in the familial group, were relatively normal (Fig. 1). Therefore, the diminished serum Hp levels in these five families with idiopathic epilepsy probably do not arise from accelerated intravascular red cell destruction, hepatic disease, medications, or seizures per se.

We hypothesize that decreased serum Hp levels may be causally related to the development of epilepsy. Specifically, cerebral "bruising" probably occurs in most people, either spontaneously or as a result of trauma. Hypohaptoglobinemic patients might fail to efficiently clear free Hb from the central nervous system. Residual, uncomplexed Hb within the brain could act as a starting point for inflammation, predisposing these patients to the development of seizures. Indeed, intracerebral injection of either iron salts or heme proteins into animals leads to the appearance of electroencephalographic abnormalities that foreshadow seizure disorders (17-23).

We have tested two corollaries of this hypothesis: namely, (a) that Hp speeds the clearance of free, intracerebral Hb, and (b) that Hb may promote inflammatory processes within brain tissue.

The first corollary was tested in hypohaptoglobinemic Swiss-Webster mice. ^{51}Cr -labeled human Hb was then injected into the left cerebral hemisphere of these animals, and into control mice that had normal serum Hp levels. An additional group of hypohaptoglobinemic mice was injected with ^{51}Cr -labeled Hb in complex with purified Hp. The results, shown in Fig. 2, indicate that

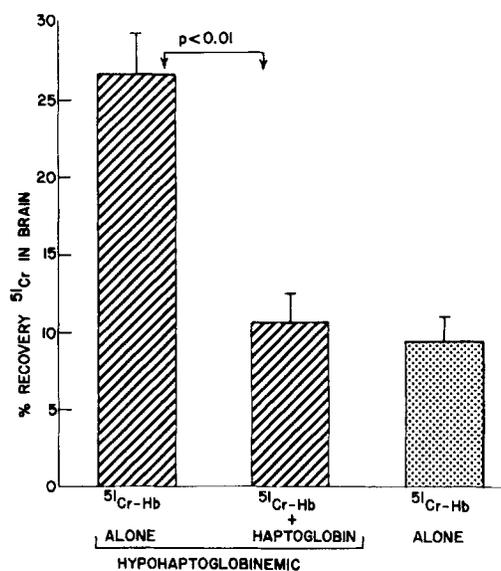


FIGURE 2. Clearance of ^{51}Cr -labeled Hb from the brains of hypohaptoglobinemic, and normal mice. 24 h after the intracerebral injection of Hb or Hb-Hp complex, the residual radioactivity within the injected hemisphere was determined as described in Materials and Methods. Shaded bars represent the mean values, and vertical lines denote one standard deviation. Hypohaptoglobinemic mice (left bar) retained almost three times as much intracerebral Hb as did the normal animals (right bar) ($P < 0.01$), or hypohaptoglobinemic mice (middle bar) injected with ^{51}Cr -Hp precomplexed with purified Hp ($P < 0.01$). $n = 6$ in all groups.

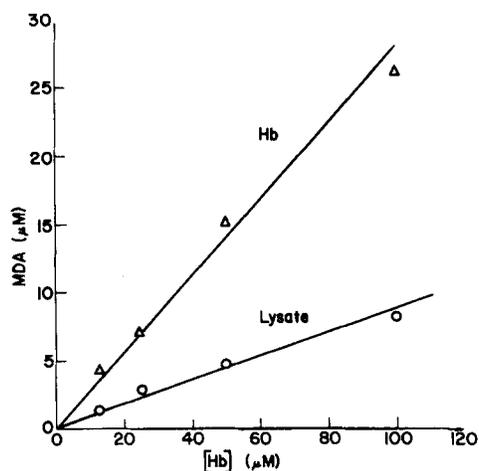


FIGURE 3. Hb-mediated generation of MDA in murine brain homogenates. Baseline MDA formation (in the absence of added Hb) was $1.2 \mu\text{M}$. The addition of progressively larger amounts of either purified human Hb (Δ), or crude red cell lysate (\circ) was associated with the production of increasing amounts of MDA over the 30-min incubation period.

hypohaptoglobinemic animals clear intracerebral Hb at a substantially slower rate, and that the defect in clearance of Hb is normalized by Hp.

