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Metabolism of the Carbocyclic Nucleoside Analogue Carbovir, an Inhibitor of Human Immunodeficiency Virus, in Human Lymphoid Cells†

Laureano L. Bondoc, Jr.,‡ William M. Shannon,§ John A. Secrist III,§ Robert Vince,∥ and Arnold Fridland*,‡

Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, 332 N. Lauderdale, P.O. Box 318, Memphis, Tennessee 38101, Southern Research Institute, Birmingham, Alabama 35455, and Department of Medicinal Chemistry, College of Pharmacy, Health Sciences Unit F, University of Minnesota, Minneapolis, Minnesota 55455

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ABSTRACT: Carbovir (CBV) is a highly selective carbocyclic nucleoside inhibitor of HIV replication in human lymphocytes and is potentially useful in the treatment of AIDS [Vince et al. (1988) *Biochem. Biophys. Res. Commun. 156*, 1046–1053]. Using human lymphoid cells severely deficient in nucleoside kinases, we were able to identify the route of activation of CBV metabolism. The present studies have demonstrated that CBV is anabolized to the mono-, di-, and triphosphates and to guanosine 5′-triphosphate in CCRF-CEM cells. Conversion to GTP amounted to 15–20% of the total analogue nucleotides formed in the cells and may arise from CBV through depurination and salvage via HGPRT. Evidence was obtained that neither deoxycytidine kinase, adenosine kinase, or mitochondrial deoxyguanosine kinase is primarily involved in the initial step of phosphorylation of CBV in CCRF-CEM cells. In contrast, earlier studies [Johnson & Fridland (1989) *Mol. Pharmacol. 36*, 291–295] showed that a cytosolic 5′-nucleotidase catalyzes the activation of CBV to the monosphosphate. Other biochemical effects examined showed that the nucleobases hypoxanthine and adenine, but not guanine, their respective nucleosides, and the dideoxynucleosides 2′,3′-dideoxyinosine, 2′,3′-dideoxyguanosine, and 3′-azido-3′-deoxythymidine produced significant increased accumulation of CBV nucleotides in CEM cells. The exact mechanism for this potentiation of CBV phosphorylation has not been elucidated but may be due to a modulating effect of intracellular nucleotides on 5′-nucleotidase activity.

The carbocyclic nucleoside analogue carbovir (carbocyclic 2′,3′-didehydro-2′,3′-dideoxyguanosine, NSC-614846, CBV)[1] is a novel guanosine derivative which has shown potent in vitro activity against the human immunodeficiency virus, the etiological agent of AIDS. CBV inhibits HIV replication and

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‡St. Jude Children's Research Hospital.
§Southern Research Institute.
∥University of Minnesota.

[1] Abbreviations: CBV, carbovir; HIV, human immunodeficiency virus; AIDS, autoimmune deficiency syndrome; AZT, 3′-azido-3′-deoxythymidine; ddC, 2′,3′-dideoxycytidine; TP, 5′-triphosphate; ddNTP, 2′,3′-dideoxynucleoside 5′-triphosphate; HPLC, high-pressure liquid chromatography; HGPRT, hypoxanthine–guanine phosphoribosyltransferase; ddG, 2′,3′-dideoxyguanosine; dGuo, 2′-deoxyguanosine; PNP, purine nucleoside phosphorylase.

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HIV-induced cytopathic effects at concentrations approximately 200–400-fold below its cytotoxic concentrations in a variety of human T-lymphoblastoid cell lines (Vince et al., 1988). Comparison with AZT and ddC, two of the most potent and selective inhibitors of the HIV virus (Mitsuya & Broder, 1986; Balzarini et al., 1987), showed CBV to be as effective as AZT or ddC in reducing the expression of viral antigen in HIV-infected CEM cells. Biochemically, the 5′-triphosphate of CBV, CBVTP, is as active as AZTTP or ddCTP against HIV reverse transcriptase but much less inhibitory than the latter two against cellular DNA polymerase β and γ. If the toxicities of AZT [i.e., myelosuppression (Richman et al., 1987)] and the dideoxynucleosides [e.g., peripheral neuropathy for ddC (Yarchoan et al., 1988)] are due to the inhibition of either or both of the said polymerases (White et al., 1989), then CBV offers a therapeutic alternative for the treatment of AIDS which should not exhibit the same toxicity. In this respect, the pathways that affect the metabolism of this type of compound in cells are of considerable interest but have not been investigated. In the present investigation, we have used mutant T-lymphoid cells deficient in nucleoside kinases to analyze the metabolism of carbovir in HIV-infectable cells. These results have revealed novel aspects of carbovir metabolism that may have important implication for the use of carbovir and other agents of this type against HIV infection.

