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Epstein-Barr Virus-Induced Increase in the Concanavalin-A Receptor Density of Established EBV-Negative Lymphoma Lines In Vitro

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Infection of two human EBV-genome negative lymphoma lines with the EBV-variants P3HR-I and B95-8 converts the lines to permanent carriers of EBV-DNA and the EBV-determined nuclear antigen. This process is accompanied by a series of biologic changes similar to those in oncogenic transformation by known tumor viruses (e.g. polyoma, SV40, Rous SV). They include altered growth properties and membrane changes, one of which is expressed in an increased agglutinability by Con-A.

Although most transformed cell systems are more agglutinable than the non-transformed counterparts it is controversial whether they possess similar or changed numbers of lectin receptors. Since little was known about lectin receptor densities of virus transformed lymphoma cells, we set out to determine the densities of Con-A receptors on the EBV-negative lymphoma lines BJAB and Ramos and their EBV-converted sublines by simultaneous recording of the cell membrane fluorescence of FITC-Con-A and the cell volume using the FLUVO-METRICELL flow cytometer.

We found that EBV infection gave rise to cell lines with a significantly elevated Con-A receptor density compared to the EBV-negative parental lines. The similar changes found in a number of independently converted sublines suggest that they are due to the direct or indirect action of the viral genome.

Key words: Con-A receptor density – Epstein-Barr Virus – flow cytometry – lymphoma lines

The EBV-negative Burkitt Lymphoma lines, BJAB and Ramos, have been converted into permanently EBV-positive sublines by in vitro infection with two different EBV-variants, B95-8 and P3HR-I (1–4). The converted sublines contain the EBV-genome and express the EBV-determined nuclear antigen (EBNA) (2–5). Beside these changes the sublines show a number of characteristic biologic alterations, which are attributed to the presence of the EBV-genome. Therefore these cell lines provide an excellent system to study EBV-re-

TABLE 1
Properties of the cell lines

	EBV-genome	EBNA	Converting EBV	Reference
Ramos	–	–	–	2
Ramos-P3HR-I	+	+	P3HR-I	3,32
Ramos-B95-8	+	+	B95-8	3,33
AW-Ramos	+	+	B95-8	2
EHR-A-Ramos	+	+	P3HR-I	2
BJAB	–	–	–	1
BJAB-P3HR-I	+	+	P3HR-I	3
BJAB-B95-8	+	+	B95-8	3

+ = present, – = absent.

lated changes, which are reminiscent of changes that accompany the transformation of culture cells by known experimental oncogenic DNA viruses (polyoma, SV40) or RNA viruses (e.g. Rous SV). They include a reduced capping of surface IgM, Con-A and β_2-microglobulin (6–8), and altered growth characteristics in culture. The converted lines were more resistant to cell saturation conditions and showed a decreased serum dependence and independence of some dialysable serum factors (9–11). Furthermore they had the potency to form colonies in soft agar, in contrast to EBV-free lines (12). It has been found that the converted sublines show a much higher activation of the alternative complement pathway and a higher complement receptor density (13). A further membrane change associated with the transformation by known experimental oncogenic viruses is expressed by the increased agglutinability of cells by lectins such as Con-A (14–16). Interestingly it was found that EBV conversion of lymphoma cells also leads to a similar rise in Con-A induced cell agglutination (8).

Although most transformed cells are more agglutinable than their non-transformed counterparts there is controversy if the agglutinability correlates with the number of lectin receptors (17). Some authors claim that there is no significant difference in lectin surface receptors in some cell lines (18–20), whereas Noonan and Burger (21,22) find several-fold increases in the number of Con-A receptors on transformed cells. Since these observations were made in animal transforming virus cell systems, we were now interested to study the effect of viral oncogenic transformation on the membrane glycoprotein composition in a human cell system.

Con-A receptor densities were determined by labeling the EBV-negative cell lines BJAB and Ramos and their corresponding EBV-converted sublines with FITC-Con-A. Simultaneous flow cytometric analysis of cell volume and fluorescence enabled us to calculate the Con-A receptor densities of the cell lines mentioned above.