Results of further experiments indicate that free Hb may promote brain lipid peroxidation. The addition of purified Hb to mouse brain homogenates causes the accumulation of MDA, generally considered an indicator of lipid peroxidation (10). This Hb-driven generation of MDA shows a clear dose dependence (Fig. 3). Stimulation of prostaglandin synthesis and MDA generation by Hb, as well as inhibition of these reactions by Hp, has been documented in other tissues (24–31). Furthermore, Hb will act as a “Fenton” reagent, promoting the generation of hydroxyl radicals from H₂O₂, supporting the possible involvement of Hb iron in the production of highly reactive oxygen species (32).

Not all subjects with seizures have hypohaptoglobinemia, however. Hypohaptoglobinemia may represent but one predisposing factor to the development of seizure disorders. Indeed, many individuals with hereditary hemolytic disease are chronically hypohaptoglobinemic, yet are not known to have a high frequency of seizure states resembling epilepsy. Nonetheless, we have found a large number of concordantly epileptic and hypohaptoglobinemic individuals in five kindreds with familial epilepsy, suggesting an association between low plasma Hp levels and the development of seizure disorders. A hereditary deficit in Hp may lead to slowed clearance of interstitial Hb from the brain. The resultant encephalic inflammation, prolonged and amplified by residual Hb, may ultimately lead to the occurrence of epileptiform seizures. Similarly, in cases of severe head trauma accompanied by acute Hp depletion (33), normal individuals may be subject to the same chain of events.

Summary

In select kindreds afflicted with familial idiopathic epilepsy, most individuals suffering seizures also have low levels of the plasma hemoglobin-binding protein, haptoglobin. This hypohaptoglobinemia may be causally associated with a tendency to develop epilepsy. Our experimental results indicate that artificially-induced hypohaptoglobinemia in mice causes retarded clearance of free hemoglobin from the central nervous system, and that such free hemoglobin may engender the peroxidation of brain lipids. We hypothesize that hypohaptoglobinemia, either inherited, or acquired via traumatic processes, may prevent efficient clearance of interstitial hemoglobin from the central nervous system, thereby predisposing these people to encephalic inflammation and the appearance of seizure disorders.

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References

1. Gumnit, R. J. 1983. *The Epilepsy Handbook*. Raven Press, New York. p. 2.
2. Loiseau, P., and P. Jallon. 1981. Post-traumatic epilepsy. *Prog. Neurol. Surg.* 10:323.

