Changes in cell surface structure by viral transformation were studied by examining changes in the binding of various lectins differing in carbohydrate specificities. Binding of lectins was assayed directly using cells grown on coverslips. The following 125I-lectins were used: Concanavalin-A (specific for glucose and mannose), wheat germ agglutinin (specific for N-acetylglucosamine), castor bean agglutinin (specific for galactose), Wistaria floribunda agglutinin (specific for N-acetylgalactosamine), and soybean agglutinin (specific for N-acetylgalactosamine).

Cells of a clone, SS7, transformed by bovine adenovirus type-3, were found to bind 5 to 6 times more Wistaria floribunda agglutinin than the normal counterpart cells (clone C31, from C3H mouse kidney). In contrast, the binding of soybean agglutinin, which has a sugar specificity similar to Wistaria floribunda agglutinin, to normal and transformed cells was similar. The binding of wheat germ agglutinin and castor bean agglutinin, respectively, to normal and transformed cells was also similar. However, normal cells bound twice as much concanavalin-A as transformed cells. Only half as much Wistaria floribunda agglutinin was bound to transformed cells when they had been dispersed with EDTA. These changes in the number of lectin binding sites on transformation are thought to reflect alteration of the cell surface structure.

The amount of lectins bound per cell decreased with increase in cell density, especially in the case of binding of Wistaria floribunda agglutinin to normal cells.

On viral transformation of cultured cells, glycoproteins and glycolipids on the cell surface are reported to change qualitatively and quantitatively. These changes are probably caused by alteration in the activities of cell surface glycosyltransferases, proteinases, and/or some yet unknown functions of the cell. There must be some correlation between these surface alterations, expressed by sugar-containing materials, and various characteristics of transformed cells, such as altered morphology, uncontrolled cell growth, antigenic alterations, etc.

Lectins are proteins of mostly plant origin which bind specifically with various sugars. Recently they have been used to examine changes of cell-surface carbohydrate groups. We have been studying the process of viral transformation of cultured cells, especially structural changes of the cell surface. We have obtained transformed cells by infecting mouse cells with an oncogenic DNA virus, bovine adenovirus type-3 (BAV-3), and have compared the cell surface structure of the transformed cells to that of normal cells by measuring their binding with radioactive lectins.

This paper reports studies carried out with 5 different lectins, concanavalin-A, wheat germ agglutinin, castor bean agglutinin, Wistaria floribunda agglutinin, and soybean agglutinin. Lectin binding and cell aggluti-
nation were examined in growing and stationary phase cells. Cells grown on coverslips were used for lectin binding assays instead of those suspended by treatment with EDTA, trypsin, or EDTA-trypsin, and maximum binding of lectins to the cells was determined kinetically by the use of the Scatchard plot.

**MATERIALS AND METHODS**

**Materials** Jack bean meal and wheat germ lipase were purchased from Sigma Chemical Co., St. Louis, and Chloramin-T from Merck, Darmstadt. Eagle's minimum essential medium (MEM) was purchased from Nissui Seiyaku Co., Tokyo, and fetal calf serum from Grand Island Biological Co., Grand Island, New York. All other chemicals used were reagent grade. Castor beans and soybeans were purchased from a local seed company. Wistaria floribunda seeds were collected in the Kyoto Herbal Garden of this firm.

**Cell Lines** The mouse cell clones C31 (normal cells) and SS7 (BAV-3 transformed cells) were used throughout. The C31 cell line was obtained by two clonings of the cell line C3H2K, derived from a newborn C3H/He mouse kidney. Clone SS7 was obtained by cloning transformed cells derived from C3H2K cells by infection with BAV-3. Clone SS7 showed the characteristics of transformed cells of altered morphology, high saturation density, weak adhesiveness to the substrate, colony formation in Methocel, and cell-surface antigen(s) which seemed specific to BAV-3 infection (unpublished).

**Cell Culture** Cells were maintained in MEM supplemented with 10% fetal calf serum and cultured in dishes under 5% CO2-95% air at 37°C. The molecular weights of Wistaria agglutinin and soybean agglutinin were calculated to be about 70,000 and 96,000, respectively. Wheat germ agglutinin gave a broad band under our labeling conditions, with one molecule of the iodinated product being calculated to bind about one molecule of 125I.