MATERIALS AND METHODS

Cell lines. Ramos, Ramos-B95-8, Ramos-P3HR-I, AW-Ramos, EHR-A-Ramos, BJAB, BJAB-B95-8 and BJAB-P3HR-I cells were examined in this study. Origins and some relevant properties are listed in Table 1. Ramos and BJAB cell lines were established from exceptional EBV-negative Burkitt Lymphomas, of American and African origin, respectively (2,23). The cells were maintained as stationary suspension cultures in RPMI 1640 with the addition of 10% fetal calf serum, penicillin (100 μg/ml) and streptomycin (100 μg/ml). They were seeded at a concentration of 3×10^5/ml and harvested at a density $8–10 \times 10^5$/ml.

Labeling with FITC-Con-A. Con-A (Pharmacia, Uppsala) was conjugated with fluorescein-isothiocyanate (Isomer I, Serva, Heidelberg) according to standard procedures (24). Briefly, 200 μg FITC were added to 1 ml Con-A (10 mg/ml) in 150 mM NaCl, 10 mM $NaHCO_3$, pH 9.5. After stirring at room temperature for 1 h under pro-

tection from light nonbound FITC was removed by chromatography on Sephadex G-25 equilibrated with phosphate buffered saline (PBS). The resulting FITC-Con-A had a protein concentration of 800 μg/ml. For FITC-Con-A labeling cells were washed 3 times with cold RPMI 1640 without fetal calf serum and suspended in the same medium at a density of 3×10^6/ml. If not indicated otherwise 20 μl FITC-Con-A corresponding to 16 μg was added to 1 ml cell suspension and incubated in ice for 2 h (25).

Flow cytometric analysis. The flow cytometric analysis of the stained cells was performed with a FLUVO-METRICELL flow cytometer (26), which combines electrical cell sizing with epi-illumination fluorescence. The orifice for electrical sizing was cylindrical with 85 μm diameter and 100 μm length. The cells were measured in serum free RPMI 1640 medium (65 Ohm cm at 25°) with an electrical current of 0.50 mA and a suction of 0.2 kg/cm^2. The fluorescence exciting light was provided by a HBO-100 mercury high pressure lamp. The excitation filter was a low pass No. 53 glass filter (Carl Zeiss, Oberkochem, FRG) with cut off points 500 and 520 nm. The cells were measured at room temperature at a rate between 500–1200 cells/sec, so that each measurement was concluded within approximately 1 min.

The two parameter histograms of cell volume versus cell fluorescence were recorded in a 64 × 64 channel array of a multichannel analyzer. After transfer of the data onto magnetic tape, the histograms were analysed with a computer program which converts the cell volume into cell surface area and plots the distribution curve of the ratio of fluorescence/μm^2 cell surface (27). It further calculates the mean and the standard deviation of the distribution. This calculation has the advantage that cells differing in size can be compared because fluorescence/μm^2 cell surface is a parameter which, in contrast to total cell fluorescence, is independent of the volume.

RESULTS

Comparison of the FITC-Con-A staining behaviour of EBV-converted and non-converted cells. Immediately after labelling, cells were analysed by flow cytometry without further treatment or washing. It is important to point out that manipulations of the cells have been reduced to a minimum and care has been taken to keep the cells at the most physiologic conditions possible so as not to disturb the receptor-ligand interactions.

The incubation temperature of 0° followed by 1 min at room temperature during the measuring process was such that ligand induced endocytosis or redistribution was reduced or prevented. Microscopically no patching or capping could be observed.

A first recording revealed that the fluorescence intensity differed greatly between the various cell lines. This turned out to be in part due to differences in cell volume (see Table 2). Ramos and its converted sublines were rather homogenous in size with the exception of EHR-A-Ramos which had a considerably higher volume. Among the BJAB cell lines, BJAB had the highest volume, whereas the converted BJAB sublines only had a volume of 79% (BJAB-P3HR-I) and 68% (BJAB-B95-8) of their EBV-negative progenitor cell line. For this reason it was necessary to correlate fluorescence with the cell surface area which was deduced from the cell volume measurement. This correlation was obtained by the simultaneous analysis of fluorescence and cell volume.