3. O'Farrell, P. H. 1975. High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007.
4. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680.
5. VanLente, F., A. Marchand, and R. S. Galen. 1979. Evaluation of a nephelometric assay for haptoglobin and its clinical usefulness. *Clin. Chem.* 25:2007.
6. Valette, I., J. Pointis, Y. Rondea, M. Waks, and R. Engler. 1979. Is immunochemical determination of haptoglobin phenotype-dependent? *Clin. Chim. Acta.* 99:1.
7. Delers, F., C. Lombart, M. Domingo, and S. Musquera. 1981. A novel and specific method for the purification of hemoglobin binding proteins. *Anal. Biochem.* 118:353.
8. Barksdale, A. D., B. E. Hedlund, B. E. Hallaway, E. S. Benson, and A. Rosenberg. 1975. The binding of azide to human methemoglobin A₀. *Biochemistry.* 14:2695.
9. Hebbel, R. P., O. Yamada, C. F. Moldow, H. S. Jacob, J. G. White, and J. W. Eaton. 1980. Abnormal adherence of sickle erythrocytes to cultured vascular endothelium: A possible mechanism for microvascular occlusion in sickle cell disease. *J. Clin. Invest.* 65:754.
10. Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. *Methods Enzymol.* 52:302.
11. Allison, A. C., B. S. Blumberg, and W. Rees. 1958. Haptoglobin types in British, Spanish Basque, and Nigerian African populations. *Nature (Lond.)*. 181:824.
12. Giblett, E. R. 1969. Genetic Markers in Human Blood. Blackwell Scientific Publications, Oxford, Great Britain.
13. Giblett, E. R. 1974. Haptoglobin. Structure and Function of Plasma Proteins. A. C. Allison, editor. Plenum Press, London, Great Britain. 1:55.
14. Lefranc, G., M.-P. Lefranc, J. Seger, J.-P. Salier, L. Chakhachiro, and J. Loiselet. 1981. Sex-limited ahaptoglobinemia. *Hum. Genet.* 59:294.
15. Putnam, F. W. 1975. In The Plasma Proteins, F. W. Putnam, editor. Academic Press, New York. p 1.
16. Javid, J. 1978. Human haptoglobins. *Curr. Top. Hematol.* 1:151.
17. Kopeloff, L. M., S. E. Barrera, and N. Kopeloff. 1942. Recurrent convulsive seizures in animals produced by immunologic and chemical means. *Am. J. Psychol.* 98:881.
18. Willmore, L. J., G. W. Sybert, J. B. Munson, and R. W. Hurd. 1978. Chronic focal epileptiform discharges induced by injection of iron into rat and cat cortex. *Science (Wash. DC)*. 200:1501.
19. Willmore, L. J., R. W. Hurd, and G. W. Sybert. 1978b. Epileptiform activity initiated by oral iontophoresis of ferrous and ferric chloride on rat cerebral cortex. *Brain Res.* 52:406.
20. Rosen, A. D., and N. V. Frumin. 1979. Focal epileptogenesis after intracortical hemoglobin injection. *Exp. Neurol.* 66:277.
21. Willmore, L. J., and J. J. Rubin. 1981. Antiperoxidant pretreatment and iron-induced epileptiform discharges in the rat: EEG and histopathologic studies. *Neurology.* 31:63.
22. Levitt, P., W. Wilson, and R. Wilkins. 1971. The effects of subarachnoid blood on the electrocorticogram of the cat. *J. Neurosurg.* 35:185.
23. Hammond, E. J., R. E. Ramsay, J. H. Villarreal, and G. J. Wilder. 1980. Effects of intracortical injection of blood and blood components in the electrocorticogram. *Epilepsia.* 21:3.
24. Brennecke, S. P., H. O. Collier, P. A. Denning-Kendall, W. J. McDonald-Gibson, S. A. Saed, and M. D. Mitchell. 1981. Prostaglandin synthesis inhibition of maternal and fetal sheep plasma and its relation to haptoglobin and albumin levels. *Prostaglandins. Med.* 6:243.
25. Denning-Kendall, P. A., and S. A. Seed. 1980. Effect of cofactor, enzyme and

- substrate concentration on inhibition of prostaglandin synthesis of bull seminal vesicles by serum albumin, haptoglobin and albumin. *Biochem. Soc. Trans.* 8:379.
26. Denning-Kendall, P. A., N. M. Butt, S. A. Saeed, and H. O. Collier. 1981. Differential effects of haptoglobin and albumin on the oxygenation of arachidonic acid during prostaglandin biosynthesis. *Biochem. Soc. Trans.* 8:79.
 27. Denning-Kendall, P. A., S. A. Saeed, and H. O. Collier. 1981. Comparison of the inhibition by serum albumin, haptoglobin, and albumin on biosynthesis of various prostaglandins. *Biochem. Soc. Trans.* 9:93.
 28. Jue, D.-M., B.-S. Shim, and Y.-S. Kang. 1983. Inhibition of prostaglandin synthase activity of sheep seminal vesicular gland by human serum haptoglobin. *Mol. Cell. Biochem.* 51:141.
 29. Kendall, P. A., S. A. Saeed, and H. O. Collier. 1979. Identification of endogenous inhibitor of prostaglandin synthesis with haptoglobin and albumin. *Biochem. Soc. Trans.* 7:543.
 30. Komoriya, K., K. Hoshina, A. Ohtsu, N. Saito, S. Kurozumi, T. Naruchi, Y. Hashimoto, K. Mizuno, and S. Yamamoto. 1980. Inhibition of prostaglandin-synthesizing enzymes by haptoglobin and plasma of rats with inflammation. *Jpn. J. Pharmacol.* 30:899.
 31. Saeed, S. A., P. A. Kendall, W. J. McDonald-Gibson, and H. O. Collier. 1978. Haptoglobin concentration and the activity of an endogenous inhibitor of prostaglandin synthetase in blood plasma or serum. *Biochem. Soc. Trans.* 6:1166.
 32. Sadrzadeh, S. M. H., E. Graf, S. S. Panter, P. E. Hallaway, and J. W. Eaton. 1984. Hemoglobin: A biologic Fenton reagent. *J. Biol. Chem.* 259:14354.
 33. Auer, L., and W. Petek. 1978. Serum haptoglobin changes in patients with severe isolated head injury. *Acta-Neurochir.* 42:229.