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A Comparison of Established Human Lymphoma Lines by Flow Cytometry: Quantitation of *Ricinus communis* Agglutinin Binding and the Effect of Specific Glycosidases*

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Abstract—*Two established cell lines of human B-cell lymphomas derived from Burkitt lymphomas and their Epstein-Barr virus-transformed counterparts were analyzed with respect to their ability to bind the β-galactoside-specific lectin* Ricinus communis *agglutinin (RCA). Native and sialidase- as well as sialidase-β-galactosidase-treated cells were compared. The method for the quantitative determination of average numbers of binding sites and of apparent affinity constants was flow cytometry with fluorescence-labeled lectin. Although with native cells there was no significant deviation of the values for virus-transformed cells from those for the parent cells, some differences could be detected after glycosidase treatment. The general procedure of the combined application of specific glycosidases and the quantitation of sugar-specific lectin binding is recommended as a general strategy for the differentiation of cells with known or putative differences in biological functions.*

INTRODUCTION

CARBOHYDRATES of cell surface glycoconjugates (glycoproteins and glycolipids) play an important role as the main determinants of many receptor structures. This function of saccharide moieties has only recently been fully appreciated, maybe because the 'central dogma' of molecular biology has dominated biological thinking in such a way that only nucleic acids and proteins were considered as informational molecules. Certainly, carbohydrates are not direct gene products; yet they contain a high degree of biological information. This information, however, is mediated by gene products, namely specific glycosyltransferases and glycosidases.

The informational role of cell surface oligosaccharides relates predominantly to recognition phenomena, such as hormone-receptor interactions, antigen-antibody reactions, adsorption of viruses and toxins, internalization of lipoproteins by macrophages, tissue-specific migration (e.g. 'homing' of lymphocytes), cell adhesion and most likely also metastatic capacities [1-7].

Thus carbohydrates will have an important bearing on cell differentiation and, conversely, on malignant aberration: the appearance or disappearance of 'differentiation antigens' or 'tumor-associated neoantigens' may be brought about by a modulation of the activity (maybe also of the specificity) of respective glycosyltransferases and/or glycosidases. The latter can also be demonstrated on cell surfaces [e.g. 8]. It is of special interest that such modulations may well be epigenetic events, leading to under- or overglycosylated oligosaccharide side chains which, in turn, receive 'wrong' signals. Thus it seems of interest to compare cells with different genetic or functional properties on the basis of the oligosaccharide side chain patterns of their surfaces. For the determination of surface sugars on living cells, the class of lectins is especially useful [9]. Lectins are di- or multivalent protein or glycoprotein molecules with selective affinities

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for carbohydrates, which at higher concentrations are able to agglutinate cells that possess a sufficient number of binding sites (= specific carbohydrate residues) for the lectin. For a review see Goldstein and Hayes [10]; a discussion of possible biological functions of lectins has been presented by Barondes [11].

One of the most vividly discussed carbohydrates in recognition phenomena in man is β-galactose [4], which in most oligosaccharides appears penultimately and is 'masked' by one or even two terminal sialic acid residues.

The β-galactoside-specific lectin *Ricinus communis* agglutinin (RCA) used in this study binds with a remarkable degree of specificity: binding is easily and completely inhibited by preincubation of the lectin with a sugar hapten, preferably lactose. Moreover, if binding studies with RCA are performed at subsaturating and non-agglutinating concentrations, an apparent equilibrium binding (affinity) constant can be extrapolated which is in the neighborhood of values for hormone-receptor interactions, i.e. the binding site reacts very avidly with monoclonal antibodies which are currently being used for cell typing purposes [12].

In addition to RCA binding studies with native cells it is of special significance to observe the changes in lectin binding patterns after treatment of the cells with specific glycosidases, notably sialidase and β-galactosidase. Since commercially available galactosidases are inefficient with mammalian cells, an enzyme was isolated from *Streptococcus pneumoniae* (see Materials and Methods) which proved to be highly effective.

The most effective way to determine binding parameters on living cells is flow cytometry with fluorescein-labeled RCA: flow cytometry enables the obtaining of information on binding properties of single intact cells which can be correlated with other parameters, e.g. size, viability, functional properties. Since all data can be retrieved as histograms, even heterogeneous cell populations may be analyzed. Moreover, if laser beam-operated instruments are used, binding parameters can be derived in equilibrium, without prior separation of bound molecules from unbound ones. In general, the important parameters R_o (number of receptors at occupancy) and K_A (the apparent equilibrium association or affinity constant in l/mol) are determined by modified Scatchard analysis. In this report the term 'receptor' is used somewhat loosely and should be regarded as synonymous with 'specific binding site'. As a rule, non-viable cells are excluded by an additional fluorescence marker. For more details refer to Materials and Methods and [13–16].

