The Presence of P-glycoprotein in L1210 Cells Directly Induces Down-Regulation of Cell Surface Saccharide Targets of Concanavalin A

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Abstract. *Overexpression of P-glycoprotein (P-gp), a plasma membrane drug transporter (ABCB1, a member of the ABC transporter family), is the most prevalent cause of multidrug resistance in cancer tissues. Lectin concanavalin A (ConA) induces massive cell death of L1210 leukemia cells (S). Cell sublines of L1210 in which P-gp overexpression was induced by selection with vincristine (R) or by stable transfection with a plasmid encoding full-length human P-gp (T) were less sensitive to ConA. Both P-gp-positive cell lines exhibited typical P-gp-mediated multidrug resistance. Resistance of R and T cells to ConA was associated with lower binding of ConA as compared to S cells when analysed by the following methods: (i) SDS PAGE and electroblotting of proteins in the crude membrane fraction followed by detection with biotinylated ConA and avidin-peroxidase, and (ii) fluorescent cytometry or confocal microscopy of the intact cells with surfaces labeled by FITC-ConA. These data indicated that the presence of P-glycoprotein in L1210 cells independently of the mode of its expression induced down-regulation of cell surface saccharide targets of ConA. Therefore, this feature may be considered as a secondary cellular response to P-glycoprotein expression.*

Multidrug resistance (MDR) of cancer tissue represents a serious problem in cancer chemotherapy and remains a significant issue despite extensive study (1). Several mechanisms of MDR have been identified (2), but the massive overexpression of P-glycoprotein (P-gp), a plasma membrane ATP-dependent drug-transporter (3), represents the most prevalent known mechanism. P-gp, the product of the *MDR1* gene, is an ABCB1 member of the ABC transporter family (4) and is responsible for the effective removal of anticancer drugs from the intracellular space of drug-resistant cancer cells (5). Overexpression of P-gp in neoplastic cells may be induced by treatment with several compounds, including vincristine (6), doxorubicin (6, 7) and apicidin, an inhibitor of histone deacetylase (8), as well as by several pathological impulses, such as hypoxia and reoxygenation (9) and finally by intracellular acidification (10). Adaptation of neoplastic cell lines by repeated cultivation in mediums with stepwise increasing concentration of anticancer drugs may confer reduced sensitivity of adapted cells to diverse substances associated with overexpression of P-gp (11). A model cell line (L1210/VCR) was obtained by treating mice leukemia L1210 cells with vincristine to select for massive overexpression of P-gp (6, 12). Other markers of MDR, such as changes in the expression of enzymes in glutathione detoxification systems and the key enzyme glutathione *S*-transferase (13) and expressions of membrane transporters linked to the glutathione detoxification system, *i.e.* MDR-associated proteins (MRPs) (14) have been shown to not play important roles in reduced sensitivity of L1210/VCR cells to several drugs (6, 15).

Overexpression of P-gp induced by cell selection with anticancer drugs confers several alterations in metabolic and regulatory pathways (*e.g.* protein phosphorylation, protein glycosylation *etc.*) (16). Recently, it was found that overexpression of P-gp in L1210/VCR cells is associated with the following changes: (i) expression or activity of

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mitogen-activated protein kinases (17) and PI3/Akt kinase (18) and in response to specific inhibitors of these kinase pathways (17-19), (ii) calcium homeostasis (20-22), (iii) response to cisplatin (not a P-gp substrate) due to alteration in cisplatin-induced apoptosis (23) and (iv) the contents of UDP-saccharides, glycogen, negatively charged cell surface binding sites (mostly sialic acid) stainable by ruthenium red and in protein glycosylation pattern (12). P-gp is glycosylated on its first extracellular loop and this glycosylation causes an increase in molecular weight from approximately 140 kDa (deduced from sequence of 1280 aminoacids) to about 170 kDa (24). Several reports demonstrate that upon development of the resistance phenotype, several changes in cell surface saccharides may be monitored using lectins like concanavalin A (ConA) (25) or LEA (26, 27), which originate from jack-bean and tomato, respectively. LEA interacts more efficiently with P-gp-positive L1210/VCR cells than with P-gp-negative L1210 cells, whereas ConA exhibites the opposite behavior (28). Additionally, LEA agglutinates to both cell sublines more effectively than ConA, but ConA induces more cell death than LEA. This indicates that the cell damage effect induced by ConA is most likely caused by interaction with specific sites on the cell surface that alter essential cellular functions important for viability, and this interaction is more effective in P-gp-negative L1210 than in P-gp-positive cells (28).