**Preparation of Lectins** Concanavalin-A, from jack bean meal, was prepared as described by Agrawal and Goldstein. Wheat germ agglutinin, from wheat germ lipase, was purified by affinity chromatography on ovomucoid-Sepharose. Ovomucoid was prepared from egg white by the method of Cuatrecasas and Anfinsen. Wheat germ lipase was dissolved in 0.1M Tris buffer (pH 7.2) and passed through an ovomucoid-Sepharose column. The adsorbed wheat germ agglutinin was then eluted with 0.1M acetic acid and further purified by CM-cellulose column chromatography. Castor bean agglutinin was purified as described by Tomita et al. Castor bean seeds were cracked and extracted with phosphate-buffered saline (PBS). The extract was adsorbed on Sepharose 4B and castor bean agglutinin was eluted with 0.1M lactose. The eluate was concentrated and subjected to gel filtration on BioGel P-150.

Wistaria agglutinin was purified in our laboratory (unpublished) from an extract of Wistaria floribunda seeds by procedures including ammonium sulfate precipitation, DEAE-Sephadex A-50 column chromatography, and Sephadex G-200 gel filtration. This preparation was homogeneous and did not contain Wistaria floribunda mitogen reported by Toyoshima et al. Soybean agglutinin was purified from soybeans as described by Lis et al.

**Purity of Lectins** The purity of lectin preparations was checked by polyacrylamide gel electrophoresis. Concanavalin-A and soybean agglutinin each gave a single band at pH 4.0. Wheat germ agglutinin gave two adjacent bands at pH 4.0. However, both seemed to have affinity for cell-surface carbohydrates because they had been purified by affinity chromatography, so that a mixture of the two was used as the wheat germ agglutinin preparation. Wistaria agglutinin and castor bean agglutinin each gave a single band on electrophoresis at pH 8.9.

**Polyacrylamide Gel Electrophoresis** Electrophoresis was carried out on 7.5% gels at pH 4.0 or 9.8 for 1 hr at 4 mA/gel (50 V/cm) at room temperature. Protein was stained with Coomassie Brilliant Blue.

**Molecular Weight Determination of Lectins** The molecular weight of purified lectins was determined by polyacrylamide disc gel electrophoresis in sodium dodecyl sulfate by the method of Weber and Osborn, using 7.5% or 10% gels. Bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), and cytochrome c (12,500) were used as molecular weight markers. The molecular weights of Wistaria agglutinin and soybean agglutinin were calculated to be about 70,000 and 96,000, respectively. Wheat germ agglutinin gave a broad band in the position corresponding to a molecular weight of about 18,000. The molecular weights of concanavalin-A and castor bean agglutinin were taken as 110,000 and 120,000 based on the reports of McKenzie et al. and of Nicolson and Blaustein. respectively.

**Preparation of 125I-labeled Lectins** The lectins were iodinated with 125I using Chloramin-T by the method of Greenwood et al. The specific monosaccharide (0.1M) known to inhibit hemagglutination by each lectin specifically was added to the reaction mixture to protect the binding sites of the lectin. Under our labeling conditions, one molecule of the iodinated product was calculated to bind about one molecule of 125I.
Binding Assay

Cells maintained in culture bottles were trypsinized and a suspension of $4 \times 10^8$ cells in 5 ml culture medium was inoculated into dishes (45 mm diameter) containing two coverslips (15 mm diameter). One or three days later, the coverslips were carefully taken out of the dishes, washed once with Tris-NaCl solution ($10mM$ Tris-HCl (pH 7.2) and $150mM$ NaCl), and placed in small vessels containing various concentrations of $^{125}$I-lectins in Tris-NaCl solution in a final volume of $0.3 \text{ ml}$. The vessels were shaken for 90 min at $22^\circ$ and then the coverslips were washed five times with Tris-NaCl solution and their radioactivities were counted in an Automatic Well Scintillation Counting System (Fujitsu, ATS-623). To measure the number of cells, the cells on the coverslips in dishes, similar to those used for binding assay, were removed with $0.125\%$ trypsin and cell number was determined in a Coulter Counter (model ZB, aperture tube, $100 \mu m$).