The values of relative fluorescence intensity/μm^2 cell surface area obtained in a number of separate experiments are compiled in Table 2. It is clearly evident that the non-converted cells, Ramos and BJAB, always show the lowest Con-A density and that EBV-converted cells exhibit a considerably higher binding density. If the values of the non-converted cells are normalized to 100%, EBV conversion leads to an increase in receptor density of at least 20% (Ramos-B95-8) and 24% in AW-Ramos which like Ramos-B95-8 has also been created

TABLE 2

Simultaneous flow cytometricanalysis of cell volume and Con-A induced membrane fluorescence

	n	Mean volume (μm^3)	%	Mean surface (μm^2)	%	FITC-Con-A/μm^2 cell surface (% of control)
Ramos	6	636 ± 44	100	357 ± 16	100	100
Ramos/P3HR-I	6	664 ± 74	104	365 ± 27	102	127 ± 7
Ramos/B95-8	4	581 ± 76	91	336 ± 29	94	120 ± 12
AW/Ramos	3	646 ± 30	102	362 ± 12	101	124 ± 5
EHR-A/Ramos	4	794 ± 59	125	415 ± 21	116	209 ± 14
BJAB	4	1137 ± 235	100	519 ± 70	100	100
BJAB/P3HR-I	4	899 ± 117	79	449 ± 39	87	177 ± 49
BJAB/B95-8	6	776 ± 52	68	405 ± 18	78	127 ± 7

n = number of experiments.
±SE = ±Standard error.

by conversion with B95-8 virus. The density increase was 27% in the case of Ramos-P3HR-I and 109% in EHR-A-Ramos. BJAB converted by B95-8 virus exhibited a relatively lower increase of 26% compared with 77% in the case of BJAB-P3HR-I.

Since the numerical differences between the various fluorescence density values are relatively low (Table 3) one might doubt their significance. That the values in fact are highly significant is shown in the following:

1) The density values given in Table 2 do not reflect the position of the peaks of the distribution profiles (Fig. 1), but are the mean values of the entire distribution which is comprised of the consecutive measurements of about 50,000 single cells.
2) It further has to be pointed out that the measurements are not disturbed by the presence of non-viable cells. Damaged cells usually have a high fluorescence and a volume different from intact cells and thereby are easily sorted out.
3) The values given for some cell lines in Table 2 show a relatively high standard error. This is caused by variations of the experimental conditions at different days. Data obtained at the same day are highly significant due to the high number of analysed cells (Table 3). The 2p values always range below 0.01.

TABLE 3

Con-A induced fluorescence density of Ramos and its converted sublines measured by flow cytometry

Cell line[a]	n	FITC-Con-A fluorescence/μm^2 cell surface (histogram channels/μm^2)	2p	% of control
I	51,139	0.0679 ± 0.0001	—	100
II	63.078	0.0749 ± 0.0001	<0.01	110
III	46,448	0.0780 ± 0.0001	<0.01	114
IV	35,767	0.0842 ± 0.0001	<0.01	124
V	12,982	0.1589 ± 0.0004	<0.01	234

[a] I = RAMOS; II = RAMOS/P3HR-I; III = RAMOS/B95-8; IV = AW/RAMOS; V = EHR-A/RAMOS.
n = number of cells/experiment.
2p according to Student test.
±SE = ±Standard error.