Changes in cell surface saccharides induced by adaptation of L1210 cells to vincristine may be directly related to P-gp overexpression as a secondary cellular response, or may be independent of the expression of P-gp as a response to drug-induced cell selection. Lavie *et al*. (7) observed higher expression of caveolin-1 and the presence of caveolae in MCF-7-Adr cells (selected for resistance by adriamycin) than in parental MCF-7 cells. They tested if this up-regulation was a consequence of overexpression of P-gp alone or a consequence of the adaptation procedure using the BC-19 cell subline of MCF-7 cells, which express similar amounts of P-gp as MCF-7-Adr cells due to stable transfection with P-gp (29). A similar principle was applied in this study to determine whether the decreased saccharide target of ConA in L1210/VCR cells is a secondary cellular response to P-gp overexpression or is a consequence of cell selection with vincristine. To determine this, L1210 cell sublines were prepared that overexpress P-gp due to stable transfection and the interactions of these cell lines with ConA measured.

Materials and Methods

Cells and cultivation conditions. P-gp-overexpressing L1210/VCR cells (R) were obtained by stepwise adaptation of parental L1210 cells (S) to vincristine (6, 12). Cells were cultured for two days in a humidified atmosphere supplemented with 5% CO_2 at 37˚C, in standard RPMI medium containing glutamine (1 mg/ml), 4% bovine fetal serum and 20 μg/l gentamycin (Invitrogen, USA).

L1210 cells overexpressing P-gp due to transfection (T) were prepared with a GenePORTER™ 2 kit (Genlantis, USA) according to the manufacturer's directions. Briefly, L1210 cells (2×10^6) were suspended in 2 ml of RPMI medium without fetal serum in a 6-cm Petri dish. A transfection mixture containing 4 μg of *pHaMDRwt* plasmid (Addgene plasmid 10957; a retrovirus carrying full-length P-gp cDNA (30)) and 0.4 μg *pSV2neo* plasmid were then added to the cells. Cells were incubated for 4 h in a humidified atmosphere supplemented with 5% CO_2 at 37˚C. Then, 2 ml RPMI medium containing 8% fetal bovine serum were added, and cells were cultivated overnight in the same conditions. After this procedure, cells were pelleted (4 min at 300 ×g) and cultivated in RPMI medium supplemented with 4% fetal bovine serum and 1200 μg/ml antibiotic G418 (Invitrogen, USA) for 14 days. During this procedure, the growth medium with G418 was replaced every two days. The presence of mRNA encoding P-gp and P-gp protein was investigated by RT-PCR and Western blotting (see below).

Detection of mRNA encoding P-gp. Total cellular mRNA was extracted from S, R and T cells using the RNA-solvent reagent concentrate R6830-02IN (OMEGA Bio-Tek, USA). mRNA was reverse-transcribed to cDNA using a First Strand cDNA Synthesis Kit (Novagen, USA). mRNA extraction and cDNA synthesis were carried out according to protocols recommended by Novagen. PCR reactions included Nova Taq PCR Master Mix (Novagen, USA). The following PCR primers were used: 5'-CCC ATC ATT GCA ATA GCA GG-3' and 5'-GTT CAA ACT TCT GCT CCT GA-3' for MDR1, which yielded a 167 bp product and 5'- TAT GTC GTG GAG TCT ACT GGT GTC -3' and 5'- GTC ATC ATA CTT GGC AGG TTT CTC -3' for GAPDH, which yielded a 492 bp product. PCR reactions were carried out using 30 cycles of 1 min denaturation at 94˚C, 1 min annealing at 57˚C (for *GAPDH*) or at 55˚C (for MDR1), and 2 min extension at 72˚C, according to the protocol recommended by Novagen. The PCR products were separated on a 1.7% agarose gel (Invitrogen) and visualised with ethidium bromide on a Typhoon 9210 (GE Healthcare, USA, formerly Amersham Biosciences).

Detection of P-gp by Western blot. Whole cell lysates were prepared by homogenisation in RIPA buffer (Pierce Biotechnology, USA) according to the manufacturer's instructions. Protein concentration was determined using the Lowry assay. Proteins in the samples were separated by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) on polyacrylamide gradient gels (8-16%). Proteins were then transferred by electroblotting to nitrocellulose (GE Healthcare). GAPDH, the internal standard, was detected using anti-GAPDH antibody (Santa Cruz Biotechnology, USA), and P-gp was detected using the C219 anti-P-gp monoclonal antibody (Calbiochem, USA). Secondary anti-mouse antibody conjugated with horseradish peroxidase was used for detection with the aid of the ECL detection system (GE Healthcare) and a Kodak (USA) scanning system CF 440.

