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Histo-blood group p: biosynthesis of globoseries glycolipids in EBV-transformed B cell lines*

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The genetic and biosynthetic basis of the histo-blood group P-system is not fully understood. Individuals with the rare p phenotype do not express the three glycolipid antigens (P^k, P and P_1) of this system, probably because of deficiencies in glycosyltransferases involved in their biosynthesis. Iiuka *et al.* [Iiuka S, Chen SH, Yoshida A (1986) *Biochem Biophys Res Commun* **137**: 1187–95], however, previously reported that detergent extracts from an EBV-transformed B cell line derived from a p individual did express the glycosyltransferase activity (P^k transferase) assumed to be missing in this blood group status. Here, we have reinvestigated the antigen expression and glycosyltransferase activities in two p individuals by analysing EBV-transformed cell lines as well as erythrocytes to confirm the blood group P status. The thin layer chromatography glycolipid profile of extracts from erythrocytes and EBV-transformed B cell lines showed characteristic accumulation of lactosylceramide and absence of P^k and P antigens. Glycosyltransferase activities of the B cell lines were analysed using glycolipid substrates and both extracts were found to contain lactosylceramide synthetase and P transferase activities but to be completely devoid of P^k transferase activity. The presented data indicate that p individuals, in contrast to previous reports, do not express a functional P^k glycosyltransferase.

Keywords: P blood group, glycolipids, glycosyltransferases, B cell lines

Introduction

The human histo-blood group P-system involves three carbohydrate antigens and five phenotypes (for structures and phenotypes see Table 1). The genetic and biosynthetic basis of the antigen polymorphism has been proposed to involve distinct genes encoding glycosyltransferases capable of synthesizing P^k (UDP-Gal: Lac-Cer α1-4-galactosyltransferase), P (UDP-GalNAc: Gb3 β1-3-*N*-acetylgalactosaminyltransferase) and P_1 (UDP-Gal: nLc4 α1-4-galactosyltransferase) (for review see [2]). The molecular genetic basis of carbohydrate defined blood group polymorphism has recently been established for the ABO, Lewis, and H/Se systems [3–5]. In these blood group systems, a null phenotype has been associated with a structurally defective enzyme protein due either to frame shift mutations or to mutations leading to unfavourable amino acid substitutions. In contrast, there is, so far, no information as to the primary structure of the glycosyltransferases involved in the synthesis of the P blood group antigens.

Studies of the P blood group system are hampered by the extremely rare incidence of the P^k and p phenotypes. Furthermore the biosynthetic scheme seems to be quite complex involving several apparently independent steps with distinct transferase activities and presumably distinct genes. The initial step in the P biosynthesis involves an α1-4 galactosyltransferase (P^k transferase) adding galactose to lactosylceramide (LacCer). In a previous study, Iizuka *et al.* [1] demonstrated that an EBV-transformed B

*Dedicated to Professor S. Hakomori in the occasion of his 65th birthday from two of his past posdoc's.

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Table 1. The P blood group system.

Phenotype	*Frequency*	*Antigens on erythrocytes*	*Antibodies in serum*
P_1	75%	P_1, P (Gb4)	none
P_2	25%	P (Gb4)	anti-P_1
$P_1{}^k$	very rare	P_1, P^k (Gb3)	anti-P
$P_2{}^k$	very rare	P^k (Gb3)	anti-P
p	very rare	none	anti-P_1PP^k

Structure of the antigens:
P_1: Galα1 → 4Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1Cer
P (Gb4): GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1Cer
P^k (Gb3): Galα1 → 4Galβ1 → 4Glcβ1Cer
p (LacCer): Galβ1 → 4Glcβ1Cer

cell line derived from a p individual did not contain appreciable amounts of P^k antigen but expressed P^k glycosyltransferase activity in a comparable amount to P_1 cells. The authors therefore suggested a regulatory mechanism for the p phenotype, i.e. the *P^k* glycosyltransferase gene was present and transcribed but the resultant transferase was, for some unknown reason, not functional *in vivo*. A similar regulatory mechanism was recently identified for the Tn polyagglutination phenomenon, where T cell clones of Tn phenotype could be reactivated to express the β1-3-galactosyltransferase leading to the synthesis of the T antigen [6]. A study by Kijimoto-Ochiai *et al.* [7], however, indicates that this might not be the case for the P blood group system since they found no P^k transferase activity in p fibroblasts.