Characterization of the Con-A binding reaction. A comparison of receptor den-

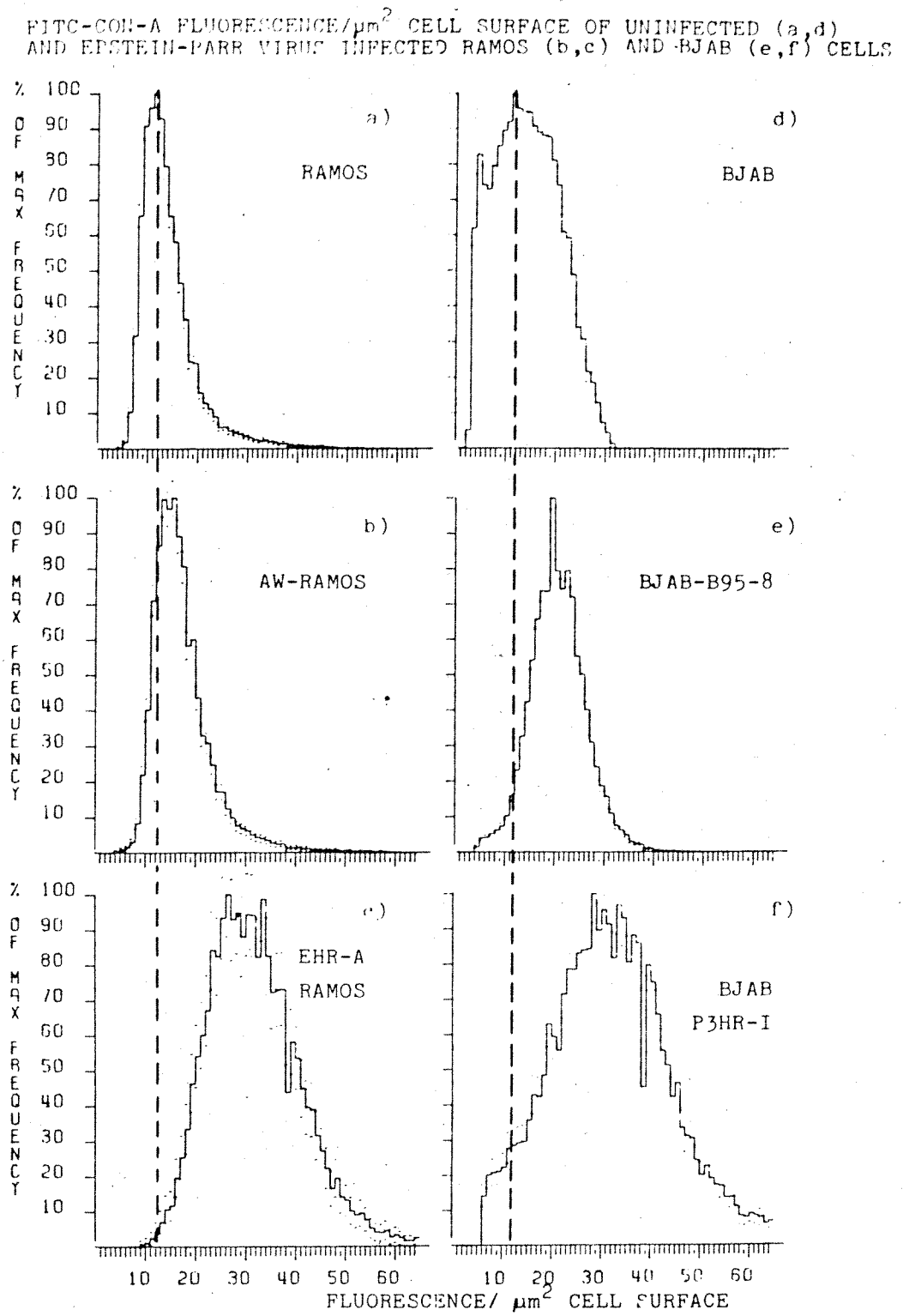

Figure 1. Distribution profiles of FITC-Con-A receptor densities of the cell lines a) Ramos, b) AW-Ramos, c) EHR-A-Ramos, d) BJAB, e) BJAB-B95-8 and f) BJAB-P3HR-I. The abscissa indicates the amount of FITC-Con-A bound/μm^2 cell surface expressed in arbitrary units. The values were calculated by the ratio of fluorescence intensity and cell surface. The ordinate gives the frequency of the cells with the indicated fluorescence densities expressed as percent of the maximal frequency. The histograms are made up of 12–63 × 10^4 single cell measurements. The short horizontal bars indicate the ±3 standard deviations of the cell content of each class. The standard deviation is calculated as square root of the class content.

