association with human T4 cells, has important ramifications from the standpoint of potential vaccine strategies. The binding of native gp120 as it might exist in a vaccine preparation to T4 cells could mask relevant epitopes that would otherwise be available for immunologic recognition. In addition, strong interactions with CD4 may have negative influences on various T4 cell functions as has been shown, for example, with the Leu-3 antibody suppression of soluble antigen proliferative responses (*29*). In view of this, the PB1 recombinant peptide might be a more attractive candidate for an HTLV-III/LAV vaccine than native viral gp120 even though both induce neutralizing antibodies. However, the efficacy of PB1 as a vaccine is still untested and final decisions must depend on immunizing chimpanzees that are susceptible to HTLV-III/LAV infection (*30, 31*). Even though many people exposed to HTLV-III/LAV do have neutralizing antibodies, it is unknown under what circumstances these are protective against viral infection. To what extent artificial immunization can mimic or improve natural immunity awaits further study.

REFERENCES AND NOTES

1. W. G. Robey *et al.*, *Science* **228**, 593 (1985).
2. F. Barin *et al.*, *ibid.*, p. 1094.
3. J. S. Allan *et al.*, *ibid.*, p. 1091.
4. B. R. Starcich *et al.*, *Cell* **45**, 637 (1986).
5. F. D. Veronese *et al.*, *Science* **229**, 1402 (1985).
6. M. Robert-Guroff, M. Brown, R. C. Gallo, *Nature (London)* **316**, 72 (1985).
7. R. A. Weiss *et al.*, *ibid.*, p. 69.
8. T. J. Matthews *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
9. W. G. Robey *et al.*, *ibid.* **83**, 7023 (1986).
10. L. A. Lasky *et al.*, *Science* **233**, 209 (1986).
11. M. Seligmann *et al.*, *N. Engl. J. Med.* **311**, 1286 (1984).
12. J. S. McDougal *et al.*, *Science* **231**, 382 (1986).
13. L. Ratner *et al.*, *Nature (London)* **313**, 277 (1985).
14. T. Mueller and S. Putney, unpublished data.
15. L. A. Ivanoff, T. Towatari, J. Ray, B. D. Korant, S. R. Petteway, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5392 (1986).
16. J. H. Elder and S. Alexander, *ibid.* **79**, 4540 (1982).
17. J. D. Lifson, G. R. Reyes, M. S. McGrath, B. S. Stein, E. G. Engleman, *Science* **232**, 1123 (1986).
18. J. Sodroski, W. C. Goh, C. Rosen, K. Campbell, W. A. Haseltine, *Nature (London)* **322**, 470 (1986).
19. H. Shida and S. Dales, *Virology* **111**, 56 (1981).
20. S. Basak and R. W. Compans, *ibid.* **128**, 77 (1983).
21. K. Olden, B. A. Bernard, S. L. White, J. B. Parent, *J. Cell. Biochem.* **18**, 313 (1982).
22. M. R. Sairam and G. N. Bhargavi, *Science* **229**, 65 (1985).
23. S. Alexander and J. H. Elder, *ibid.* **226**, 1328 (1984).
24. R. L. Willey *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5038 (1986).
25. B. H. Hahn *et al.*, *Science* **232**, 1548 (1986).
26. M. Alizon, S. Wain-Hobson, L. Montagnier, P. Sonigo, *Cell* **46**, 63 (1986).
27. M. Robert-Guroff *et al.*, *J. Immunol.*, in press.
28. S. Modrow *et al.*, *J. Virol.*, in press.
29. E. Engleman *et al.*, *J. Immunol.* **130**, 2623 (1983).
30. D. C. Gajdusek *et al.*, *Lancet* **1985-I**, 55 (1985).
31. P. N. Fultz *et al.*, *J. Virol.* **58**, 116 (1986).