Materials and Methods

The human T-lymphoblast CEM and its subline CARA were previously described (Johnson et al., 1988; Verhoef et al., 1981). The mutant subline CEM/HGPRT$^-$ was obtained from Dr. B. Ullman (Oregon Health Sciences University, Portland, OR).

The syntheses of carbovir (Vince & Hua, 1990) and CBV-TP (White et al., 1989) have been described previously. [8-^{3}H]CBV was obtained from Moravek Biochemicals (Brea, CA). Repurified [8-^{3}H]CBV was prepared by reverse-phase HPLC as described below. All other chemicals were of the highest grade available.

Exponentially growing cells were incubated at 1×10^6 cells/mL in culture flasks for 6 h in the presence of 10 μM drug. Incubation times of 3, 5, 6, and 24 h were initially tested on CEM cells, and 6 h proved to be optimal for the formation of CBV metabolites. Concentrations of 1 and 40 μM CBV were also tried, and these yielded metabolic profiles comparable to that for 10 μM CBV. After incubation, the cells were spun at 235g for 5 min, resuspended in supernatant medium, and dispensed over nyosil. The resulting mixture was microfuged, and the supernate was carefully removed. The pellet was resuspended in ice-cold 70% methanol–25 mM Tris-HCl, pH 7. Extraction was done at −20 °C for 30 min. The mixture was microfuged, and the supernate was stored at −20 °C until analyzed by HPLC. Nucleotides were separated on a Partisil SAX anion exchange column as described before (Johnson & Fridland, 1989). Nucleosides were separated on a Partisil 5 ODS-3 reverse-phase column (10 cm × 3.5 mm; Whatman) and eluted with water for 20 min followed by a linear gradient to 20% methanol over 50 min. For radioactivity measurements, samples were collected directly into scintillation vials, and Sc̸INT-A XF scintillation cocktail (Packard Instrument Co., Inc., Downers Grove, IL) was added to each vial. Nucleotides and nucleosides were identified by comparison of their retention times with those of available authentic standards.

The radiolabeled nucleotides formed from [^{3}H]CBV in cells were purified from the cell extracts by HPLC, desalted by adsorption onto charcoal, and eluted from the charcoal with

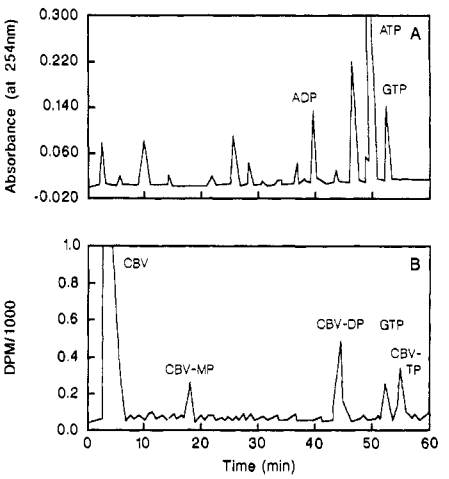

FIGURE 1: SAX ion-exchange HPLC elution profile of extracts of CEM cells: (A) based on absorbance; (B) based on radioactivity. Cells were incubated for 6 h with 10 μM [8-^{3}H]CBV (2 μCi/mL).

5% ammonium hydroxide in 50% ethanol. The eluate was evaporated to dryness and reconstituted with 200 mM Tris-HCl, pH 8.9, 180 mM NaCl, and 60 mM $MgCl_2$. The desalted extract was incubated with 6.6 mg/mL *Crotalus atrox* snake venom (Sigma Chemical Co., St. Louis, MO) and 5 units/mL *Escherichia coli* alkaline phosphatase (Pharmacia) for 1 h at 37 °C. The reaction was terminated with 60% perchloric acid and neutralized with 10 N KOH. The mixture was microfuged, and the supernate was stored at −20 °C until analyzed by HPLC.