The cells compared in this study with respect to their ability to bind RCA and to react with glycosidases are established human lymphoma cells with B cell characteristics, derived from Burkitt lymphomas that are EBV (Epstein-Barr virus)-negative (Ramos, BjAB) and their transformed counterparts, i.e. cells infected with two different strains of EBV which render them EBV-positive [17–20].

MATERIALS AND METHODS

Cells

Two different established human lymphoma lines (Ramos, BjAB) and their EBV-transformed derivatives were used: the Ramos line originates from an 'American' Burkitt lymphoma, while BjAB is derived from an 'African' Burkitt-like lymphoma. The lines were established by Klein and co-workers [17, 18]. Although negative with respect to the Epstein-Barr nuclear antigen (EBNA), these cells possess EBV receptors and could be permanently transformed into EBV-positive cells by different strains of EBV with differences in the infectibility according to the EBV strains B95/8 and P3HR-1 [17, 18]. At the same time, Fresen and co-workers [19, 20] were able to establish EBNA-positive lines from both lymphomas with both virus strains. These cells were used for our experiments and will be designated Ramos, RAM-B95/8, RAM-HR1K, BjAB, BjA-B95/8 and BjA-HR1K. The cells were routinely passaged three times weekly. The suspension medium consisted of RPMI 1640 medium, supplemented with 10% fetal calf serum and 100 U/ml penicillin plus 100 μg/ml streptomycin.

Fluorescent Ricinus communis *agglutinin (RCA-FITC)*

RCA was prepared according to the procedure described by Nicolson and Blaustein [21], with slight modifications and additional purification steps. This resulted in ricin-free RCA_I of molecular weight 120,000. Covalent labeling of the lectin with fluorescein isothiocyanate (FITC) was achieved by a modified 'Celite' method [22, 23]. After column chromatography, the lectin was lyophilized and refrigerated. The retention of binding properties was demonstrated by competitive displacement studies with native lectin, as described previously [13–16].

Enzymes and cell treatment

Sialidase from *Vibrio cholerae* (EC 3.2.1.18) was from Behringwerke AG, Marburg, F.R.G. Sialidase treatment of cells was performed with 100 mU/ml in Tris-maleate buffer, pH 6.8,

containing 20 mM $CaCl_2$, adjusted to 300 mOsm, for 20 min at 37°C.

β-Galactosidase was obtained from *Streptococcus pneumoniae* type I, strains 1425 and B8019 [24, 25], which had been isolated in the Institute of Medical Microbiology, University of Heidelberg. In preliminary experiments strain B8019 was shown to be especially suitable for production of large quantities of the desired enzyme. β-Galactosidase was, briefly summarized, isolated as follows. Bacteria were removed from the culture medium by centrifugation. The medium was fractionated with $(NH_4)_2SO_4$ and the precipitate obtained with 60% salt saturation was dialyzed against 0.3 M Na_2HPO_4 buffer, adjusted to pH 6.2, containing 3 mM $MgCl_2$. This starting material contains a mixture of β-galactosidase and other glycosidases, in particular sialidase. In the past the latter enzyme had eluded many efforts with respect to separation.

A decisive step in further purification was affinity chromatography on immobilized sialic acid. The affinity material consisted of 2-aminoethyl-α-glycoside of *N*-acetylneuraminic acid which was attached to CH-Sepharose 4B. Sialidases of other origin have already been purified by this method [Brossmer and Eschenfelder, unpublished results]. The column was loaded using 50 mM Na_2HPO_4 buffer, adjusted to pH 6.8. Most of the sialidase remained affinity-bound on the column, whereas the other glycosidases appeared quantitatively in the effluate. For final purification an affinity column was applied which consisted of 6-*N*-β(4-aminophenyl)-ethylamino-3-*o*-β-D-galactopyranosyl-6-deoxy-L-gulitol (ultimately prepared from lactose) coupled to cyanogen bromide-activated Sepharose 4B [26].