Detection of P-gp activity by calcein/AM assay. S, R and T cells (5×10^5) were washed twice in PBS containing 0.1 % bovine serum albumin, filled up to 500 μl with the same buffer. Calcein/AM (Sigma–Aldrich, resulting concentration 1 μM) and propidium iodide (resulting concentration 0.6 μg/ml) were added directly to the incubation medium and then the samples were incubated for 20 min

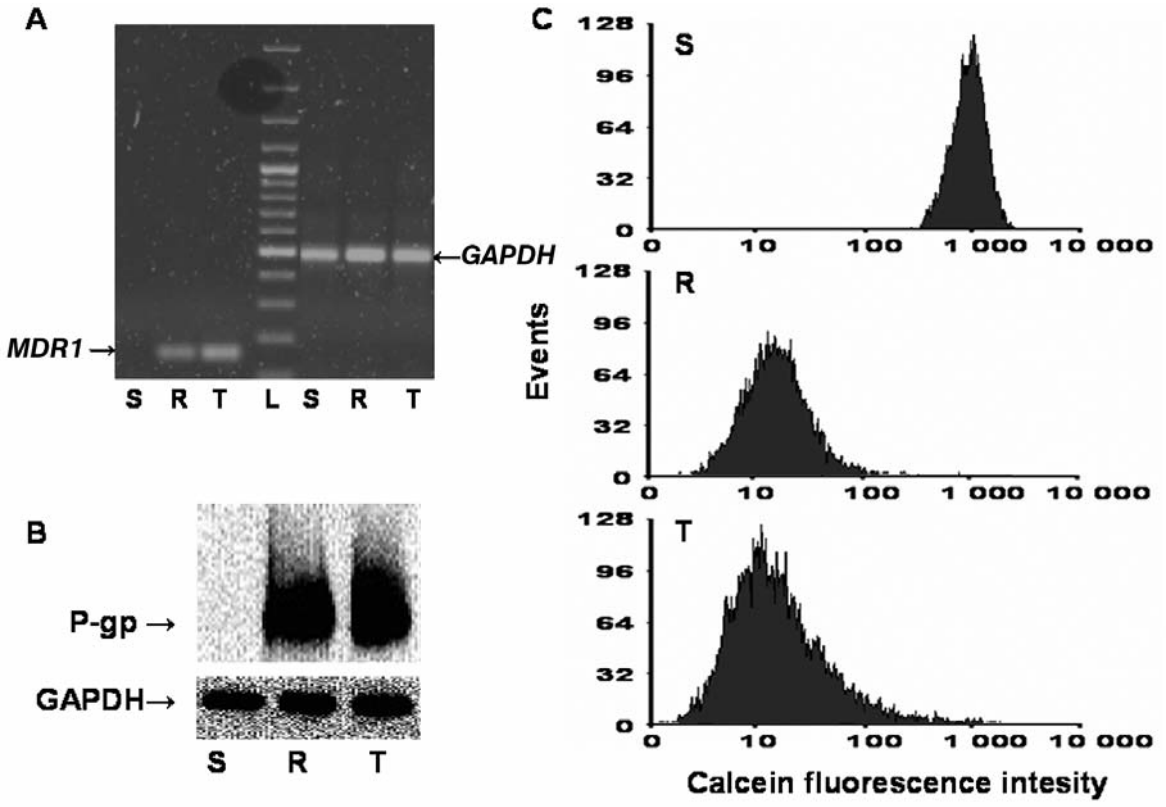

Figure 1. *Detection of P-gp expression and activity in S, R and T cells. A: Detection of mRNA encoding P-gp by RT-PCR. B: Detection of P-gp in whole cell lysates by Western blotting. GAPDH was used as internal standard in both procedures. C: Detection of P-gp transport activity by calcein/AM assay. Results are characteristic of three independent experiments that gave similar results.*

at 37˚C. After incubation, cells were washed twice in ice cold PBS. Fluorescence measurements were made using a Coulter Epics Altra flow cytometer (USA).