Pelleted mitochondria from CBV-treated CEM cells were prepared according to the procedure of Sarup and Fridland (1987) or Bestwick et al. (1982). There were no significant differences in the results from either method. The mitochondrial extract was prepared from a portion of the pellet according to the method of Sarup and Fridland (1987) and assayed for citrate synthase by the method of Srere (1969). Protein concentrations were determined by Bio-Rad protein microassays with bovine γ-globulin as standard. Most (>80%) of the total cellular citrate synthase was present in the said mitochondrial extract, thus confirming that the extract is of mitochondria. The rest of the mitochondrial pellet was extracted with 70% methanol buffer and analyzed by HPLC as described above.

Results

Figure 1A shows a representative HPL profile based on the absorbance of extracted nucleotides from CEM cells incubated in 10 μM [^{3}H]CBV. At the bottom of Figure 1 are profiles of the radioactivity associated with the various absorbance peaks after incubation with [^{3}H]CBV. A major peak, corresponding to that of authentic CBV was seen at about 2-min elution time. Three radioactive peaks at 18-, 46-, and 57-min elution time corresponded to the mono-, di-, and triphosphate derivatives of CBV, on the basis of the retention times of authentic drug standards. One additional peak with a retention time identical with that of GTP was also always detected. Of the total intracellular radioactivity, 4.29% was found in CBV nucleotides (recovery during HPLC separation was >80%) and 0.71% in GTP while the peak containing unmodified [^{3}H]CBV contained approximately 95% of total intracellular radioactivity. At exogenous CBV concentrations of 1, 10, and 40 μM, the ratio between CBV nucleotides and that incorporated into GTP remained constant.

To confirm the identity of each nucleotide peak, the nucleotides purified from the cells were treated with alkaline

Table I: Metabolism of CBV in CEM vs CARA Cells[a]

cell line	pmol/10^6 cells			
	CBV-MP	CBV-DP	CBV-TP	GTP
CEM	0.075 ± 0.009	0.091 ± 0.004	0.039 ± 0.002	0.031 ± 0.002
CARA	0.094 ± 0.012	0.093 ± 0.014	0.026 ± 0.004	0.029 ± 0.002

[a] The data represent the average of two trials. Cells in logarithmic growth phase were incubated with 10 μM CBV (2 μCi/mL) for 6 h. Cells were collected, extracted with 70% methanol buffer, clarified by centrifugation, and analyzed by ion-exchange HPLC on Partisil SAX as described under Materials and Methods.

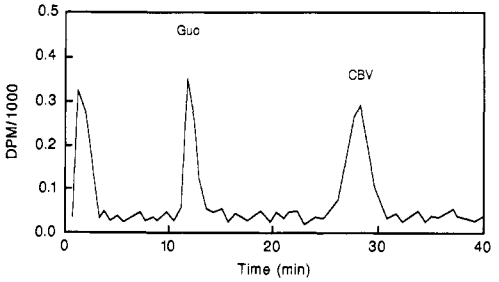

FIGURE 2: Reverse-phase HPLC elution profile of the nucleoside analogues obtained by the enzymatic dephosphorylation of the nucleotide derivatives arising from CBV. Nucleotides were extracted from cells after incubation with 10 μM [^{3}H]CBV (2 μCi/mL for 6 h) and analyzed as described under Materials and Methods by reverse-phase HPLC.

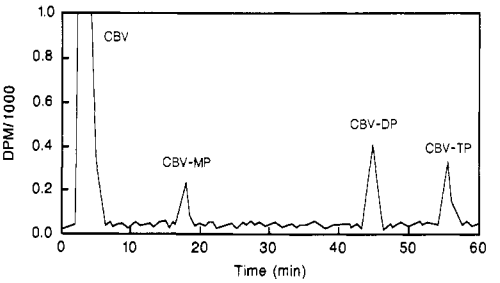

FIGURE 3: SAX ion-exchange HPLC radioactivity profile of extracts of HGPRT-deficient CEM cells incubated with repurified [8-^{3}H]CBV. Cells were incubated for 6 h with 10 μM CBV (2 μCi/mL).