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Oligodendrocyte Adhesion Activates Protein Kinase C–Mediated Phosphorylation of Myelin Basic Protein

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When isolated adult oligodendrocytes adhere to a substratum myelinogenesis occurs. Investigation of the mechanism by which this happens indicated that the oligodendrocyte-substratum interaction (i) activated protein kinase C–dependent phosphorylation of myelin basic protein and (ii) promoted the synthesis of myelin basic protein. In addition, when agents that activate protein kinase C (second messenger diacylglycerol or a tumor-promoting phorbol ester) were added to nonattached oligodendrocytes, they mimicked the influence of the substratum by inducing phosphorylation of myelin basic protein; and reagents that increase cellular adenosine 3′, 5′-monophosphate (cyclic AMP) inhibited phosphorylation of myelin basic protein. Thus, at least in vitro, the interaction between oligodendrocytes and the substratum may mediate myelinogenic events, and phosphorylation of myelin basic protein may be an early requirement in the sequence of steps that ultimately results in myelin formation.

CIRCUMSTANTIAL EVIDENCE INDICATES that cell-cell and cell-substratum interactions are key elements in central nervous system (CNS) myelination, but the origin and nature of the signals that initiate and control assembly and maintenance of myelin are unknown. In oligodendrocytes (OLG), the cells that assemble and maintain CNS myelin (*1*), myelination is preceded by the synthesis of myelin basic protein (MBP) and other myelin proteins and lipids. Myelin basic protein is phosphorylated (*2*); the functional significance of this modification is unclear, although it may be a signal for myelination (*3*). The fact that isolated OLG in culture express myelinogenic properties (*4, 5*) makes them suitable for investigating mechanisms that control myelin assembly. We have shown that, upon attachment onto a polylysine substratum, mature OLG re-enact events associated with myelinogenesis (*6–8*) and, over time in culture and without neurons, reform myelin, a process termed myelin palingenesis (*9*). We have now extended this work to investigate the mechanism by which the substratum influences myelinogenesis by observing early changes in metabolism after cell-substratum interaction. Our results indicate that the substratum transmits a signal that activates protein kinase C (PKC)–mediated phosphorylation of MBP, as well as promoting the synthesis of MBP.

Oligodendrocytes are isolated from ovine white matter (Fig. 1) and maintained in culture (*10*) as floating clusters (B3.f) or attached to a polylysine substratum (B3.fA). The appearance of live B3.f OLG, as viewed by phase microscopy, is shown in Fig. 1A. When B3.f OLG are plated onto a polylysine substratum, after having been maintained in vitro for 4 days as floating clusters, they adhere within 6 hours and undergo modifications in metabolism and morphology (*10*). Over time in culture, changes in morphology become readily apparent by light microscopy (Fig. 1, B and C). We can maintain B3.fA cultures for 120 days and longer with 99% purity, as assessed by immunocytochemical criteria (*9, 10*).

We then examined the influence of attachment on the synthesis and phosphorylation of MBP. B3.f and B3.fA cultures were labeled with $^{32}PO_4^{3-}$ for 30 minutes, MBP was immunoprecipitated, and the amount of radiolabel incorporated into MBP was assessed by autoradiography of the proteins separated by SDS–polyacrylamide gel electrophoresis (Fig. 2). The uptake of $^{32}PO_4^{3-}$ into MBP was low in B3.f cells but was considerably higher in B3.fA cultures (Fig. 2, lanes a and b, respectively). This differ-

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ence between the two cultures occurred after only 24 hours of attachment for the B3.fA cultures; total time in culture was the same. Parallel cultures were labeled with [^{14}C]leucine for 16 hours, and the net synthesis of MBP in B3.f and B3.fA cultures assessed from autoradiograms of immunoprecipitated MBP that had been resolved on SDS polyacrylamide gels (Fig. 2, lanes c and d, respectively). Although the net rate of MBP synthesis is greater in B3.fA than in B3.f OLG, B3.f OLG clearly synthesize MBP (Fig. 2). Thus, the lack of significant $^{32}PO_4^{3-}$ incorporation into MBP by B3.f OLG must be the consequence of factors other than synthesis. Moreover, incorporation of $^{32}PO_4^{3-}$ into other cellular phosphoproteins by B3.f OLG is similar to that of B3.fA OLG.