Here, we have analysed the antigen and transferase expression involved in the P-blood group system in erythrocytes and newly established EBV-transformed B cell lines from two p individuals. We show that these cells lack the P^k and P antigens but contain and express LacCer on the cell surface. We also report that EBV-transformed cell lines of the p individuals are completely devoid of P^k glycosyltransferase activity but that they do have a high P glycosyltransferase activity.

Material and methods

ESTABLISHMENT OF EBV-TRANSFORMED B CELL LINES AND CELL CULTURE

Fresh peripheral blood lymphocytes (PBL) from p individuals were purified by Ficoll-Hypaque density centrifugation. After washings, 3×10^6 cells were cultured with the B95-8 strain of Epstein-Barr virus (contained in 1 ml of concentrated cell-free supernatant from the B95-8 marmoset cell line) in 5 ml of RPMI 1640 medium containing 2 mM L-glutamine, 50 μg ml^{-1} gentamycin, 20 mM glucose, 1 mM pyruvate and supplemented with 10% foetal calf serum (FCS). The flasks were incubated at 37 °C in an humidified atmosphere of 5% CO2 in air and cells were fed weekly with fresh RPMI-10% FCS. Transformation was then assessed visually and emerging cell lines were expanded before being subjected to immunofluorescence analysis with various mAb directed against B cell differentiation antigens.

The Ramos Burkitt's lymphoma (BL) cell line was a gift from Professor G. Klein (Stockholm, Sweden) and the myeloma cell line U266 was obtained from Dr B. Klein (Montpellier, France). IARC 174 (an EBV-transformed cell line from a P individual) was established at the International Agency for Research on Cancer (Lyon, France) and kindly given by Dr G. Lenoir.

All cell lines were cultivated in RPMI 1640 medium containing 2 mM L-glutamine, 50 μg ml^{-1} gentamycin, 20 mM glucose, 1 mM pyruvate and supplemented with 5% FCS.

ANTIBODIES

B9E9 (anti-CD20) was purchased from Immunotech (Marseille, France). T5A7 (anti-LacCer) was established and kindly given by Dr I. Bernstein [8] and CLBery-2 (anti-P/Gb4) by Dr A.E.G. Kr. von dem Borne [9]. 38.13 hybridoma (anti-P^k/Gb3) was prepared in one of our laboratories as previously described [10, 11].

FLOW CYTOMETRY

Cells were washed twice in cold phosphate-buffered saline (PBS) and 5×10^5 cells were transferred to 96 well round-bottomed microculture plates and resuspended in 100 μl of PBS containing 5% FCS and the monoclonal antibodies. After 45 min incubation at 4 °C, cells were washed twice with cold PBS and resuspended in 100 μl of PBS containing 5% FCS and fluorescein isothiocyanate-labelled goat anti-mouse antibody (GAM-FITC). Cells were incubated for 45 min at 4 °C in the dark, followed by two washes in cold PBS and fixation in 2% paraformaldehyde prior to analysis. Samples were analysed by flow cytofluorometry using an Epics C cell sorter (Coulter, France).