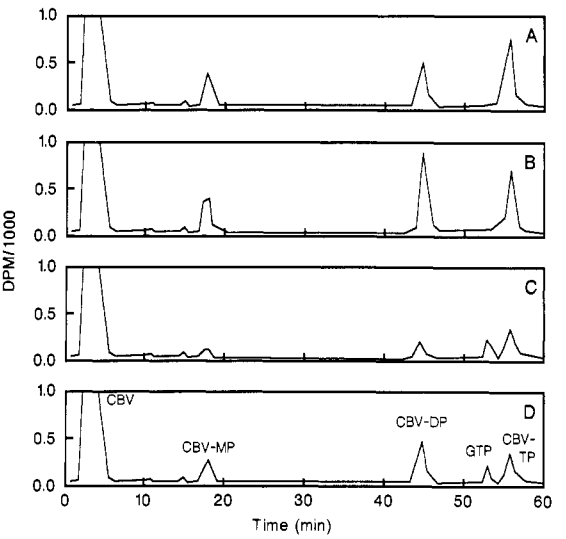

FIGURE 4: SAX ion-exchange HPLC elution profiles of extractions of CEM cells incubated with 10 μM [^{3}H]CBV (2 μCi/mL) and 100 μM adenine (A), hypoxanthine (B), guanine (C), and no purine base added (D).

phosphatase and phosphodiesterase to degrade all the nucleotides to nucleosides, which were subsequently analyzed on a reverse-phase HPLC column. As shown in Figure 2, degradation of the putative radioactive nucleotides resulted in two peaks, which correspond to those of authentic guanosine and CBV.

To further determine the source of the isotope that was incorporated into the various nucleotides, an additional experiment was performed in which cells were incubated with HPLC-repurified [8-^{3}H]CBV just prior to use. The radioactivity incorporated into the nucleotides was found associated with CBV mono-, di-, and triphosphate and GTP, as obtained before (Figure 1B), indicating that the radioactivity incorporated into GTP derives from some intracellular catabolism of CBV during the incubation with CEM cells. To test this further, repurified [^{3}H]CBV was also incubated with an HGPRT-deficient mutant CEM cell (Figure 3). Under these conditions, the radioactivity incorporated into the nucleotides was found associated exclusively in CBV mono-, di-, and triphosphate.

In the extracellular medium, CBV remained essentially in the form of unreacted drug. We did not observe any significant degradation or other CBV metabolites arising from transformation of the drug. The initial 10 μM concentration of CBV decreased to ca. 8.5 μM after 6-h incubation with cells. Thus, approximately 15% of the drug was taken up and metabolized in CEM cells.

In human lymphoid cells, nucleosides are converted to their phosphorylated products via several enzymes of varying specificities. When it became apparent that CBV was converted to nucleotides in CEM cells, we sought to determine whether CBV was phosphorylated by the same enzymes that convert deoxyguanosine to the phosphorylated products. Cytosolic dCyd kinase in human lymphoidal cells has been shown to be the primary pathway for the phosphorylation of dGuo to the monophosphate (Sarup & Fridland, 1987). To investigate the possible role of this enzyme in CBV activation, CARA, a mutant deficient in both dCyd and Ado kinases, was incubated with the drug. As shown in Table I, the pattern of nucleotide accumulation in mutant CEM cells was not altered in any significant way from that seen in wild-type CEM cells. From these results we conclude that neither of these two cytosolic enzymes is essential for CBV phosphorylation.

Another candidate for the phosphorylation of CBV is dGuo kinase, a purine-specific enzyme previously shown to be localized in mitochondria in CEM cells. To determine if the phosphorylation of CBV was mediated via this enzyme, CEM cells were fractionated into mitochondrial and cytosolic fractions after incubation with [^{3}H]CBV and then analyzed for the presence of drug metabolites. The results showed that essentially all CBV nucleotides were recovered in the cytosol (data not shown). Moreover, when CBV was tested as a substrate for mitochondrial dGuo kinase, purified from the double mutant CARA, no phosphorylation activity was detected (data not shown). From these data we have concluded that neither dCyd kinase nor dGuo kinase is primarily responsible for CBV phosphorylation. Earlier studies (Johnson & Fridland, 1989) in our laboratories with CEM cells have shown that a cytosolic 5′-nucleotidase converts CBV to its monophosphate, and this is likely to be its activation pathway.

Table II: Effect of Select Nucleosides and Bases on the Accumulation of the Metabolites of CBV in Human Cells[a]

addition (μM)	% of control		
	CBVMP	CBVDP	CBVTP
none	100	100	100
hypoxanthine (100)	86	115	156
adenine (100)	85	153	268
guanine (100)	35	65	106
inosine (100)	150	159	156
adenosine (100)	126	200	157
guanosine (100)	1	51	34
AZT (10)	61	130	126
ddI (10)	76	112	134
ddG (10)	76	47	120

[a] CEM cells in logarithmic growth phase were incubated with 10 μM CBV (2 μCi/mL) for 6 h in the presence or absence of the listed compounds. Cells were collected, extracted with 70% methanol buffer, clarified by centrifugation, and analyzed by ion-exchange HPLC on Partisil SAX as described under Materials and Methods.