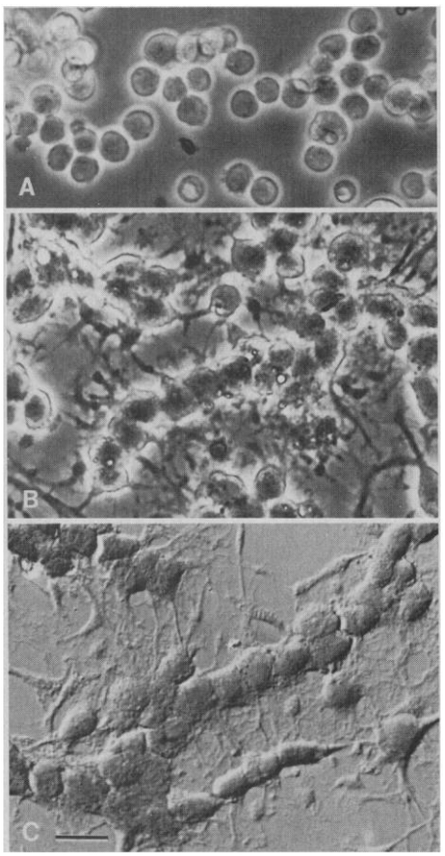

Fig. 1. Cultured oligodendrocytes. The procedures for cell isolation (*24*) and culture (*5, 10*), as well as the characterization of these cells (*25*), have been described. OLG are isolated as follows. White matter from lamb brain is trypsinized, disrupted by passage through nylon and metal screen from a pore size of 350 μm to 30 μm; myelin is removed by taking advantage of its low density; and the crude cell suspension is resolved on a linear sucrose gradient into three bands (1 to 3 from top to bottom). Only cells from band 3 (*24*) were used for our experiments. We maintain OLG in two culture conditions (*10*). B3.f OLG are OLG from band 3 plated onto tissue culture dishes and kept floating for 3 to 5 days; they do not adhere to culture plates. B3.fA OLG are B3.f OLG transferred to polylysine-coated petri dishes to which they adhere. After attachment, these cells undergo drastic changes in their morphology. Except where indicated, cultures are kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% horse serum and 2 m*M* L-glutamine, and fed twice weekly. (A) Phase-contrast micrograph of live B3.f OLG 4 days after isolation. The cells are round, phase bright, and gathered in clusters. Neither cellular processes nor redundant membranes are seen in these cultures, even if cells are kept for 2 weeks. (B) Phase-contrast micrograph of live B3.fA OLG after 21 days in culture. Note that cells are aligned in rows as they are in vivo and have extended a network of processes. (C) Fixed B3.fA OLG after 21 days in culture, viewed by Nomarski optics. Note the very fine processes covering the surface; these cannot be seen by ordinary phase microscopy. Bar = 20 μm.

To identify the kinase responsible for the phosphorylation of MBP in OLG cultures, we used compounds known to activate, directly or indirectly, specific protein kinases in intact cells (*11*). Twenty-one-day-old B3.fA cultures were labeled with $^{32}PO_4^{3-}$ for 20 minutes and then exposed to various drugs for another 20 minutes. Total radioactivity incorporated into immunoprecipitated $^{32}PO_4^{3-}$-MBP was measured on bands excised from SDS polyacrylamide gels. Two compounds that elevate cellular adenosine 3′,5′-monophosphate (cAMP), isobutylmethylxanthine (IBMX), a cAMP phosphodiesterase inhibitor, and forskolin, an activator of adenylate cyclase, both inhibited $^{32}PO_4^{3-}$ incorporation into MBP by 50% when compared to controls (Fig. 3A). An even greater suppression than that achieved with either IBMX or forskolin was attained with trifluoperazine (TFP), an inhibitor of Ca^{2+}-dependent protein kinases. No significant effect was obtained with the Ca^{2+} ionophore A23187, which is known to affect Ca^{2+}-calmodulin dependent protein kinase, but 4β-phorbol 12-myristate 13-acetate (TPA), which activates PKC, induced a fourfold increase in the uptake of $^{32}PO_4^{3-}$ into MBP (Fig. 3A).

The stimulation of MBP phosphorylation by TPA, in conjunction with the inhibitory action of elevated intracellular cAMP; the lack of effect of the Ca^{2+} ionophore; and the nearly complete suppression of MBP phosphorylation by TFP, suggest that PKC is the sole mediator of MBP phosphorylation in intact OLG. These results contrast with those obtained from cell-free systems, where MBP has been found to be a substrate for other kinases (*12–14*), as well as for PKC (*15*). This finding supports the suggestion by Turner *et al.* (*16*) that PKC catalyzes MBP phosphorylation in vivo.