GLYCOLIPID PURIFICATION AND CHARACTERIZATION

Glycolipid were prepared as previously described [12]. In brief, 2–5 ml of packed cells (approximately 2×10^9 to 5×10^9 cells) were homogenized, extracted twice with 20 volumes of isopropanol:hexane:water (55:25:20, by vol) and then submitted to Folch's partition [13]. The lower phase (LP) glycolipids were purified and freed from contaminating lipids and phospholipids by acetylation procedure: glycolipids were acetylated according to the method of Saito and Hakomori [14] and subjected to Florisil column chromatography (Sigma, France) and deacetylation. Folch's LP contains neutral glycolipids having short carbohydrate chains (less than five sugars) and also part of the GM3 ganglioside content.

LP glycolipids were then taken in chloroform:methanol (2:1, by vol) in a quantitative manner: 100 μl for 10^9 cells. Glycolipids were chromatographed on High Perfor-

mance Thin Layer Chromatography (HPTLC) plates (J.T. Baker Chemicals, France) and then visualized by spraying with 0.5% orcinol in 10% sulphuric acid and heating at 120 °C for 10 min.

TLC immunostaining was performed as previously described [15]. Briefly, glycolipids were chromatographed on HPTLC plates and incubated overnight with 38.13 mAb. After washing, plates were incubated for 2 h with rabbit anti-rat IgM and then for 1 h with ^{125}I-labelled protein A. After drying, the plates were submitted to autoradiography.

GLYCOSYLTRANSFERASE ASSAYS

After culture, the cells were washed twice with PBS and the pellets were homogenized at 4 °C in two volumes of buffer (20 mM HEPES buffer, pH7, 0.5 M sucrose, 1 mM EDTA) with a Dounce homogenizer. Protein concentrations of these crude homogenates, which were used as enzyme source, were determined by using the Bio-Rad protein assay reagent with bovine serum albumin as a standard.

The optimal requirements of the glycosyltransferases in B cells were previously determined in our laboratory [16, 17]. The reaction mixtures were incubated for 2 h at 37 °C and the reaction was stopped by the addition of 900 μl of H_2O. The radioactive glycolipids formed were separated from the radioactive sugar-nucleotides by a reverse phase C-18 Bond elut cartridge (Varian, Les Ulis, France) as described previously [18]. After evaporation of the solvent, the glycolipids were dissolved in 40 μl of chloroform:methanol (2:1, by vol) and chromatographed on HPTLC plates in a solvent composed of chloroform:-methanol:water (60:35:8, by vol). Labelled glycolipids were located by autoradiography and identified by co-migration with standard glycolipids which were visualized by orcinol spray. The labelled products were scraped from the plates and quantified by liquid scintillation counting. Control assays without substrate acceptors were used to correct for endogenous acceptors and were thus substracted in the calculation of activities.

Assay of Gb3 and LacCer synthetase activities

The reaction mixtures for the Gb3 synthetase (or UDP-galactose: lactosylceramide $\alpha 1 \rightarrow 4$ galactosyltransferase) or LacCer synthetase (or UDP-galactose: glucosylceramide $\beta 1 \rightarrow 4$ galactosyltransferase) contained 5 μmol of sodium cacodylate buffer, pH 5.9, 50 nmol of LacCer (for Gb3 synthetase) or 50 nmol of GlcCer (for LacCer synthetase), 300 μg of Triton X-100, 1 μmol of $MnCl_2$, 0.5 μmol CDP-choline, 0.5 μmol of galactonolactone, 310 nmol of phosphatidyl glycerol, 30 nmol of UDP[^{14}C]-galactose (325 mCi mmol^{-1}) and 100–500 μg of cellular protein in a total volume of 100 μl. The incorporation of [^{14}C]galactose into glycolipids was determined as described above.

Assay of Gb4 synthetase

The reaction mixtures for the Gb4 synthetase (or UDP-*N*-Acetylgalactosamine: Gb3 $\beta 1 \rightarrow 3$ *N*-acetylgalactosaminyltransferase) contained 5 μmol of sodium cacodylate buffer, pH 6, 42 nmol of Gb3, 300 μg of Triton X-100, 1 μmol of $MnCl_2$, 1 μmol CDP-choline, 0.5 μmol of galactonolactone, 310 nmol of phosphatidyl glycerol, 31 nmol of UDP[^{14}C]*N*-Acetylgalactosamine (60 mCi mmol^{-1}) and 100–500 μg of protein in a final volume of 100 μl. The incorporation of [^{14}C]*N*-acetylgalactosamine into glycolipids was determined as described above.

