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# Cell-Surface Changes in Cadmium-Resistant *Euglena*: Studies Using Lectin-Binding Techniques and Flow Cytometry

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Most *in vitro* studies on contaminants only focus on the short-term effects of pollutants on cells, without regard to long-term effects and the ability of cells or microorganisms to develop a specific resistance to a pollutant. Indeed, the knowledge of these resistance mechanisms allows a more holistic approach to the problem of environmental pollution.

We have previously reported on the ability of unicellular *Euglena gracilis Z* algae to develop cadmium (Cd) resistance (Bariaud *et al.* 1985, Bonaly *et al.* 1978). Cadmium is ubiquitous environmental contaminant. This heavy metal enters the aquatic environment mainly through vapor emissions and fallout during smelting operations (Trevors *et al.* 1986). Diverse mechanisms of algal resistance to toxic metals are known (Wood and Wang 1983). Among these, the most general mechanism is the development of metal-binding proteins. For example, animal cells develop metalloproteins, whereas plant cells and algae acquire phytochelatins (Rauser 1991). Concerning our cadmium-resistant *Euglena* cells, the metal did not appear to be sequestered on soluble metal-binding ligands. On the contrary, previous experiments have shown that resistance development is related to a diminution of cadmium penetration into cells, implicating cell surface or membrane alteration (Bariaud *et al.* 1985).

The purpose of this research was to investigate further the mechanisms of development of cadmium resistance in *Euglena* cells at the cell-surface level. Sugar chains of glycoproteins and glycolipids are a predominant feature of the surface of cells. Moreover, it is becoming increasingly clear that the cell-response to environmental changes is often orchestrated through surface macromolecules such as glycoproteins.

One method for analyzing membrane glycoproteins is the use of plant lectins as specific probes of cell-surface sugars. These proteins interact specifically with monosaccharides and oligosaccharides (Lis and Sharon 1973). Lectins can be labeled with fluorescein-isothiocyanate (FITC). The relative amount of cell-bound lectins can be either observed microscopically or determined by a flow cytometric measurement. In this study, we applied this lectin method to investigate surface carbohydrate expression during and after resistance development. Our interest was twofold: (1) to learn more about the carbohydrate composition of the cell-surface of *Euglena*; and (2) to determine whether transition from wild cells to Cd-resistant cells changes the expression of cell-

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surface carbohydrates.

## MATERIALS AND METHODS

Experiments were performed on the wild chlorophyllous *Euglena gracilis* Klebs strain Z and on a Cd-resistant strain adapted from the first one. Cells were grown at 23°C in a synthetic medium containing minerals, lactic acid and vitamins B1 and B12 (Bonaly *et al.* 1978). Cadmium chloride (0.5m*M* final concentration) was aseptically added to the medium.

After two washings in a phosphate buffer 0.2*M* (pH 7.4), cells ($5x10^6$ per sample) were incubated for 1hr with 1mL (100µg) of the various FITC-lectins (Sigma) in the same phosphate buffer added with $CaCl_2$ (1m*M*) and $MgCl_2$ (0.5m*M*). To establish the specificity of lectins for their appropriate sugars, aliquots of culture were incubated first with 0.5 mL of specific sugar (10 mg/mL). Cells were analyzed within 1 hr. Lectins included *Helix pomatia* agglutinin (HPA) and *Glycin max* agglutinin (SBA), specific for N-acetyl-D-galactosamine (D-galNAc), concanavalin A (Con A) and *Lens culinaris* agglutinin (LCH), specific for mannose residues, peanut agglutinin (PNA), specific for D-galactose (D-gal) and *Lotus tetragonolobus* agglutinin (LTA), specific for α-L-fucose.

Flow cytometry measurements were performed with a FC 200 cytofluorograf (Biophysics) connected to a 1024 channel analyzer.