The physiological activation of PKC involves a receptor-mediated breakdown of phosphoinositides (PtdIns) by phospholipase C that results in a transient liberation of the second messenger diacylglycerol (DAG), which in turn activates PKC (*17, 18*). We therefore hypothesized that, upon adhesion of OLG to a substratum, there is an increase in the turnover of PtdIns with the subsequent release of DAG. This possibility suggests an explanation for the lack of MBP phosphorylation in B3.f OLG—the absence of a DAG signal. Hence, we treated B3.f cultures with either the DAG, 1-oleoyl 2-acetylglycerol, or TPA for 5 minutes after a 25-minute initial labeling with $^{32}PO_4^{3-}$. A dramatic (> tenfold) increase in MBP phosphorylation resulted from the action of these drugs (Fig. 3B), suggesting that DAG is the limiting factor in MBP phosphorylation in the cultures of nonattached OLG.

Diacylglycerol or TPA is thought to mobilize PKC from cytosol to a membrane compartment (*19*), where PKC is activated. This is accompanied by a reduction in cytosolic PKC activity (*20*). Measurement of PKC activity in OLG homogenates, assayed by transfer of $^{32}PO_4^{3-}$ from [γ^{32}P]adenosine triphosphate (ATP) to histone III-S, revealed no significant differences between

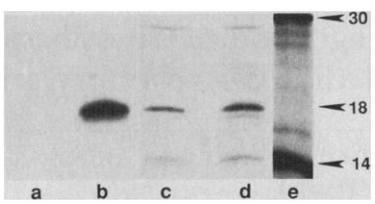

Fig. 2. Comparison of the extent of phosphorylation and synthesis of myelin basic protein by B3.f and B3.fA OLG cultures. Cultures of B3.f and B3.fA OLG after 5 days in vitro (1 day of adhesion for B3.fA; see Fig. 1) were labeled with either $^{32}PO_4^{3-}$ (50 μCi/ml) in Hepes-buffered phosphate-free DMEM (Hepes-PFM) for 30 minutes or [^{14}C]leucine (1 μCi/ml) in leucine-free DMEM for 16 hours. Exposure to label was terminated by aspiration of medium and addition of 10% trichloroacetic acid (TCA) containing 1 m*M* EDTA. TCA precipitates were solubilized in buffer A (50 m*M* tris-HCl, 0.15*M* NaCl, 1% Triton X-100, 0.4% SDS, *p*H 7.4). To each solubilized sample containing 60 μg of protein (Bio-Rad protein assay), 2 μl of rabbit polyclonal antibody to bovine MBP (18,500 daltons) were added, and samples were incubated with rotation for 16 hours at 4°C to immunoprecipitate MBP (*26*). Immune complexes were precipitated by the addition of protein A–Sepharose beads (Pharmacia) and centrifuged for 6 minutes at 10,000*g* (*26, 27*). Immunoprecipitates were washed six times with buffer A, boiled for 1 minute in sample buffer [3% SDS, 1 m*M* 1,4 dithio-L-threitol, 60 m*M* tris-HCl, *p*H 6.8, 10% glycerol (weight to volume), 0.05% bromophenol blue (weight to volume)], and resolved by SDS–polyacrylamide gel electrophoresis (*28*). Autoradiograms were obtained by exposing the dried gel to Kodak XK-5 film. Representative autoradiograms: lane a, $^{32}PO_4^{3-}$-labeled MBP from B3.f OLG; lane b, $^{32}PO_4^{3-}$-labeled MBP from B3.fA OLG (24 hours of attachment); lane c, [^{14}C]leucine-labeled MBP from B3.f OLG; lane d, [^{14}C]leucine-labeled MBP from B3.fA OLG; lane e, ^{14}C molecular weight standards. Numbers on the right indicate $M_r \times 10^{-3}$. MBP has a M_r of 18,000.