Results

Glycolipid content of erythrocytes and EBV-transformed B cell lines of p individuals

Blood samples were obtained from three p individuals (EB, LS, OC). In order to confirm their phenotype, glycolipids were extracted from erythrocytes of these three individuals and from two EBV-transformed B cell lines, derived from B lymphocytes of EB and LS individuals. Furthermore, glycolipids were also obtained from Ramos cells, derived from a Burkitt's lymphoma. Folch's LP glycolipids were then chromatographed with standard glycolipids and orcinol stained. As shown in Fig. 1A, EB and LS B cell extracts contained a major component which had the same mobility as LacCer. In contrast, this compound was very minor in LP extracted from Ramos cells where the major glycolipid co-migrated with Gb3, the P^k antigen. In LP extracts obtained from erythrocytes, the major band co-migrated with LacCer and other compounds migrating slightly below Gb4 were also present. Since a faint band with the mobility of Gb3 was detectable in these samples, we performed a TLC immunostaining with 38.13, a mAb recognizing Gb3, to determine if this compound could be Gb3. It can be seen in Fig. 1B, that 38.13 mAb reacted strongly with the standard Gb3 and with the compound present in the Ramos LP glycolipids whereas an extremely faint band could also be seen in the lane corresponding to LS erythrocyte glycolipids. However, comparison of this staining with the clear detection by 38.13 mAb of the very low amount of Gb3 present in the Gb4 standard lane (see very faint orcinol staining in Fig. 1A) strongly suggests that the glycolipid expressed by LS cells is not Gb3. We therefore have, so far, no explanation for this faint staining. No reaction with the anti-Gb3 mAb could be observed in the LP obtained from p EB and OC individuals.

Cell surface expression of glycolipid antigens on EBV-transformed B cell lines from p individuals

The two EBV-transformed B cell lines derived from B lymphocytes of EB and LS individuals were analysed for