This column was loaded using 10 mM Na_2HPO_4 buffer, adjusted to pH 7.0. β-Galactosidase was eluted with borate buffer, adjusted to pH 9.2 and immediately dialyzed against 0.3 mM Na_2HPO_4 buffer, adjusted to pH 6.2 and containing 3 mM $MgCl_2$. Concentration by Amicon with a PM 10 membrane yielded β-galactosidase in a 45% yield which contained only negligible hexosaminidase but still about 2% sialidase activity, as determined with 4-methylumbelliferyl glycosides as substrates [Brossmer and Nehrbass, unpublished results].

Incubation of cells with the β-galactosidase preparation was carried out in sodium phosphate buffer, adjusted to pH 6.7, for 30 min at 30°C, under gentle agitation. The enzyme concentration was 1.2 mU/ml.

Because of the sialidase contamination, control experiments with β-galactosidase alone were not possible. After completion of these studies, we were able to prepare a β-galactosidase from *S. pneumoniae*, strain B8019, by affinity chromatography on immobilized *N*-acetylneuraminic acid coupled to divinylsulfone-activated Sepharose 6B. This preparation is free of any other glycosidase activity and has a high specific activity [Brossmer and Nehrbass, unpublished results].

Flow-cytometric binding studies

For the quantitative determination of binding parameters with RCA-FITC we used a 'Cytofluorograf FC 210' with sorting equipment H-50 (Ortho Instruments, Westwood, MA), containing a 4-W argon-ion laser from Lexel. The instrument was interfaced with the computer system 'Plurimat Multi 20' (Deutsche Intertechnique), programmed for biparametric data acquisition and on-line data processing. The protocol for the flow-cytometric extrapolation of binding data and the calibration procedure for the conversion of fluorescence signals into a mean number of bound molecules per cell has been described in detail [13–16]. Briefly, cells are incubated for 60 min in the cold (to avoid endocytotic processes) and in the dark (to avoid fluorescence bleaching) under gentle continuous concentrations of fluorescent ligand or a mixture of fluorescent and native RCA in different ratios. Then the cells are passed through the flow cytometer without previous perturbation of the binding equilibrium, i.e. unbound molecules remain in the incubation medium without being registered. The flow rate is approximately 1000 cells/sec. As the cells pass through the focused laser beam by 'hydrodynamic focusing', both green fluorescence emission (proportional to the number of RCA molecules bound per cell) and low-angle laser light scatter signals (proportional to cell volume) are detected, remaining coordinated with one and the same cell. In many cases an additional parameter is recorded, namely red fluorescence emission from erythrosin B, a marker for cells with impaired membrane barrier function [14, 15]. With homogeneous populations, as was the case in this study, this parameter can be substituted by scatter-light gating: by setting an appropriate threshold, non-viable cells can be excluded by their property to produce considerably lower scatter-light signals than live ones. All signals are converted into histograms, and a mean number of bound molecules per cell can be computed after appropriate calibration experiments with a spectrofluorimeter. It should be noted that under normal conditions (not too high concentration of fluorescent ligand) the fluorescence signals from unbound molecules remain ignored since only signals associated with the proper scatter-light

signal (volume-related) are registered.

The determination of bound molecules at different incubation concentrations can be used for a Scatchard analysis [27], with the following precautions: if B is the molar concentration of bound, F that of free ligand (i.e. the difference between the molar incubation concentration and B), K_A the apparent equilibrium association (affinity) constant in l/mol and R_o the extrapolated molar concentration of bound ligand at occupancy (i.e. the 'molar receptor concentration'), the following equation holds:

$$\frac{B}{F} = K_A\,(R_o - B).$$

In most cases, especially for the determination of K_A, we use a double-reciprocal transformation of this equation [13]:

$$\frac{1}{F} = \frac{1}{B}\,(R_o - K_A).$$

A plot of $1/F$ vs $1/B$ yields a line with the ordinate intercept K_A and the abscissa intercept $1/R_o$. The latter can easily be converted into a 'per cell' calculation (see Figs 1-4). The computerized construction of such plots can be achieved with a non-linear least-squares fitting program [28].

The cytometric procedure, unlike radiolabel studies, thus permits the derivation of 'on-line' binding data from unperturbed living cells, with automatic omission of non-viable cells, either by a fluorescence or by the light-scatter criterion.