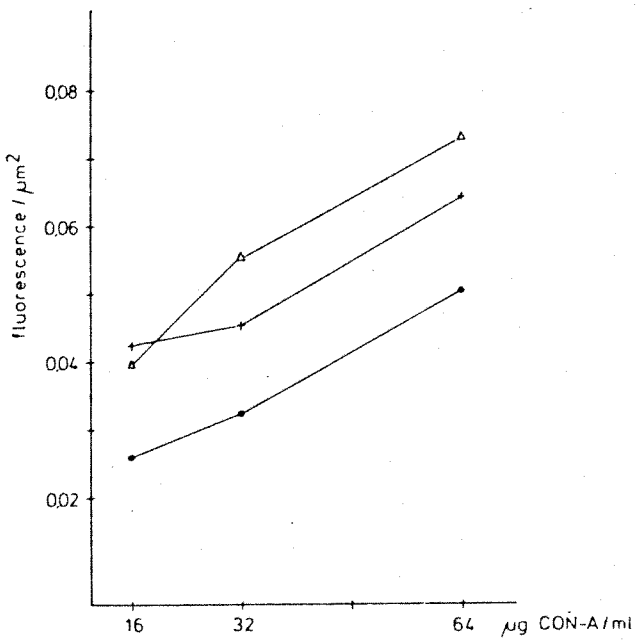

Figure 2. Concentration dependence of FITC-Con-A binding to Ramos (●—●), Ramos-B95-8 (+—+) and Ramos-P3HR-I (△—△). Fluorescence density is expressed in arbitrary units.

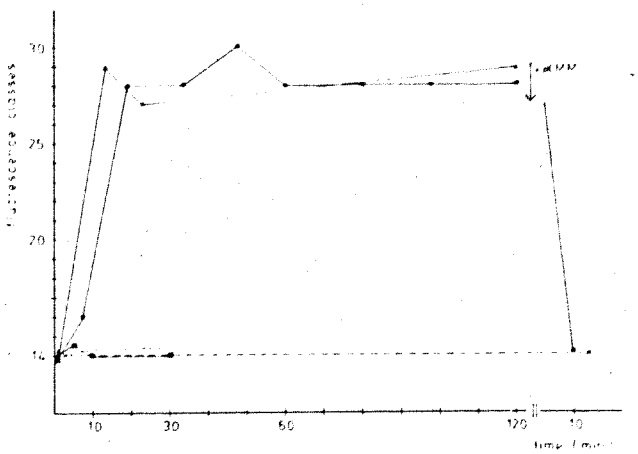

Figure 3. Time dependence of binding of FITC-Con-A to native Ramos-P3HR-I cells (●—●) and displacement of bound FITC-Con-A by the addition of 50 mM α-methyl-mannoside (arrow). Binding of FITC-Con-A to formaldehyde fixed cells (▲—▲) and to native cells in the presence of 50 mM α-methyl-mannoside (○- - - -○) and unlabeled Con-A (■- - - -■). The ordinate gives the fluorescence density in arbitrary units. The background fluorescence is indicated by the dotted line (×- - -×).

sities is only valid if the ligand concentration is below the saturation of the receptor under study.

To prove that the conditions used did fulfill this requirement cells were exposed to increasing concentrations of FITC-Con-A (Fig. 2). Labeled Con-A was added at the final concentrations of 16, 32 and 64 µg/ml to Ramos and two converted Ramos lines. The increase of fluorescence density was almost linear and in no case a plateau was reached. Since the routinely applied concentration was 16 µg/ml this concentration is clearly below the saturating concentration.

In order to show that we were measuring specific Con-A receptor interactions binding and displacement experiments were performed (Fig. 3). Labeled Con-A was added to Ramos-P3HR-I cells at time zero and the fluorescence intensity was followed for 120 min. Addition of labeled Con-A resulted in a linear increase of bound fluorescent dye which reached a plateau after 20 min. Thereafter no change in fluorescence could be observed. The addition of 50 mM α-methyl-mannose led to a total removal of the fluorescent dye within 10 min. No labeled Con-A was bound in the presence of the unlabeled ligand and of 50 mM α-methyl-mannose during the incubation period.

To exclude the possibility that we were not measuring membrane receptors but fluorescent dye trapped by endocytosis we analysed formaldehyde fixed cells in parallel. However, fixation of the membrane did not lead to a change in FITC-Con-A induced fluorescence density, indicating that the obtained receptor density values were not influenced by ligand induced endocytosis.