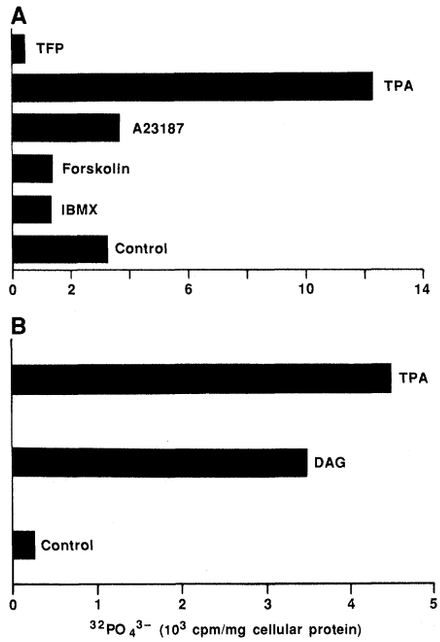

Fig. 3. Effect of various drugs on the incorporation of $^{32}PO_4^{3-}$ into MBP. OLG cultures were labeled as described below, and MBP was immunoprecipitated and resolved by SDS–polyacrylamide gel electrophoresis as described in Fig. 2. Labeled MBP bands were identified by autoradiography, gels were cut accordingly, and radioactivity quantitated as described by Aloyo (*29*). Values given are cpm per mg of total cellular protein and are averages from triplicate experiments. Individual values differed by no more than 6%. (A) 21-day-old B3.fA OLG cultures were initially labeled with 50 μCi/ml of $^{32}PO_4^{3-}$ in Hepes-PFM for 20 minutes; drug or vehicle alone was added and incubation continued for another 20 minutes (40 minutes of total labeling time). Drug concentrations were: IBMX, $5 \times 10^{-4}M$; forskolin, $1 \times 10^{-7}M$; A23187, $5 \times 10^{-8}M$; TPA, $1 \times 10^{-7}M$; and TFP, $1 \times 10^{-7}M$. Reactions were terminated by aspirating the media and adding ice-cold 10% TCA containing 1 m*M* EDTA. Samples were processed as described above. (B) Four-day-old B3.f OLG cultures were initially labeled for 25 minutes with 50 μCi/ml of $^{32}PO_4^{3-}$ in Hepes-PFM, then incubated with either DAG ($1 \times 10^{-7}M$), TPA ($1 \times 10^{-7}M$), or vehicle alone for an additional 5 minutes. Cells were centrifuged for 10 seconds at 8000*g*, supernatants removed, and the reaction terminated by the addition of ice-cold 10% TCA with 1 m*M* EDTA. Samples were then processed (see above).

B3.f and B3.fA OLG. However, when PKC activities in cytosolic fractions of B3.f and B3.fA OLG were compared, a marked decrease of activity in B3.fA cytosol was detected (Fig. 4). Furthermore, treatment of B3.f OLG with TPA for 15 minutes resulted in the total abolition of PKC activity in the cytosolic compartment (Fig. 4). Interpretation of these results must be tentative in view of (i) our and others (*21*) inability to totally account for the lost PKC activity (only approximately 50% was found in the membrane fraction) and (ii) a recent report that shows a TPA-induced reduction in PKC activity toward histone III-S as a substrate, but not with other substrates (*22*). Independent of the mechanism, our observations demonstrate distinct behavior of PKC in B3.f and B3.fA OLG.

We have shown that (i) OLG-substratum adhesion initiates a signal that activates PKC-mediated phosphorylation of MBP [as well as a previously observed increased uptake of [^{3}H]glycerol into phosphatidylinositol (*6*)]; (ii) adhesion of OLG also results in an increased synthesis of MBP; (iii) exogenously added DAG or TPA mimics the influence of substratum by stimulating nonattached OLG to phosphorylate MBP; and (iv) reagents that elevate intracellular cAMP have an inhibitory effect on the incorporation of $^{32}PO_4^{3-}$ into MBP, possibly by blocking inositol trisphosphate and DAG release (*23*). These observations are consistent with the hypothesis that an initial event following attachment of OLG to the substratum involves generation of DAG. Whether or not DAG is a signal for myelinogenesis in vivo remains to be shown. This work substantiates our earlier observations on the plasticity of mature oligodendrocytes and provides new evidence on the mechanism of substratum effects on myelinogenesis.