The maximum amount of lectin bound per cell was estimated from the Scatchard plot. If the binding sites of a lectin on the cell surface are all of one kind, with respect to the association constant ($K$, see later paragraph), the reaction between lectin and its binding site should obey the law of mass action.

$$\frac{n-\nu}{[\text{Free lectin}]} = K \nu$$

where $\nu$ represents the amount of lectin bound per cell, $[\text{Free lectin}]$, the free lectin concentration, $n$, the maximum binding of lectin per cell, and $K$, an association constant. A plot of $\nu/[\text{Free lectin}]$, calculated by measurement of radioactivity, against $\nu$ gives a straight line (Scatchard plot). The intercept of the $\nu$ axis is $n$. An example of a plot of results in a typical binding assay is shown in Fig. 1. In this case, the cell (C31) number on the coverslip was $1.7 \times 10^4$ and the maximum binding of concanavalin-A was determined as $4.0 \times 10^{-5} \mu g/cell$.

Inhibition of Lectin Binding to Cells by Sugars

The specific sugars for each of the lectins (final concentration, $0.05 \sim 0.2M$) were added to the reaction mixture and their inhibition of binding of the lectin to the cells (C31) was measured after incubation at room temperature for 90 min. Addition of a specific sugar is known to inhibit hemagglutination by the lectins. The sugars used were $\alpha$-methylglucose for concanavalin-A, N-acetylglucosamine for wheat germ agglutinin, N-acetylgalactosamine for Wistaria agglutinin (unpublished), and galactose for castor bean agglutinin. The concentration of lectin was $25 \mu g/0.3 \text{ ml}$.

Determination of Wistaria Agglutinin Binding to Dispersed Cells

Cells in the growing phase in bottles were washed twice with cold Tris-NaCl solution and once with $0.02\%$ EDTA solution. Then $10 \text{ ml}$ of $0.02\%$ EDTA solution was added and the bottles were stood for 30 min at room temperature. The dispersed cells were harvested by low-speed centrifugation ($1,000 \text{ rpm, } 5 \text{ min}$), washed twice with cold Tris-NaCl solution, and suspended at a concentration of $10^6$ cells/0.05 ml. Aliquots of 0.05 ml of the cell suspension were added to vessels each containing 0.05 ml of the desired concentration of $^{125}$I-Wistaria agglutinin. The mixtures were incubated with shaking for 90 min at room temperature (about $22^\circ$), 3 ml of cold Tris-NaCl solution was added, and the mixtures were centrifuged ($1,000 \text{ rpm, } 5 \text{ min}$). The supernatant was discarded and the precipitated cells were washed five times with 3 ml each of Tris-NaCl buffer by centrifugation. Their radioactivities were measured and the maximum amount of lectin bound to the cells was determined from the Scatchard plot.

Determination of Cell Agglutination by Lectins

After culture for 1 or 3 days, cells were removed from the bottles by treatment with $0.02\%$ EDTA solution and washed with $3/4 \times \text{ PBS}$ by centrifugation. They were suspended in $3/4 \times \text{ PBS} (2 \times 10^6 \text{ cells/ml})$, 50 $\mu l$ of the suspension was mixed with serial dilutions of lectin solution (50 $\mu l$), and the mixture was incubated with shaking for 30 min at $22^\circ$. The initial concentration of lectin was $1.0 \text{ mg/ml}$. Cell agglutination was scored on a qualitative...
scale of — to ++ (no agglutination to complete agglutination) under a microscope.

**Protein Determination** Protein was determined by the method of Lowry et al.\(^{22}\) with crystalline bovine serum albumin as a standard.

## Results

**Comparison of the Binding of Various Lectins to Normal and Transformed Cells** The maximum binding of lectins to normal or transformed cells grown on coverslips was determined at different cell densities (at different growing phase with respect to cell-to-cell contact) (Fig. 2). After culture for 1 day, there were about $5 \times 10^4$ cells on each coverslip, while after 3 days there were $10$ to $15 \times 10^4$ cells forming nearly confluent monolayers.

Normal cells bound nearly twice as much concanavalin-A as transformed cells at all cell densities while normal and transformed cells bound almost equal amounts of castor bean agglutinin and wheat germ agglutinin, respectively. In contrast, transformed cells bound about five to six times more *Wisteria* agglutinin than normal cells and the difference in binding was especially marked at a low cell density (early growing phase). N-Acetyl-galactosamine is known to inhibit hemagglutination by both *Wisteria* agglutinin and soybean agglutinin, but the binding of soybean agglutinin was less than that of *Wisteria* agglutinin, and the difference between the amount of soybean agglutinin bound to normal cells and that to transformed cells was hardly detectable.