DISCUSSION

The results presented above show that conversion of EBV-negative cells into permanent carriers of EBV-DNA and the EBV-determined nuclear antigen (EBNA) by infection with two different EBV-variants is accompanied by a clear increase of Con-A receptor density. Depending on the cell line and possibly on the converting virus variant the increase varies between a minimum of 20% and about 100%. The cell line with the highest receptor density is EHR-A-Ramos.

The data suggest that there may be a specific difference in the increase of Con-A receptor density depending on the EBV variant used for conversion. B95-8 virus converted sublines of both Ramos and BJAB seem to have a lower Con-A receptor density, compared with P3HR-I converted sublines. This observation may be explained by differences in the genetic information of the two EBV substrains. B95-8 virus, in contrast to P3HR-I, has the capacity to transform cord blood B lymphocytes from cells with a limited lifespan to cells with permanent growth in vitro.

Molecular evidence, including partial denaturation mapping (28) and restriction analysis of viral DNA (29) suggests that P3HR-I virus contain particles with two different types of viral DNA, in contrast to B95-8 virus which harbours one homogenous DNA species.

The biologic differences between the two EBV strains are further confirmed by the recent finding that P3HR-I virus converted sublines exhibit a significantly reduced EBV-receptor concentration in comparison with the B95-8 virus converted sublines (30).

Control experiments clearly demonstrate the specificity of the Con-A binding reaction. These tests include binding and displacement reactions of FITC-Con-A in the presence of unlabeled Con-A or the competitive sugar hapten, α-methyl-mannoside.

The high statistical significance and experimental reliability of the reported data are in part due to methodological advantages offered by the flow cytometric two-parameter measurements. One advantage resides in the possibility to determine simultaneously fluorescence and volume in a high number of consecutive single cell measurements. Thus the considerable variation in cell size between the different cell lines can be neglected because surface receptor densities are calculated for each single cell.

The second advantage is that the flow cytometry is performed under biochemical equilibrium conditions. No centrifugations or washings are required. This is in contrast to binding studies with radiolabeled lectins where unbound lectins have to be removed prior to counting. This eventually results in underestimation of lectin receptors due to desorption.

Furthermore, two-parameter measurements offer additional security, because dead cells which have an altered volume and an increased non-specific uptake of fluorescent dye are sorted out and not taken into account by the calculations. Finally it should be mentioned that in flow cytometry cells are investigated under physiological conditions, and thereby cell damage by a changed environment is almost excluded.

It has to be pointed out also that we were purposely measuring cells in their

native state in order to obtain a true picture of the physiological membrane composition. To exclude the possibility that the determined fluorescence density values were influenced by ligand induced redistribution or endocytosis, formaldehyde fixed cells were investigated in parallel. Fixed cells bound Con-A to the same extent as native cells. Furthermore, Con-A bound during the 120 min incubation at 4° could be totally displaced within 10 min from native cells by the addition of α-methyl-mannoside.

The availability of EBV-negative lymphoma lines and their virus converted EBV-positive sublines offers the opportunity to study transformation associated events in vitro in a lymphoma cell system of human origin. Although BJAB and Ramos were isolated from malignant lymphomas they exhibit the criteria of non-malignant cells in vitro and acquire after EBV-infection the behaviour of malignant cells according to the in vitro criteria of malignancy. They include a reduced capping of IgM, Con-A and other surface receptors, an increased resistance to saturation conditions in culture, a decreased dependence on serum concentration and independence of some dialysable serum factor(s). Further, EBV conversion leads to a change in cell membrane sugars, which has been detected by an increased agglutinability by Con-A and by an increase in Con-A receptor density.

Changed membrane sugar composition associated with EBV-conversion was confirmed recently by gel chromatography of glycopeptides obtained by protease digestion of fucose labeled membranes. Converted cells bear fucosyl-glycopeptides that elute ahead of the corresponding molecules obtained from non-converted cells, changes which were identically seen in other neoplastic cell systems (31).

The fact that practically similar changes of the phenotype were observed in various kinds of cells originating from different species after oncogenic transformation with different oncogenic viruses, including the human lymphoma cell system described here, suggests that they are general expressions of viral induced transformation.

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