Approximately 15–20% of the radioactivity from [^{3}H]CBV converted to the physiological nucleotide GTP in CEM cells. As shown in Figure 4, addition of 100 μM hypoxanthine or adenine in the incubation with [^{3}H]CBV eliminated almost completely the incorporation of radioactivity into GTP, although guanine was somewhat less effective. Interestingly, the addition of hypoxanthine or adenine, but not guanine, also resulted in the increased accumulation of CBV mono-, di-, and triphosphate in the CEM cells. To test whether the stimulation affected by adenine and hypoxanthine required utilization of these bases for the synthesis of nucleotides, we incubated the HGPRT-deficient cell line with CBV in the presence of 100 μM adenine or hypoxanthine. Under these conditions, no significant potentiation of CBV nucleotide accumulation was observed.

Table II shows the effect of different bases, nucleosides, and dideoxynucleosides on CBV nucleotide accumulation from CBV. As shown by the results, hypoxanthine or adenine and their nucleosides promoted the formation of levels of CBVTP that were up to 170% above control level. This effect was also seen with the dideoxynucleosides AZT, ddI, and ddG.

Discussion

Carbovir is a new carbocyclic guanosine analogue which is regarded as a promising clinical candidate for AIDS treatment because of its selectivity in vitro against HIV (Vince et al., 1988). Moreover, the fact that its triphosphate lacks significant inhibitory activity against the cellular DNA polymerases β and γ offers the potential of a less toxic agent (White et al., 1989). The main results of these experiments are the identification of the metabolic products arising from CBV in human lymphoid cell CEM, the possible enzyme pathway involved in the initial activation of the drug, and the effect of nucleobases and nucleosides in potentiating the accumulation of the putative active metabolites of CBV.

Our study shows that CBV is phosphorylated to CBV mono-, di-, and triphosphate and a small amount is also converted to GTP in CEM cells. With one batch of [^{3}H]CBV, we found that some radioactivity appeared in the adenine nucleotides; but this incorporation could later be attributed to some contamination with [^{3}H]adenosine (<5%) present in the same commercial preparation, which was utilized much more effectively than [^{3}H]CBV for labeling the corresponding adenine nucleotides during the incubation with the cells.

Like several other purine ddNs, the intracellular phosphorylation of CBV was rather inefficient. During 6 h of incubation, the amount of CBV mono-, di-, and triphosphate accumulated in CEM cells was approximately 0.2 pmol/10^6 cells (which is equivalent to 0.08 μM) when the exogenous concentration of CBV was 10 μM. The remainder of the intracellular radioactivity was retained primarily as unmodified CBV (>90%). Nevertheless, the amount of CBVTP formed, which is ca. 0.03–0.10 pmol/10^6 cells (ca. 0.015–0.050 μM), is near its ID_{50} (0.03 μM) for inhibition of HIV-1 reverse transcriptase (White et al., 1989).

The demonstrated conversion of CBV to the various nucleotides is the first demonstration that this analogue can be phosphorylated by intact human cells. The fact that CBVTP is formed in the CEM double mutant deficient in both dCyd kinase and Ado kinase to about the same extent as in wild type indicates that neither of these two enzymes is responsible for its phosphorylation. This result is also consistent with the lack of detectable substrate activity with highly purified dCyd kinase from CEM cells and the failure of CBV to block the phosphorylation of dCyd with the enzyme (data not shown). Studies with mitochondria showed that CBV was taken up into this organelle (ca. 5–7% of the total intracellular CBV), but no phosphorylation of the drug could be detected in mitochondria or with purified dGuo kinase isolated from the cells. In contrast, a cytosolic 5′-nucleotidase that can transfer the phosphate group of a nucleoside 5′-monophosphate to inosine and guanosine analogues has been shown by our laboratory to catalyze the phosphorylation of CBV (Johnson & Fridland, 1989). This enzyme has recently also been shown to phosphorylate other purine antivirals including 2′,3′-dideoxyinosine (Johnson & Fridland, 1989) and the guanosine analogue acyclovir (9-[(2-hydroxyethoxy)methyl]guanine) (Keller et al., 1985). Taken together, these results suggest that the 5′-nucleotidase provides an alternative route for the initial step of activation of guanosine analogues such as CBV, which are not substrates for the known cellular kinases. Subsequent phosphorylation to analogue di- and triphosphates occurs presumably via the normal nucleotide kinases.