Detection of membrane glycoprotein by ConA blots. Membrane glycoproteins interacting with ConA were quantified in crude membrane fractions that were prepared with a ProteomeExtract Subcellular Proteome Extraction Kit (Calbiochem). After SDS-PAGE of proteins in the crude membrane fraction and electroblotting (as described above), proteins were specifically visualised with ConA conjugated with biotin and avidin conjugated with horseradish peroxidase (Sigma, USA) using the ECL detection system (GE Healthcare) (28) and a Kodak CF 440 scanning system.

Quantification of membrane glycoprotein-targets of ConA by lectin dot blot. Crude membrane fractions (6 μg of membrane protein) in 100 μl of 0.02 mol/l Tris-HCl buffer (containing 0.5 mol/l NaCl, pH 7.5) were applied to nitrocellulose membranes with a vacuum dot blot apparatus (BioRad, USA). The nitrocellulose membranes were then treated according same protocol for Western blot.

Detection of FITC-ConA binding onto S, R and T cells by confocal microscopy and flow cytometry. After cultivation, cells were washed three times with PBS, resuspended in RPMI medium without fetal bovine serum (5×10^5 cells/ml) and incubated for 30 min with fluorescein isothiocyanate (FITC)-labeled ConA (Sigma, USA) at a concentration of 0.1 mg/l in a humidified atmosphere supplemented with 5% CO_2 at 37˚C (28). After incubation, cells were washed three times with PBS, and specific labels were evaluated as green fluorescence with a confocal microscope (Leica TCS SP-2 AOBS, Germany) or were counted with a Coulter Epics Altra flow cytometer (USA).

Effect of ConA on cell survival. Cells were cultivated under the conditions described above in 96-wells plates (5×10^4 cells/well in 200 μl cultivation medium) in the absence or presence of ConA (0.1-30 mg/l). After cultivation, cell viability was analysed by MTT test (using thiazolyl blue tetrazolium bromide) (31).

Statistical analysis. Data are expressed as the mean±S.E.M. Statistical significance was assessed using an unpaired Student's *t*-test using SigmaPlot Graphing Software (version 2.01).

The concentration dependence of the ConA cytotoxic effect on cell viability was described by an equation of exponential decay (28):

$$N=A \times \exp[\ln(0.5) \times (c/IC_{50}c)^n] \qquad \text{Eq. (1)}$$

where A and N represent the viable cells after cultivation in the absence or presence of ConA at concentration c. IC_{50} is the concentration of lectin when N=0.5×A; n is the order exponent. Experimental data were fitted according this equation using SigmaPlot Graphing Software (version 2.01).

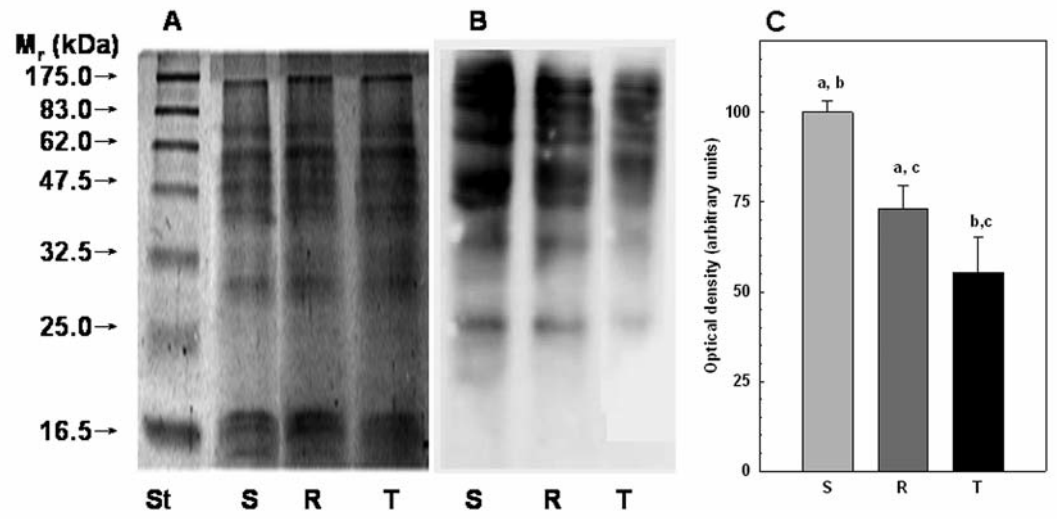

Figure 2. *Detection of ConA-interacting material in crude membrane fractions isolated from S, R and T cells. A: Coomassie blue staining of the acrylamide gel. B: staining with ConA in the blot. The data are characteristic of three independent measurements that gave similar results. C: Quantification of bound ConA using dot blot. Data represent the mean±S.E.M. from three independent experiments. Significance: a, labeled columns differ at the p<0.05 level; b, labeled columns differ at the p<0.01 level; c, labeled column do not differ significantly, p>0.1.*