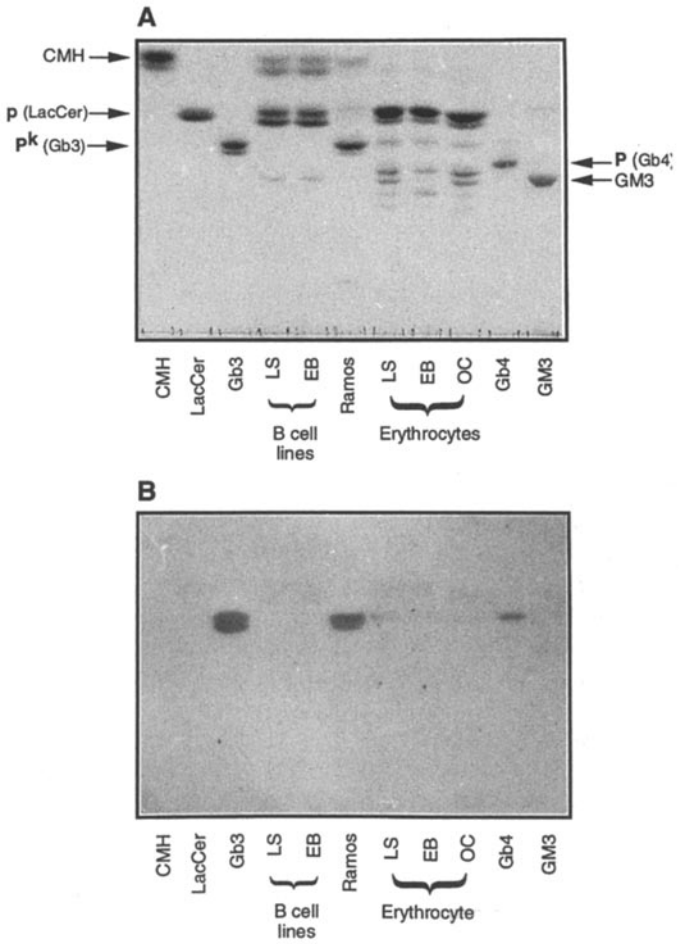

Figure 1. Immunostaining pattern of p cell glycolipids. Folch's LP glycolipids were extracted from EB and LS cells (EBV-transformed cells from p individuals) and from Ramos cells (Burkitt's lymphoma cells). (A) Chemically stained with orcinol-H2SO4 reagent. (B) immunostained with 38.13 anti-Gb3 mAb. Solvent system for TLC was chloroform:methanol:water (60:35:8, by vol.

cell surface expression of CD20 (a pan-B cell differentiation antigen), LacCer, P^k (Gb3) and P (Gb4) antigens. Two other B cell lines, Ramos and IARC 174 were used as controls. As shown in Fig. 2, EB and LS cells as well as Ramos and IARC 174 expressed CD20 antigen. In contrast, the carbohydrate antigen profile of the four cell lines greatly differed: EB and LS cells were strongly labelled by T5A7, the anti-LacCer mAb whereas Ramos and IARC 174 were negative or very weakly stained; as previously described, 38.13, the anti-P^k mAb gave a very strong staining of the Ramos BL cells [10] and was negative with EB, LS and IARC 174 cells; CLBery2, the anti-P mAb was only positive on IARC 174 cells. Together with the results on the glycolipid content, these results demonstrate that the EB and LS B cell lines have retained the p phenotype.

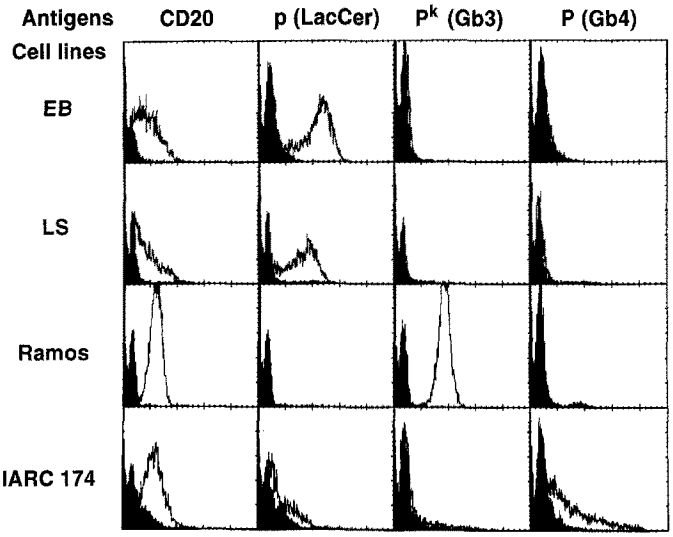

Figure 2. Immunofluorescence analysis of p B cell lines. EB and LS cells (EBV-transformed cells from p individuals), Ramos cells (Burkitt's lymphoma cells) and IARC 174 cells (EBV-transformed cells from a P individual) were labelled with B9E9 anti-CD20 mAb, T5A7 anti-LacCer mAb, 38.13 anti-P^k mAb and CLBery2 anti-P mAb followed by GAM-FITC (unshaded) or labelled with GAM-FITC alone (shaded).

Glycosyltransferase activities of EBV-transformed B cell lines from p individuals

In order to elucidate the biosynthetic mechanism leading to the accumulation of LacCer in B cell lines of p individuals, we have measured the activities of the three glycosyltransferases involved in the synthesis of LacCer, P^k (Gb3) and P (Gb4) antigens. Results of the specific activities of these three enzymes in crude cell homogenates are shown in Table 2. It can be seen that EB and LS cell lines exhibit a high specific activity of the LacCer synthetase whereas the P^k transferase is undetectable in these cells. Interestingly, EB and LS cells also contain an elevated specific activity of the P transferase which is even higher than the activity observed for most of the EBV-transformed cell lines that we previously tested [17]. The specific activities of the three glycosyltransferases

Table 2. Specific activities of glycosyltransferases in crude homogenates of various B cell lines.