Of course, simple Scatchard analysis may be too simple, especially if the ligand is di- or multivalent. Since the RCA molecule most probably binds divalently, at low concentrations two equilibrium binding constants should be considered: a single site constant and an 'equilibrium cross-linking constant', as pointed out in a recent paper by Dower *et al.* [29], analyzing the binding of monoclonal antibodies. In order to learn more about the binding kinetics, dissociation kinetics experiments should be performed. On the other hand, the possibility of non-monovalent binding, especially at low concentrations, has largely been taken into account by our calibration procedure which uses fluorescence spectroscopy at the same low concentrations as with the binding assays in the flow cytometer. However, the following precautions should be observed: R_o should be interpreted as the 'extrapolated number of bound molecules at occupancy', and not as the 'number of receptors' or the 'number of binding sites', and K_A as a 'lumped' average equilibrium binding constant.

RESULTS AND DISCUSSION

As can be seen in Fig. 1(a-c) (double-reciprocal Scatchard plots) and the compiled numerical data (K_As and R_os) in Fig. 2, there are only slight

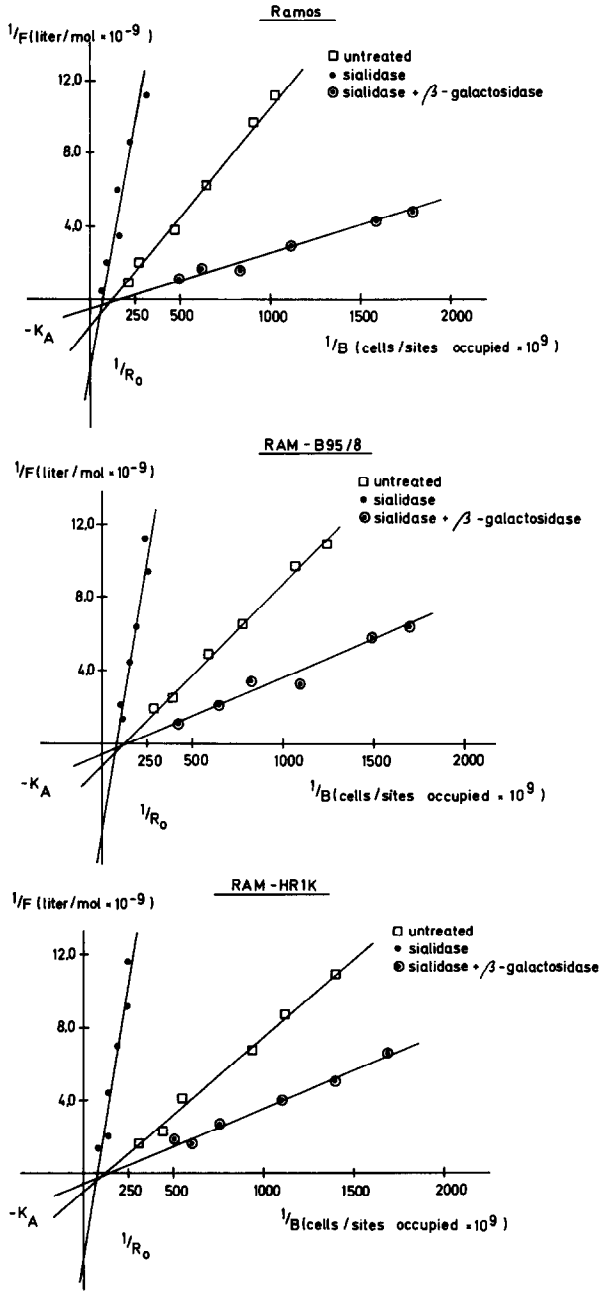

Fig. 1.(a) Ramos lymphoma. Extrapolated number of lectin molecules bound at occupancy (R_o) *and apparent affinity constants of untreated, sialidase-treated and sialidase + α-galactosidase* (S. pneumoniae)*-treated cells. Double-reciprocal Scatchard plots. Abscissa: reciprocal number of binding sites occupied; ordinate: reciprocal of molar concentration of unbound lectin. Intersection with* x*-axis: extrapolated reciprocal number of lectin molecules bound per cell at occupancy* ($1/R_o$); *intersection with* y*-axis: negative value of apparent association constant in* l/mol ($-K_A$). *(b) Ram-B95/8: Ramos cells transformed by EBV strain B95/8. Extrapolated number of lectin molecules bound at occupancy* (R_o) *and apparent affinity constants of untreated, sialidase-treated and sialidase + β-galactosidase* (S. pneumoniae)*-treated cells. Double-reciprocal Scatchard plots [for explanation see (a)]. (c) Ram-HR1K: Ramos cells transformed by EBV strain P3HR-1. Extrapolated number of lectin molecules bound at occupancy* (R_o) *and apparent affinity constants of untreated, sialidase-treated and sialidase + β-galactosidase* (S. pneumoniae)*-treated cells. Double-reciprocal Scatchard plots [for explanation see (a)].*