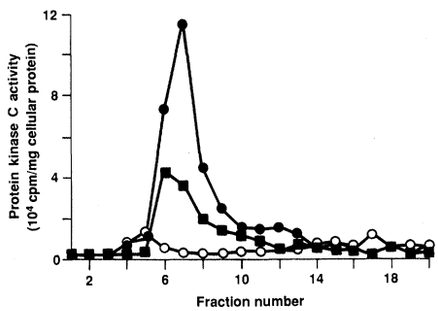

Fig. 4. Effect of adhesion to the substratum or of TPA treatment on PKC activity in cytosolic fractions of cultured OLG. Subcellular fractions were obtained as described (*30*) with minor modifications. Briefly, OLG were harvested in buffer B (20 m*M* tris, *p*H 7.5, 50 m*M* β-mercaptoethanol, 2 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride, and leupeptin) (0.1 mg/ml), disrupted by passing three times through a cell disrupter (*31*) at 4°C, centrifuged for 1 hour at 100,000*g*, and the supernatant used to measure PKC activity (*20*). Supernatants were applied to a DE-52 column (1.8 cm by 0.5 cm) that had been equilibrated with buffer B. The column was washed with four column volumes of buffer B and PKC was eluted with a 0 to 400 m*M* linear NaCl gradient (0.8 ml per fraction). 50-μl aliquots from each fraction were assayed for PKC activity (*32*) with an assay system that contained: histone III-S (320 μg/ml), 20 m*M* tris at *p*H 7.5, 500 μ*M* Ca^{2+}, 400 μ*M* [^{32}P]ATP, in the presence or absence of phosphatidylserine and diolein. Incubation time was 4 minutes at 30°C. The reaction was terminated by the addition of 1 ml of ice-cold 25% TCA. Precipitated proteins were collected by filtration through 0.45-μm Millipore filters and washed three times with 2 ml of 5% TCA. Filters were placed in vials, 4 ml of aqueous scintillation fluid added, and radioactivity determined in a Hewlett-Packard spectrophotometer. Values represent differences in cpm between corresponding samples with and without phosphatidylserine and diolein. Plots show distribution of PKC activities in cytosolic fractions from (●) 5-day-old B3.f OLG; (■) 5-day-old B3.fA OLG (1 day of attachment); and (○) 5-day-old B3.f OLG treated with TPA ($1 \times 10^{-7}M$) for 15 minutes before subcellular fractionation. Values are means from triplicate experiments. Individual values differed by no more than 5%.

REFERENCES AND NOTES

1. C. S. Raine, in *Myelin*, P. Morell, Ed. (Plenum, New York, 1984), pp. 1–50.
2. A. J. Steck and S. H. Appel, *J. Biol. Chem.* **249**, 5416 (1974).
3. J. B. Ulmer and P. E. Braun, *Dev. Neurosci.* **6**, 345 (1984).
4. R. Mirsky *et al.*, *J. Cell Biol.* **84**, 483 (1980).
5. S. Szuchet, K. Stefansson, R. L. Wollmann, G. Dawson, B. G. W. Arnason, *Brain Res.* **200**, 151 (1980).
6. S. Szuchet, S. H. Yim, S. Monsma, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7019 (1983).
7. T. Vartanian, S. H. Yim, S. Szuchet, G. Dawson, in *Biochemistry of Glial Cells*, T. Grisar, Ed. (Pergamon, New York, in press).
8. S. Szuchet, in *ibid.*
9. S. Szuchet, in *Glial-Neuronal Communication in Development and Regeneration*, A. Althaus and W. Seifert, Ed. (Plenum, New York, in press).
10. S. Szuchet and S. H. Yim, *J. Neurosci. Res.* **11**, 131 (1984).
11. A. C. Nairn, H. C. Hemmings, Jr., P. Greengard, *Annu. Rev. Biochem.* **54**, 9799 (1985).
12. Y. Takeyama and Y. Nishizuka, *J. Biol. Chem.* **260**, 12492 (1985).
13. P. V. Sulakhe, E. H. Petrali, R. E. Davis, B. J. Thiessen, *Biochemistry* **19**, 5363 (1980).
14. E. Miyamoto and S. Kakiuchi, *J. Biol. Chem.* **249**, 2769 (1974).
15. R. S. Turner, C.-H. J. Chou, R. F. Kibler, J. F. Kuo, *J. Neurochem.* **39**, 1397 (1982).
16. R. S. Turner, C.-H. J. Chou, G. J. Mazzei, P. Dembure, J. F. Kuo, *ibid.* **43**, 1257 (1984).
17. K. Hirasawa and Y. Nishizuka, *Annu. Rev. Pharmacol. Toxicol.* **25**, 147 (1985).
18. M. J. Berridge, *Biochem. J.* **220**, 345 (1984).
19. A. S. Kraft and W. B. Anderson, *Nature (London)* **301**, 621 (1983).
20. ———, H. L. Cooper, J. J. Sando, *J. Biol. Chem.* **257**, 13193 (1982).
21. W. B. Anderson, A. Estival, H. Tapiovaara, R. Gopalakrishna, *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**, 287 (1985).
22. C. Cochet, C. Sourignet, M. Keramidas, E. M. Chambaz, *Biochem. Biophys. Res. Commun.* **134**, 1031 (1986).
23. T. Takenawa, J. Ishitoya, Y. Nagai, *J. Biol. Chem.* **261**, 1092 (1986).
24. S. Szuchet, B. G. W. Arnason, P. E. Polak, *J. Neurosci. Methods* **3**, 7 (1980).
25. L. Hertz, B. H. J. Juurlink, S. Szuchet, in *Handbook of Neurochemistry*, A. Lajta, Ed. (Plenum, New York, 1985), pp. 603–661.
26. L. S. Reidl, C. W. Campagnoni, A. T. Campagnoni, *J. Neurochem.* **37**, 373 (1981).
27. A. T. Campagnoni, C. W. Campagnoni, J.-M. Bourre, C. Jacque, N. Baumann, *ibid.* **42**, 733 (1984).
28. U. K. Laemmli and M. Faure, *J. Mol. Biol.* **80**, 595 (1983).