## RESULTS AND DISCUSSION

Fluorescence microscopic observations were performed first to examine the

Table 1. Microscopic observations of FITC-lectin binding on *Euglena* cell-surface during exponential growth.

| Lectin used | Wild cells binding | Cd-resistant cells binding |
|---|---|---|
| Con A | - | - |
| LCH | - | - |
| HPA | + + + | - |
| SBA | + + + | - |
| PNA | + + + | 2 subpopulations: +++ / - |
| LTA | - | + |

- no lectin binding; + minor green fluorescence; +++ bright green fluorescence

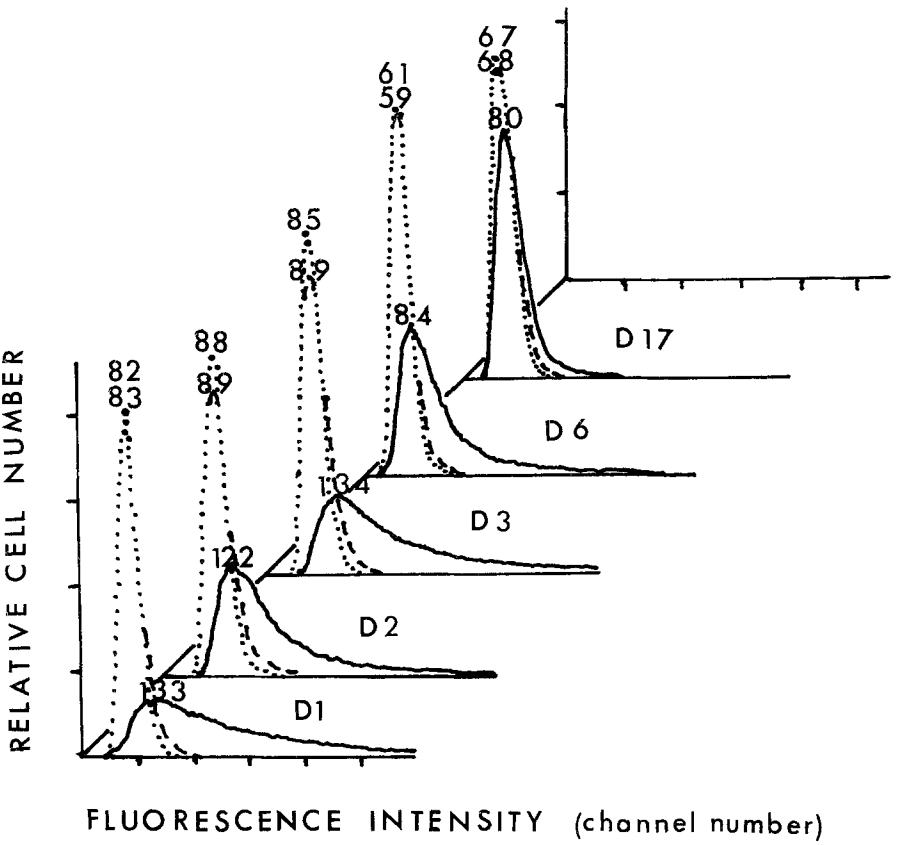


Figure 1. FITC-HPA binding to wild *Euglena* cells as measured by flow cytometry. Fluorescence histograms reveal more FITC-HPA binding during exponential growth (days 1 to 3)
— FITC-HPA only; --- with N-acetyl-Dgalactosamine and FITC-HPA; ..... without FITC-HPA; D= day.

ability of the various lectins to bind on wild and on Cd-resistant *Euglena* cell-surface. All experiments were conducted during exponential growth. Results are summarized in Table 1.

For wild cells, only HPA, SBA and PNA produced a strong microscopically detectable cell-surface fluorescence. In contrary, Con A, LCH and LTA did not bind to wild *Euglena* cells. The binding of lectins was different for Cd-resistant cells: no green fluorescence was detected after either HPA or SBA incubation. After exposure to PNA, both labeled and unlabeled cells were observed. After exposure to LTA, all cells were labeled but with just a minor green fluorescence. Exposure to Con A and to LCH produced unlabeled cells. Moreover, Cd-resistant cells showed a more intense red chlorophyll fluorescence than wild cells in all experiments. Binding of these lectins to *Euglena* cell surface was found to be specific since it was blocked by competition with appropriate sugars.