Results

Expression and activity of P-gp in R and T cells. In contrast to S cells that did not contain any measurable amount of P-gp mRNA, this transcript was detected in both R and T cells using RT-PCR (Figure 1A). Consistent with these results, P-gp was detected by Western blotting of cell lysates from R and T but not S cells (Figure 1B). The transport activities of P-gp in R and T cells were measured by flow cytometry using calcein/AM as fluorescent P-gp substrate. The presence of P-gp in R and T cells was able to protect them against loading with calcein (Figure 1C). However, massive calcein loading was observed in S cells.

Binding of ConA to crude membrane fractions isolated from S, R and T cells. More intensive staining of glycoproteins by ConA (using the lectin blot method) was obtained after SDS-PAGE and electroblotting of crude membrane fractions isolated from S cells than from R or T cells (Figure 2B). Signal of GAPDH or β-actin in crude membrane fraction was very weak and could not be used as housekeepers. Therefore, the accuracy of protein loading to the respective lines was proved by Commassie blue staining of separate gel (Figure 2A). Crude membrane fractions isolated from R and T cells compared with S cells did not contain any new protein bands detectable with ConA in the 140-180 kDa range, which corresponds to material detected as P-gp with the c219 antibody as documented in Figure 1B. Therefore, ConA did not bind P-gp in crude membrane fractions isolated from R and T cells. Similar results were recently observed with R cells (28).

Quantitative lectin dot blotting was next used to demonstrate decreased lectin binding on R and T cells compared to S cells. The crude fraction isolated from both P-gp-positive cell lines also bound lower amounts of ConA than the corresponding fraction isolated from S cells (Figure 2C).

Binding of FITC-labeled ConA on the cell surface of S, R and T cells. Decreased binding of FITC-labeled ConA to R and T cells was observed compared to S cells by fluorescence cytometry (Figure 3A). This trend was also visible by confocal microscopy (Figure 3B). ConA was not able to enter the cells under these experimental conditions because FITC-labeled ConA was localised directly on the cell surface and not in the intracellular space of cells (Figure 3C). Cell surface binding of ConA was described also for LAN5 neuroblastoma cells (32).

Cytotoxic effect of ConA on S, R and T cells. It was previously demonstrated that R cells were less sensitive to ConA than S cells (28). Therefore, it was necessary in this study to determine if T cells are also less sensitive to ConA than S cells. Figure 4 clearly shows that both P-gp-positive cell sublines (R and T) were less sensitive to ConA. PZR (protein zero related protein) was identified as major target of ConA (33). Therefore, it was attempted to determine if PZR was involved in the mechanism by which ConA induced cell death in S, R and T cells. Unfortunately, RT-PCR failed to detect mRNA encoding PZR in S, R and T cells using primers: 5'-GTT GGT GTT CCT GGT TTG GG -3' and 5'-TTT CTG CAC GAG AGT CCA GTT TG-3' (data not shown). However, using RT-PCR under the same