Cell lines	*LacCer synthetase*	*P^k transferase* ($pmol\,h^{-1}\,mg^{-1}$)	*P transferase*
EB	50 ± 4	<1	64 ± 7
LS	41 ± 6	<1	52 ± 6
U266	8 ± 1	326 ± 35	<1
Ramos	ND	408 ± 17	<1
IARC174	ND	5 ± 3	44 ± 6

Results are given as mean ± SD of three or four independent experiments. ND, not determined.

were also measured in control cell lines and were similar to previous results [17].

Discussion

The P blood group system comprises five phenotypes defined by the expression of three different glycolipids on the erythrocytes. Two of these antigens are biosynthetically related with a direct precursor-product relationship, therefore loss of P^k synthetase will result in loss of both P^k and P antigens (see Table 1).

It is generally assumed that the p and P^k phenotypes result from the lack of the appropriate glycosyltransferase activities similarly to what has been observed for other carbohydrate defined histo-blood group antigens [2]. In the ABO, Lewis, and H/Se systems, for example, the null phenotypes are associated with structurally defective genes leading to no protein or enzymatically inactive proteins [4, 5]. The molecular genetic basis for the lack of glycosyltransferase activities, however, is not resolved for the P blood group system and a previous report has even suggested that p individuals could carry an intact, but *in vivo* non-functional, P^k glycosyltransferase gene [1]. Interestingly, it has recently been established that the Tn mosaicism, in affected T cells of Tn individuals, is the result of a regulatory change since the β1-3-galactosyltransferase leading to the T antigen can be induced by transcriptional activators [6].

Purification of glycosyltransferases is cumbersome and often unsuccessful. Transfection cloning, as introduced to glycosyltransferase cloning by Lowe *et al.* [19], is a good alternative but generally requires a host cell to be transfected, which lacks the glycosyltransferase activity in question. Cell lines from p individuals could potentially be used for this purpose although the report of Iizuka *et al.* [1] raised the problem that an endogenous P^k transferase gene may be activated *in vitro*.

These observations have prompted us to reinvestigate the P^k transferase activity in p cells. We have established EBV-transformed cell lines from B lymphocytes of two p individuals and examined their cell surface expression and total content of LacCer, P^k (Gb3) and P (Gb4) glycolipids as well as the specific activities of the glycosyltransferases implied in the synthesis of these compounds. We now report that the erythrocytes and B cell lines of these p individuals contain high amounts of LacCer and that this glycolipid is expressed on the surface of the B cells. On thin layer chromatograms, LacCer appears as a doublet band due to heterogeneity in its lipid moiety but, as previously reported by Marcus *et al.*, the increase in LacCer, in p erythrocytes, is almost entirely restricted to the upper band [2]. Interestingly, this is not the case for the B cell lines where both components are almost equally accumulated. Such a diversity in the fatty acid moiety of glycolipids between erythrocytes and other tissues has previously been observed [20] and is likely due to differences in the biosynthetic pathway of ceramide in the various types of cells.

In this work, we also demonstrate that the B cell lines completely lack P^k transferase activity but contain P transferase activity. The reason for the apparent conflict of our data with those of Iizuka *et al.* [1] showing that EBV transformed cells from one p individual have the capacity to synthesize the P^k antigen *in vitro*, is at present, unknown. One has to notice, however, that the P^k transferase assay performed by these authors involved a 24 h incubation time which is rather unusual for a glycosyltransferase test. Furthermore, in previous studies, where this enzyme was tested in various types of cells, it was shown that its activity was maximum after 2 h of incubation [7, 16, 21]. It must also be underlined that in contrast to the patients studied by Iizuka *et al.* [1] we have confirmed the p phenotype of our two patients by analyzing the glycolipid profile of erythrocytes. Thus, either several subgroups of p individuals exist or the patient studied by Iizuka *et al.* [1] was not a genuine p individual.