These results suggest that wild *Euglena* cells in exponential growth expressed

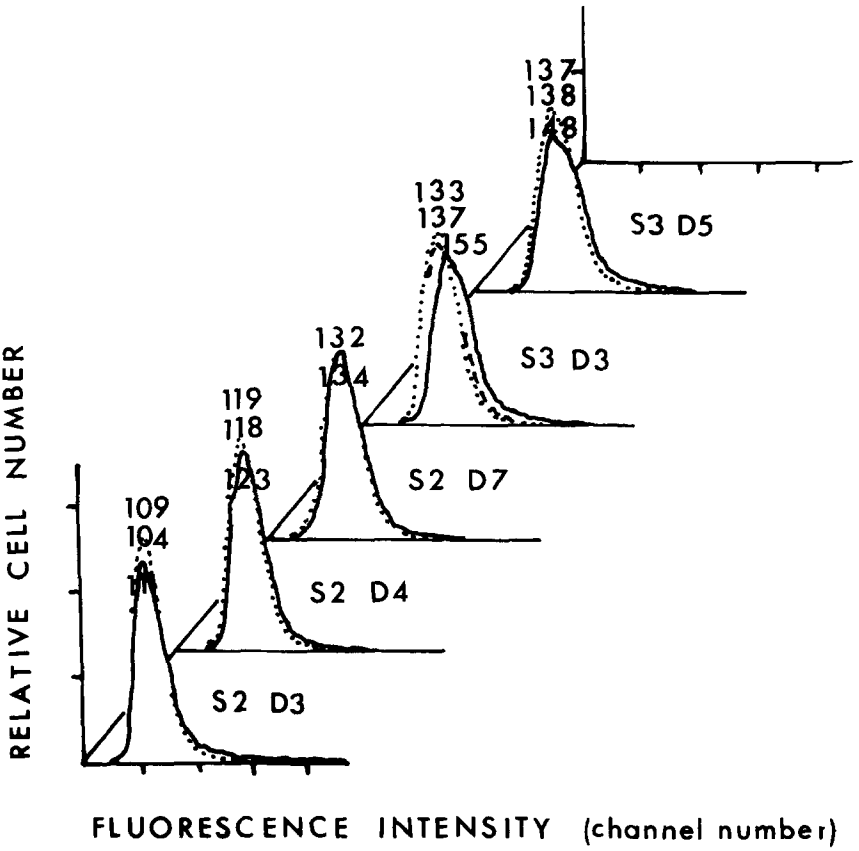


Figure 2. FITC-HPA binding to Cd-treated *Euglena* cells as measured by flow cytometry. Fluorescence histograms reveal the lack of specific FITC-HPA binding all along the two subcultures. S2= second Cd subculture; S3= third Cd subculture — FITC-HPA only; --- with N-acetyl-Dgalactosamine and FITC-HPA; ..... without FITC-HPA; D= day.

galactose and N-acetyl-galactosamine, but did not express fucose and mannose (since cells did not bind either LTA, Con A, or LCH), PNA, SBA and HPA showing the same pattern of binding. Cd-resistant cells did not express the same oligosaccharide binding sites as did wild cells. D-galNAc was not detected. Only half of the cell population contained galactose groups on their surface accessible to PNA. Fucose, not detected on wild cells, became slightly expressed as visualized by LTA binding. In contrast, mannose remained undetected. Therefore, Cd-resistance was related to a modification of membrane glycoconjugate expression, revealing hidden or lost D-galNac receptors and a development of fucose sites.

The second phase of this study was to investigate cell-surface carbohydrate expression during Cd-resistance adaptation. For this purpose, D-galNAc expression was analyzed during serial Cd-exposures. Since the fluoresceinated lectin HPA has homogeneous cell-surface binding, it was used for further experiments. The analyses of fluorescence intensity were performed after flow cytometric measurements.

Investigations were first carried out throughout the growth cycle of wild cells. The growth cycle of *Euglena* consists of a short lag phase followed by an exponential phase (day 1 to day 4 in our experimental conditions) and by a plateau stage (from day 5). Fluorescence-intensity histograms (Figure 1) show heterogeneity of lectin binding on an individual basis. Binding of HPA to the cell surface was found to be specific since competition with the appropriate sugar blocked binding and reduced fluorescence to autofluorescence levels (see overlap of correspondant histograms). Most cells during exponential growth (days 1 to 3) bound FITC-HPA. In contrast, cells in the stationary stage (days 6 and 17) revealed a progressive decrease in FITC-HPA binding: at least 50% at day 6, and more than 80% at day 17.