differences both in the number of binding sites and in apparent affinity constants between the different strains of Ramos cells, particularly with untreated cells. (In a similar study with mouse lymphoma lines of different metastatic potential [30] we found significant differences, especially in affinity constants.) The number of binding sites (or, rather, the 'number of bound RCA molecules at occupancy', see above) is about 8×10^6/cell (probably 16×10^6 binding sites, considering the divalent binding at the low concentrations used).

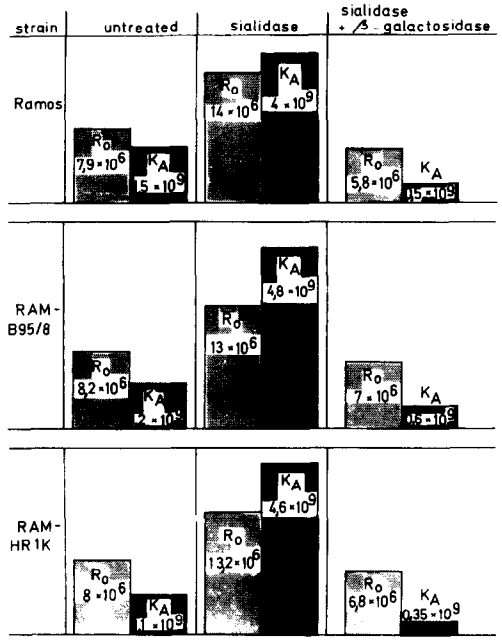

Fig. 2. Compiled numerical data (R_os and K_As) of Ramos lymphoma and two derived EBV-transformed cell lines. For explanation of symbols see Fig. (a).

The apparent affinity constant is above 10^9 l/mol, which seems a remarkably high value for a 'non-physiological' ligand like a plant lectin. Both the numbers of binding sites and the 'affinity constants' are raised by sialidase treatment, which seems plausible, since new galactoside residues, formerly 'masked' by sialic acid, become available. While the number of sites is not even doubled, the 'affinity' between lectin and binding sites increases by a factor of about four, indicating an especially favorable exposition of the sugar residues to the lectin after removal of sialic acids. The better 'availability' may also relate to the loss of negative surface charge carriers after sialidase treatment. Conversely, the sequential action of sialidase and β-galactosidase lowers the number of binding sites to below the values for untreated cells. Again, the effect on the apparent affinity constant, probably reflecting the accessibility of unremoved 'buried' galactoside residues, is more pronounced than the reduction of binding sites. In the case of RAM-HR1K, the affinities of sialidase treated and sialidase- and galactosidase-treated cells differ even by a factor above 13!

In the case of BjAB cells (Figs 3a–c and 4), a slightly different behavior is encountered; again the HR1K-transformed subline exhibits the lowest affinity for RCA, in this case significantly lower than the parent cells. Also this subline is the