Of additional interest is the demonstration that in CEM cells metabolism of CBV to the physiological nucleotide GTP also occurs. This metabolism could be a result of some degradation of CBV presumably by PNP during the incubation. This conclusion is consistent with the data showing that incubation of CEM mutant lacking in HGPRT activity or coincubation of [^{3}H]CBV with the base hypoxanthine or adenine in wild type almost completely inhibited this conversion of CBV to GTP. However, it remains unclear why addition of 100 μM guanine did not similarly prevent this incorporation in GTP.

The mechanism by which the physiological purines or their corresponding nucleosides potentiate the accumulation of CBV nucleotides from CBV remains to be elucidated, but these results suggest that the anti-HIV activity of CBV in vivo may be enhanced by their presence. The studies with an HGPRT-deficient mutant indicate that the effect of the nucleobases depends on their conversion to the nucleotides. A consideration of the nucleotidase as the anabolic pathway for this agent suggests a possible metabolic basis for this effect. 5′-Nucleotidase is a regulatory enzyme, and its activity is controlled by the size of IMP and ATP pools (Johnson & Fridland, 1989). Addition of hypoxanthine, adenine, inosine, or adenosine leads to an increase in the intracellular levels of IMP and ATP. ATP has been shown to stimualte this enzyme activity, and we have shown that the catalytic activity of the enzyme is increased by increasing the concentration of the phosphodonor IMP (Johnson & Fridland, 1989). Increases in CBV anabolism may be explained by a stimulation in nucleotidase activity by increasing levels of IMP or ATP in the

presence of exogenous hypoxanthine, adenine, or their respective nucleosides.

In conclusion, CBV is a new anti-HIV compound endowed with selective and potent antiretrovial activity. Our current studies suggest strongly the strategy of exploring the effect of nucleobases, nucleosides, or other compounds to enhance the antiretroviral effectiveness of CBV.

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NMR and Computational Studies of Interactions between Remote Residues in Gangliosides†

J. Neel Scarsdale,*,‡ James H. Prestegard,§ and Robert K. Yu‡

Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Box 614 MCV Station, Richmond, Virginia 23298-0614, and Department of Chemistry, Yale University, Box 6666 Yale Station, New Haven, Connecticut 06511

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ABSTRACT: Conformational preferences of the gangliosides GM1, GM1b, and GD1a have been investigated by using a systematic combination of NMR distance constraints and molecular mechanics calculations. These gangliosides share a common four-sugar core but differ in the number or placement of sialic acid residues attached to the core. Placement of the sialic acid residues is shown to influence the preferred core conformation. The origin of these effects is postulated to be intramolecular interactions of the sialic acid residues with other remote residues. In the case of GM1, hydrogen bonds between the internal sialic acid and an *N*-acetyl group on GalNAc are suggested. In the case of GD1a, a hydrogen-bonding network between the terminal and internal sialic acids is suggested to play a role.

Cell surface oligosaccharides, such as those which occur in the headgroups of glycolipids, display a diversity in both primary structure and tertiary conformation that makes them useful mediators of cell-specific interactions. Although there is general recognition of the importance of sequence-specific recognition of these cell surface receptors, there have been few attempts to delineate direct recognition of residue functionality from recognition of conformational properties of specific regions. It is not always easy to distinguish between these possibilities because alteration of primary structure at a point well removed from the oligosaccharide segment involved in recognition may in principle affect the conformation of the recognition site. Only recently has evidence begun to accumulate to suggest the importance of conformation and modulation of this conformation by remote residue substitution.

Biochemical data on the specificity of some closely related antibodies can be interpreted in terms of remote group induced alterations in the conformation of primary recognition sites. One example occurs in the case of type-specific streptococcal polysaccharides (Jennings & Kasper, 1981; Schifferle et al., 1985). Antisera raised against type III group B streptococci were determined to contain two antigenically distinct populations of antibodies (Kasper et al., 1979; Jennings et al., 1980). One class of antibodies is specific for the native type III antigen, which has a β1→4Glc(β1–4)[NeuAc(α2–6)]Gal(β1–4)GlcNAc(β1–4)Gal repeating unit, while a second class of

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* Author to whom correspondence should be addressed.
‡ Virginia Commonwealth University.
§ Yale University.

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