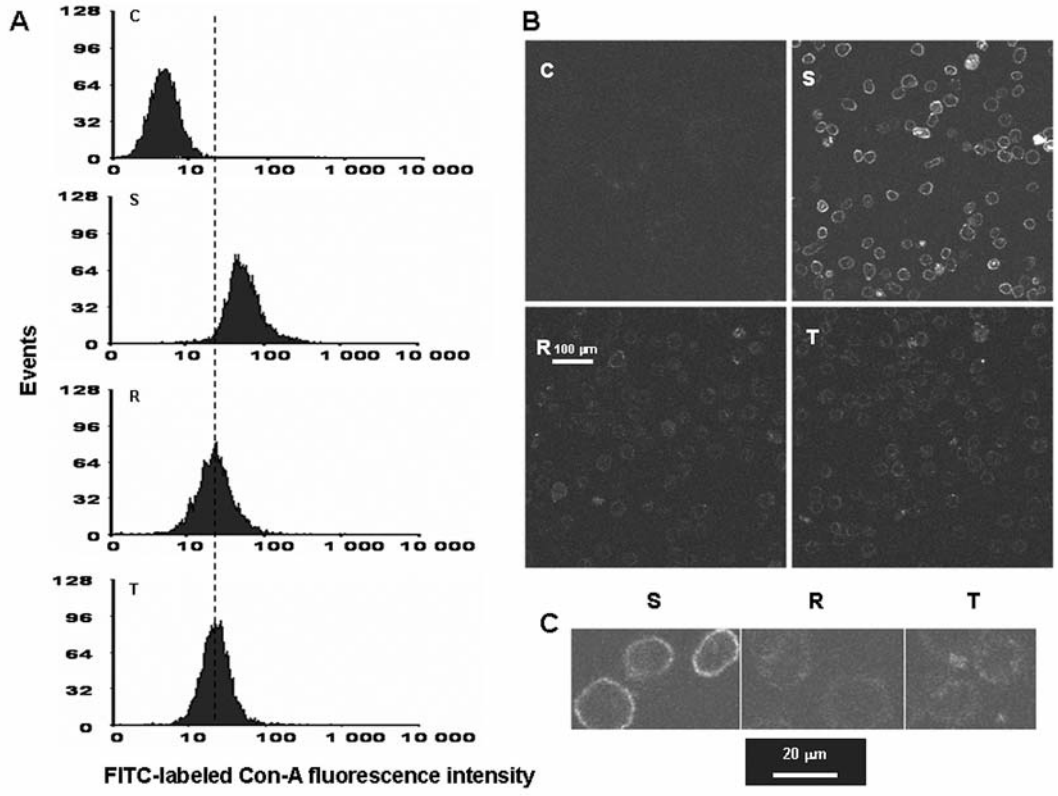

Figure 3. *Detection of ConA binding to S, R and T cells using flow cytometry (A) and confocal microscopy (B and C). Cells were incubated without FITC-labeled lectins in control experiments (C, shown for S cells, R and T cells gave similar records). The data are characteristic of six independent measurements that gave similar results.*

conditions, it was possible to detect this mRNA in mouse embryonic fibroblast cell line NIH 3T3 (data not shown) as a positive control (33; 34). Thus, expression of PZR in S, R and T cells was absent.

Discussion

Massive expression of P-gp and manifestation of its activity in R and T cells using calcein/AM as P-gp substrate is documented on the Figure 1. Presence of P-gp in R and T cells protect them against loading with calcein. Massive loading with calcein was observed in P-gp-negative S cells. Retention of calcein in R and T cells (similar to that in S cells) could be induced by the presence of P-gp inhibitors (*i.e.* verapamil and cyclosporine A) but not with probenecide, an inhibitor of several anion transporters including MRPs (data not shown). These results indicated that active P-gp is present in R and T cells. Consequently, R and T cells were much less sensitive than S cells to vincristine, vinblastine, doxorubicin, actinomycin D, mitomycin C, cyclophosphamide and dexamethasone (data not shown), which were previously described as P-gp substrates (35).

Lower amounts of ConA binding to glycoproteins present in crude membrane fraction isolated from R and T cells as compared with S cells are documented in Figure 2. The fact that P-gp expression (in both cases, *i.e.* selection with vincristine or transfection with human gene encoding P-gp) induced changes in the composition of membrane-bound saccharides of L1210 cells detectable by the interaction with ConA indicated that these changes were induced simply by the presence of P-gp alone in R and T cells. Therefore, the decrease in membrane bound saccharide targets of ConA in R and T cells as compared with S cells may be considered as secondary cellular response to P-gp overexpression.

The lower intensity of ConA induced agglutination of a vincristine-colchicine-resistant subline of L1210 cells relative to a sensitive subline of L1210 cells was detected as early as 1976 (25) in the same year that Juliano and Ling discovered P-gp (36). Although the former authors did not prove presence of P-gp in resistant cells, they showed that previous treatment of sensitive cells with vincristine (which induces P-gp overexpression in this cell model (6)) caused a marked decrease of their agglutinability. However, under the same treatments, agglutinability of the resistant cells was lowered only slightly (25). In the current experiments,