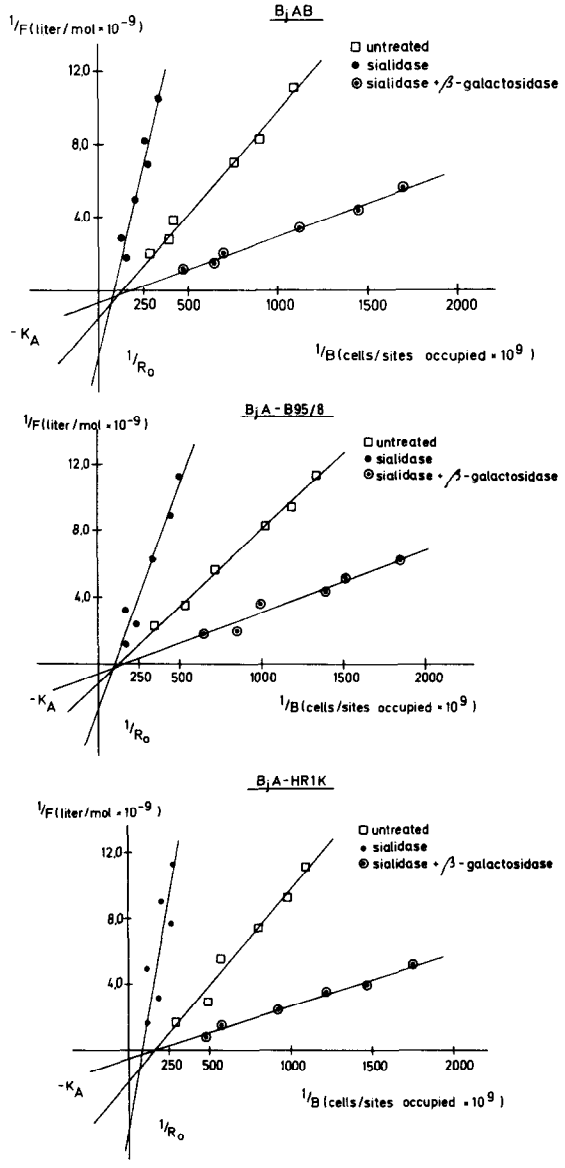

Fig. 3.(a) BjAB lymphoma. Extrapolated number of lectin molecules bound at occupancy (R_o) and apparent affinity constants of untreated, sialidase-treated and sialidase + β-galactosidase (S. pneumoniae)*-treated cells. Double-reciprocal Scatchard plots [for explanation see Fig. (a)]. (b) BjA-B95/8: BjAB cells transformed by EBV strain B95/8. Extrapolated number of lectin molecules bound at occupancy (R_o) and apparent affinity constants of untreated, sialidase-treated and sialidase + β-galactosidase* (S. pneumoniae)*-treated cells. Double-reciprocal Scatchard plots [for explanation see Fig. 1(a)]. (c) BjA-HRK: BjAB cells transformed by EBV strain P3HR-1. Extrapolated number of lectin molecules bound at occupancy (R_o) and apparent affinity constants of untreated, sialidase-treated and sialidase + β-galactosidase* (S. pneumoniae)*-treated cells. Double-reciprocal Scatchard plots [for explanation see Fig. 1(a)].*

one showing the largest difference in affinity ('availability' or 'accessibility') when sialidase-treated and sialidase- and β-galactosidase-treated cells are compared. While sialidase-treated RAM-B95/8 cells have the highest apparent affinity constant, the BjAB counterpart, transformed with the same virus, possesses the lowest affinity of all sialidase-treated sublines studied. Although these differences are not dramatic, they show at least that in cases where native cells of the various strains are practically indistinguishable, differences become apparent after specific enzyme treatment, whatever the molecular bases of such differences: spatial arrangement, availability to both the lectin and the respective enzymes, etc.

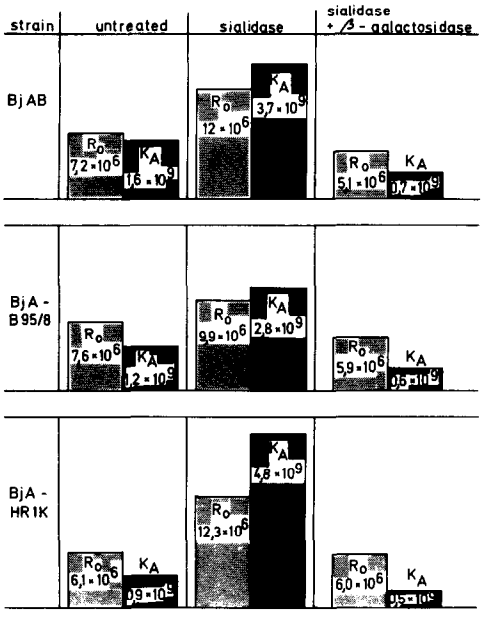

Fig. 4. Compiled numerical data (R_os and K_As) of BjAB lymphoma and two derived EBV-transformed cell lines. For explanation of symbols see Fig. 1(a).

It is conceivable that further differentiation becomes possible by the use of additional glycosidases, notably *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase and respective lectins with high sugar specificity. Moreover, this approach will also lend itself to the monitoring of the efficiency of glycosyltransferases on intact cells. Finally, the lectin binding studies should be complemented by direct chemical microprocedures for the determination of liberated sugar residues [31].

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