29. V. J. Aloyo, *Anal. Biochem.* **99**, 161 (1979).
30. S. Szuchet, P. E. Polak, S. H. Yim, *J. Cell Biol.* **101**, 435a (1985).
31. S. Szuchet and P. E. Polak, *Anal. Biochem.* **128**, 453 (1983).
32. K. Hirota, T. Hirota, G. Aguilera, K. J. Catt, *J. Biol. Chem.* **260**, 3243 (1985).
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The Nucleocapsid of Hepatitis B Virus Is Both a T-Cell–Independent and a T-Cell–Dependent Antigen

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One characteristic of the immune response during hepatitis B virus (HBV) infection in humans is the vigorous production and subsequent persistence of antibodies of immunoglobulin (Ig) classes M and G to the nucleocapsid antigen (HBcAg). In this study HBcAg was shown to be similarly immunogenic in mice. When injected into athymic (nude) B10.BR and athymic BALB/c mice, HBcAg induced IgM and IgG class antibodies to HBc in spite of the absence of T cells in nude mice. In euthymic mice, HBcAg efficiently stimulated T-cell proliferation in vitro and helper T-cell function in vivo. The dual functions of HBcAg as a T-cell–independent and a T-cell–dependent antigen may explain its enhanced immunogenicity. Denaturation of HBcAg yields a nonparticulate antigen designated HBeAg; when HBeAg was used as the immunogen, antibody production required helper T-cell function. Although HBcAg and HBeAg are serologically distinct, they are structurally related, and in these experiments were highly cross-reactive at the T-cell level. These results suggest that the elevated levels of IgM antibodies to HBc and the enhanced immunogenicity of HBcAg during HBV infection in humans reflect the ability of HBcAg to directly activate B cells to produce antibodies to HBc in the presence or absence of HBcAg- or HBeAg-sensitized T cells.

DURING INFECTION WITH HEPATITIS B virus (HBV), at least four antigen-antibody systems are observed: hepatitis B surface antigen (HBsAg) and its antibody (anti-HBs); the pre-S antigens associated with HBsAg particles and their antibodies; the particulate nucleocapsid antigen (HBcAg) and anti-HBc; and an antigen structurally related to HBcAg, namely, HBeAg and its antibody (anti-HBe). When HBcAg is treated with proteolytic enzymes or mild detergent it is converted to HBeAg (*1, 2*). This has been confirmed by the finding that cryptic HBeAg determinants are present on recombinant HBcAg (rHBcAg) (*3*).