Kijimoto-Ochiai *et al.* [7] also tested the P^k transferase activity in p individuals and found, in agreement with the data presented herein, that p fibroblasts were devoid of P^k transferase activity. In this study, P transferase activity was also tested and was not detected in p fibroblasts whereas we found a quite high activity of this enzyme in p B cell lines. One might suggest that this discrepancy is due to the difference in cell types tested. Our present results are, however, in accordance with a previous report by Fellous *et al.* [22] showing that somatic hybrids, created by the fusion of fibroblasts from p and P^k individuals, expressed the P antigen and assuming that the p cells supplied the P transferase.

The absence of P^k transferase activity in the p cells is not the only intriguing and controversial issue concerning the P blood group system. The P^k and P_1 antigens, which share the terminal immunodominant disaccharide Galα1 $\rightarrow$ 4Gal, are independently expressed in P^k and P individuals but the unexplained finding that p persons lack both of these antigens suggests that the biosynthesis and genetic regulation of P^k and P_1 antigens are related (for review see [23]). Two main hypotheses have been proposed. In one case, it is considered that the P^k and P_1 genes would code for two different α-galactosyltransferases but that these two genes are linked and affected by a single genetic event in the p persons. In the other case, it is suggested that the P^k gene would code for an α-galactosyltransferase using only LacCer as substrate and that the P_1 gene product would alter the substrate specificity of this enzyme such as it would become able to use LacCer and nLc4 as substrates. Inactivation of the P^k gene would then cause the p phenotype. Analysis of

the glycolipid profile of EBV-transformed B cell lines indicated that these cells contain globo- and ganglio-series glycolipids but no lacto-series [17], which makes the analysis of expression and biosynthesis of P_1 antigen in this cell type probably not relevant. We have, however, assayed the galactosyltransferase activity of our p B cell lines using nLc4 as substrate (data not shown) but since the majority of product formed in this case was a β-linkage we were unable to conclude about the presence or not of a P_1 synthetase (α-galactosyltransferase) activity in p B cells.

The molecular genetic basis of carbohydrate defined histo-blood group systems has long remained unresolved due to the inherent difficulties in obtaining purified transferase proteins for amino acid sequencing and cDNA cloning. Attempts to clone the P^k transferase have involved classical purification of the enzyme [24] as well as cDNA transfection experiments [25], as yet without success. The cDNA transfection approach was apparently partly unsuccessful because of lack of a suitable cell line which was truly deficient in the P^k transferase. The two EBV-transformed cell lines from the p individuals will be important tools for further studies aimed at cloning the P^k transferase gene.

Insights into the P^k transferase gene and its regulatory elements would be of special interest for studies of the biological role that the P^k antigen appears to play in the late stages of B cell maturation. During B cell differentiation, cell surface expression of Gb3 is restricted to B lymphocytes located in germinal centres [26–29], the site where the maturation of the humoral immune response takes place. During this process, germinal centre B cells randomly diversify their *Ig* genes by a somatic hyper-mutation mechanism, after which, B lymphocytes containing high affinity variants are positively selected whereas B cells with either non-functional or low affinity Ig rapidly die by apoptosis [30]. Since we have recently demonstrated that Gb3 is able to transduce a signal leading to apoptosis of the cells [31], one can assume that this glycolipid is directly involved in this selection process. To further investigate the function of Gb3, we plan to test the expression of this glycolipid on germinal centre B lymphocytes of p individuals. If, as most probable, these cells do not express Gb3, we will study how the maturation of B lymphocytes arises in the p individuals.

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