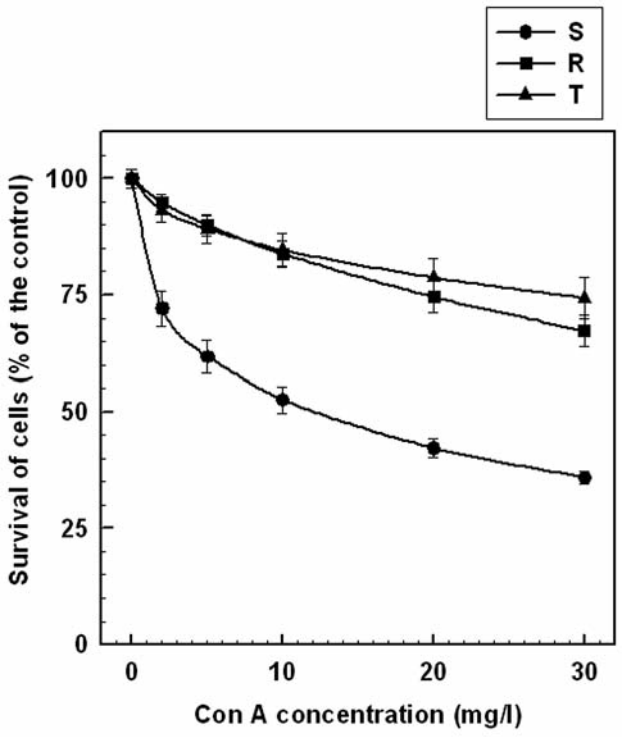

Figure 4. *Effects of ConA on viability of S, R and T cells. The number of cells in the absence of lectins was arbitrarily taken as 100%. The data represent the mean±S.E.M. from six independent experiments. Curves were obtained by nonlinear regression according to Eq. (1).*

minimal agglutinating ConA concentrations were between 0.5-1.0 mg/l, >2.5 mg/l and >3 mg/l for S, R and T cells, respectively. All of the above facts indicated that P-gp-positive R and T cells differ from S cells by decreased levels of saccharide-targets of ConA directly on the cell surface.

More pronounced FITC-labeled ConA binding to cell surface of S cells as to R and T could be observed by confocal microscopy or fluorescence cytometry (Figure 3). The presence of P-gp alone in R and T cells was responsible for such alteration in the composition of cell surface saccharides and was independent of the way in which P-gp expression was established. Non-differentiating rat L6 myoblast cell lines selected for resistance to ConA were bound significantly less [^{3}H]-ConA than their parental wild-type line (37). Therefore, resistance to ConA exists in several cell models and is connected with defective biosynthesis of glycoproteins (38). Moreover, resistance to ConA was associated with loss of the ability to undergo normal morphological or biochemical differentiation. The current experiment proved that both P-gp-positive cells R and T exerted differences in cell surface saccharide targets of ConA compared to S cells. Therefore, alterations in the biosynthesis of glycoproteins in R and T cells relative to S cells were hypothesised.

Lower expression of cell surface saccharide targets of ConA in R and T cells consequently depressed sensitivity of these cells to ConA as compared to S cells (Figure 4). This indicated that P-gp-mediated drug resistance might exist in cells together with a reduction in cell surface saccharide-targets of ConA, which also induced resistance to ConA. Moreover, alterations in cell surface saccharide composition were directly related to the presence of P-gp.

However, the mechanism by which the presence of P-gp in R and T cells affects the biosynthesis of cell surface saccharides is still unknown. Zhao *et al*. (33) identified PZR (protein zero related) as a major mediator of ConA-induced cell signaling. PZR is an integral plasma membrane protein that may anchor tyrosine phosphatase SP-2 (39). The extracellular portion of PZR contains a single immunoglobulin-like domain displaying 46% sequence identity to that of myelin P0, a major structural protein of peripheral myelin (40). However, S, R and T-cells did not contain any measurable amount of mRNA encoding PZR and therefore, the idea that PZR is the target that is responsible for the cell death effect of ConA in the cells used in this study is improbable.

Recently, Wang *et al*. (41) showed that a phosphoinositide 3-kinase (PI3K) gamma inhibitor blocked ConA-induced hepatic injury in mice. Interestingly the possible involvement of the PI3K/AKT kinase pathway in modulation of P-gp-mediated MDR in the R cell line has been described previously (18). However, the involvement of the PI3K/AKT kinase pathway in ConA-induced cell death remains to be determined.

The present study suggests that expression of P-gp in L1210 cells is causally associated with massive remodelation of cell surface sugars that could be monitored by conA and other lectins. Alteration in surface saccharides may be considered as a secondary cellular response on P-gp expression.

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