The reactivities of both normal and transformed cells with all the lectins tested decreased with increase in the cell density.
Among them, the decrease in binding of *Wistaria* agglutinin to normal cells was especially remarkable.

**Inhibition of Lectin Binding by Sugars**

The binding of these lectins to the cells was inhibited by the presence of a specific sugar which inhibits their hemagglutination reaction (Fig. 3). The binding of wheat germ agglutinin and concanavalin-A was inhibited about 80~90% by 0.2M N-acetylglucosamine and α-methylglucose, respectively, while that of *Wistaria* agglutinin and castor bean agglutinin was inhibited about 50% by N-acetyl galactosamine and galactose, respectively. Stronger inhibition was found if lactose was used as an inhibitor of castor bean agglutinin binding to the cells (Fig. 3).

These results indicate that these lectins bind mostly to the corresponding carbohydrate chains or moieties exposed on the cell surface.

**Comparison of Cell Agglutination by Various Lectins**

For the following experiments cells were detached from the bottles with EDTA. Transformed cells were agglutinated by much lower concentration of lectins than that needed to agglutinate normal cells (Table I). The difference in the lectin concentration required to agglutinate normal cells and transformed cells was greater with cells at an early stage of growth than with cells at subconfluent cultures. Similar observations have been made by Goto *et al.* in the case of 3T6 cell agglutination by con-
canavalin-A. Of the five lectins tested, *Wistaria* agglutinin showed the greatest preferential agglutination of our transformed cells (SS7). Soybean agglutinin had weak agglutinating activity and the difference of soybean agglutinin concentration needed to agglutinate normal and transformed cells was less than that of *Wistaria* agglutinin.

**Binding of *Wistaria* Agglutinin to Cells Harvested by EDTA Treatment** Of the five lectins, *Wistaria* agglutinin showed the greatest difference in binding to normal and transformed cells. Since *Wistaria* agglutinin also caused the strongest cell agglutination of transformed cells, it seems probable that cell agglutination by *Wistaria* agglutinin is correlated with a higher degree of lectin binding. The cells used for agglutination test were harvested by EDTA treatment, and the binding of *Wistaria* agglutinin to EDTA-dispersed cells was measured (Table II), using cells in the growing phase. As expected, 1.1 to 1.7 times more *Wistaria* agglutinin bound to EDTA-dispersed transformed cells than to EDTA-dispersed normal cells. The maximum binding of *Wistaria* agglutinin to dispersed normal cells and to normal cells grown on coverslips was similar, whereas the binding to EDTA-dispersed transformed cells was only one-half of that to transformed cells on coverslips. These results indicate that the cell surface of transformed cells is damaged to some extent by EDTA treatment and some of the *Wistaria* agglutinin receptors are modified or lost by this treatment.

### Table I. Agglutination of Cells by Various Lectins

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<th>Lectin</th>
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<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
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<td></td>
<td>RCA</td>
<td>N</td>
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<td>WGA</td>
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<td>+</td>
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</tr>
</tbody>
</table>

(a) Cells grown for one day (sparse, $1.0 \times 10^4$ cells/cm$^2$) and three days (subconfluent, $3.2 \times 10^4$ cells/cm$^2$) were used. N=normal cells, T=transformed cells, Con-A=concanavalin-A, WGA=wheat germ agglutinin, RCA=castor bean agglutinin, WFA=Wistaria floribunda agglutinin, SBA=soybean agglutinin.

### Table II. Maximum Binding of $^{125}$I- *Wistaria* Agglutinin to Normal and Transformed Cell Dispersed by EDTA Treatment

<table>
<thead>
<tr>
<th>Cells</th>
<th>Bound lectin ($\times 10^{-5}$ µg/cell)</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Mean</th>
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</thead>
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<tr>
<td>Normal</td>
<td></td>
<td>0.55</td>
<td>0.45</td>
<td>0.45</td>
<td>0.48</td>
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<tr>
<td>Transformed</td>
<td></td>
<td>0.60</td>
<td>0.80</td>
<td>0.55</td>
<td>0.65</td>
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</table>

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LECTIN BINDING TO VIRAL TRANSFORMED CELLS

DISCUSSION

Cell-surface glycoproteins, glycolipids, and some cell-surface components are known to change or disappear during viral transformation. In the present work, we examined these surface changes in a BAV-3 transformant, SS7, and its normal counterpart cells using five different lectins. Results showed that there were more Wistaria agglutinin binding sites on SS7 cells than on normal cells. Unexpectedly, there were only about one-half as many concanavalin-A binding sites on transformed cells as on normal cells. The binding site of soybean agglutinin, which has a similar sugar specificity as Wistaria agglutinin, was also similar on normal and transformed cells.