We then attempted to establish if the HPA receptor (D-galNAc) on the surface of *Euglena* could be altered by the presence of cadmium. As previously reported, a primary culture in cadmium showed an altered growth curve (Bonaly *et al.* 1978). In brief, a first, short exponential growth was followed in our experimental conditions (1) by a block stage (days 4 to 14), (2) by a second exponential growth (days 15 to 17) and a stationary stage (from day 17). Fluorescence intensity histograms after HPA exposure (not included) gave a decrease in lectin binding all along the growth. However, histograms were difficult to interpret because (1) autofluorescence level was increased as compared to controls (since the chlorophyll quantity was more abundant as aforesaid) and (2) cell population was heterogeneous as microscopically observed. Moreover, the heterogeneity increased throughout growth; for example, at day 15 (the end of the second period of exponential growth) microscopic observations showed five distinct cell subpopulations: (1) dead cells (about 50% of the total population); (2) HPA-labeled cells showing a more abundant quantity of chlorophyll than controls; (3) voluminous lectin-unlabeled cells full of chlorophyll; (4) cells labeled only on the ridges of the pellicle ornementation showing a reduced chloroplastic system; and (5) small achlorophyllous cells showing many HPA receptors.

In order to study the development of cadmium-resistance, 17-d old cadmium cells from a primary culture were cultured into a further medium with $CdCl_2$. A third subculture was then performed with some 7-d old cells from this second subculture. During these second and third subcultures, the slopes of the growth curve were not high compared to controls. As previously reported, the slopes of the growth curves rise throughout the serial propagations, up to the control curve slope, after about 20 subcultures in Cd-medium (Bariaud *et al.* 1985).

During the second and third subcultures, flow cytometric analyses revealed a significant lack of HPA-binding sites (Figure 2). The overlap of histograms indicated no differences of intensity between autofluorescence and FITC green fluorescence. Moreover, FITC-HPA treated cells exhibited insignificant differences between cells in the exponential phase (S2 day 3, S3 day 3) and cells in the stationary stage (S2 day 7, S3 day 5). Because D-galNAc remained accessible for HPA during the first subculture in the presence of Cd, cell-surface accessibility was not cluttered up by Cd ions. Microscopic observations revealed heterogeneous population, but with fewer subpopulations. During the third culture, the major subpopulation (80% of the total population) was made up of voluminous FITC-unlabeled cells showing a red chlorophyll fluorescence. Earlier, we indicated that Cd-resistant cells gave similar pictures. Consequently, it appears that Cd-resistant cells probably resulted from this major chlorophyllous HPA-unlabeled cell subpopulation. Moreover, this HPA-

unlabeled cell subpopulation appeared rapidly selected when exposing cells to Cd.

This study showed that acquiring resistance in *Euglena* cells was correlated with the characteristic of their cell surface as probed by specific lectins: D-galNAc and D-gal were only accessible on wild cells, but fucose was only accessible in Cd-resistant cells. It is well known that complex cell-surface oligosaccharides can be branched many times and vary in their substitution pattern as well as in the sequence of sugars beyond the core (Kornfield and Kornfield 1980). Therefore, undetected sugars are either lacking or masked for their appropriate lectin. Many cell perturbations have been correlated with changes in cell-surface glycoproteins: cell differentiation (Notter and Leary 1987, Christensen *et al.* 1992), malignant transformation (Mannori *et al.* 1991), chemical or physical stress (Grillon *et al.* 1991, Schreiber *et al.* 1989) or vitamin B12 starvation (Bré *et al.* 1986). However, mediated changes are quite different according to cells and stress: either only one sugar or several sugars become undetected or overexpressed.

In summary, we have shown that the development of Cd-resistance in *Euglena* was correlated with changes in expression of D-galNAc, D-gal and fucose. Interestingly, after vitamin B12 starvation, *Euglena* cells were modified in lectin binding for the same sugars: D-galNAc and D-gal were undetected at the surface level too (Bré *et al.* 1986). So, in *Euglena*, these macromolecules seem to be good candidates for mediating biological events in environmental responses. Further studies might clarify the mechanisms and the meaning of these changes in sugar expression.

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