The HBsAg, including the pre-S region, is an early marker of HBV infection. Although the development of antibodies to HBsAg may be delayed, antibodies to pre-S antigens may occur quite early in infection (*4*). Nevertheless, the humoral responses to the envelope antigens may vary considerably from patient to patient during infection and after vaccination, ranging from high to nonresponder phenotypes (*5*). Despite being internal components of virions, nucleocapsid antigens of a number of human viruses are efficient immunogens (for example, HTLV-I, HTLV-III, hepatitis Delta virus, and Epstein-Barr virus). The HBcAg is no exception since high titers of anti-HBc are regularly produced by virtually 100% of HBV-infected patients. Furthermore, anti-HBc of the immunoglobulin M (IgM) class appears early in acute hepatitis B, and IgM and IgG anti-HBc can persist with slowly decreasing titers for many years (*6*). In contrast, antibodies to HBeAg may not develop or may appear at various times after the appearance of anti-HBc, and are correlated with viral clearance (*7*).

Table 1. Comparison of primary antibody responses after immunization with HBsAg and HBcAg.

Strain*	H-2	Anti-HBs (titer)	Anti-HBc (titer)
B10	b	256	40,960
B10.D2	d	1024	81,920
B10.S	s	0†	163,840
B10.BR	k	32	163,840
B10.M	f	0†	20,480
$C_3H.Q$	q	2048	327,680
BALB/c	d	1024	327,680

*Groups of five mice from each strain were immunized with 4.0 μg of HBsAg or HBcAg in CFA, and pooled sera were analyzed by solid-phase RIA for IgG antibodies of the indicated specificities at day 24. Data are expressed as the reciprocal of the highest serum dilution to yield four times the counts of the sera before immunization (titer). †The $H\text{-}2^s$ and $H\text{-}2^f$ haplotypes are nonresponsive to HBsAg even after secondary immunization (*21*).

Although HBcAg and HBeAg are serologically distinct, the primary amino acid sequences show significant identity [serum HBeAg lacks the COOH-terminal 35 residues of HBcAg (*8*)]. One might therefore predict that these antigens cross-react at the T-cell level. Why then do the immune responses to HBcAg and HBeAg appear to be regulated independently? This apparent contradiction, the enhanced immunogenicity of HBcAg, and reports that vaccination with HBcAg confers protection against HBV infection prompted us to examine the murine humoral and cellular immune responses to HBcAg.

A number of inbred murine strains, including a series of H-2-congenics, were immunized with 4 μg of rHBcAg or HBsAg (both particulate antigens) in complete Freund's adjuvant (CFA), and the primary IgG antibody responses were analyzed by solid-phase radioimmunoassays (RIA) of approximately equal sensitivities. The results of these assays correlated with results obtained with commercially available anti-HBs and anti-HBc assays (Abbott), and were of equal to greater sensitivity. All strains immunized with HBcAg showed a vigorous, primary, IgG anti-HBc response (Table 1). The influence of H-2–linked genes on the anti-HBc response is apparent (B10.S, B10.BR>B10, B10.D2, B10.M), although no nonresponder strains were identified. The anti-HBc responses were significantly greater (at least 80-fold) than the anti-HBs responses in all strains tested. Furthermore, high-titered anti-HBc persisted in these mice a year after this single dose of HBcAg. The comparative magnitudes of the primary anti-HBc and anti-HBs responses, and the lack of nonresponsiveness to HBcAg are, in general, consistent with the human immune responses to these HBV antigens.

The enhanced immunogenicity of HBcAg in mice prompted us to examine the ability of HBcAg to activate B cells directly. Therefore, B10.BR euthymic (+/+) and B10.BR athymic (nu/nu) mice were immunized with various doses (0.5, 1.5, and 4.0 μg) of rHBcAg in CFA, and sera were analyzed for anti-HBc at 10 and 24 days after immunization. As expected, the B10.BR +/+ mice produced dose-dependent anti-HBc at 10 days and a 4- to 16-fold increase in the anti-HBc titer at 24 days (Fig. 1A). However, the B10.BR nu/nu mice also produced dose-dependent, anti-HBc antibody at 10 days after immunization (Fig. 1B), but showed no increase in the anti-HBc titer at 24 days. Complete Freund's adjuvant was not re-

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