Table III summarizes the number of lectin binding sites on the cell surface in growing and stationary phases, calculated from the molecular weight of each lectin. Of the five lectins, the number of binding sites for wheat germ agglutinin was greatest on transformed and normal cells. There were more binding sites for Wistaria agglutinin on transformed cells and their number was similar to that of concanavalin-A binding sites on the cell surface. There were fewer binding sites for soybean agglutinin than for other lectins on both normal and transformed cells.

Table III. Lectin Binding Sites of Sparse and Subconfluent Cells

<table>
<thead>
<tr>
<th>Lectin*</th>
<th>N=normal cells, T=transformed cells. Sparse, 3×10^4 cells/coverslip; subconfluent, 12-16×10^4 cells/coverlsp.</th>
<th>T Subconfluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con-A</td>
<td>2.3</td>
<td>1.7</td>
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<td>WGA</td>
<td>13.4</td>
<td>20.9</td>
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<td>1.2</td>
<td>1.6</td>
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<td>WFA</td>
<td>0.63</td>
<td>2.5</td>
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<tr>
<td>SBA</td>
<td>0.43</td>
<td>0.08</td>
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</table>

Table III. Lectin Binding Sites of Sparse and Subconfluent Cells

* Same as the footnote in Table I.

Inhibition of lectin binding to the cells by sugars was about 50% in the case of castor bean agglutinin and Wistaria agglutinin, but if lactose was used to inhibit castor bean agglutinin binding instead of galactose somewhat higher inhibition was observed. If complex carbohydrates containing N-acetylgalactosamine were used to inhibit Wistaria agglutinin binding to the cells, much stronger inhibition would be observed. In lectin binding to tumor cells, Kaneko et al. also reported in their sugar inhibition experiment that castor bean agglutinin seemed to bind to cells more tightly than concanavalin-A. Internalization of the lectins by the cells...
might occur to some extent, especially in castor bean agglutinin binding to the cells.

The binding of all the lectins with both normal and transformed cells decreased with increase in cell density. This was especially marked in the case of Wistaria agglutinin binding to normal cells. A possible reason for this difference is that the carbohydrate chains on the cell surface which bind with Wistaria agglutinin might be modified as the cells begin to come into contact, so that they no longer bind with Wistaria agglutinin. That is, "contact-sensitive" changes, as reported by Hakomori et al.\textsuperscript{13,14} or thickening of cell "coating" materials such as acid mucopolysaccharides or collagen, might prevent the binding of Wistaria agglutinin as the cells grew. Nicolson and Lacorbiere observed that the confluent 3T3 cells possessed 2.5 times the number of castor bean agglutinin binding sites per cell compared to growing phase cells.\textsuperscript{26} In contrast to their results, castor bean agglutinin binding to our normal cells (C31) decreased abruptly as the cells came in contact (Fig. 2). Some of the reasons for this discrepancy may be due to the difference of cells used and the different methods of lectin binding assays. Nicolson and Lacorbiere used EDTA-dispersed cells in lectin binding assays.

When transformed cells were dispersed by EDTA, their binding of Wistaria agglutinin decreased by about 50%. This suggests that materials binding Wistaria agglutinin on the transformed cell surface are removed by EDTA. In support of this, we obtained glycopeptides which inhibited Wistaria agglutinin binding to transformed cells from a pronase digest of the EDTA-soluble fraction from transformed cell surface (unpublished data). There seem to be fewer of these materials on the surface of normal cells because the binding of Wistaria agglutinin by normal cells did not change when the cells were dispersed from coverslips with EDTA. It remains to be clarified whether this increase of Wistaria agglutinin binding materials on the cell surface during transformation is specific to SS7 cells or whether it is seen with all BAV-3-infected or -transformed cells. Studies are also required on whether transformation by other tumor viruses or carcinogens also increase the binding of Wistaria agglutinin. We have some preliminary data that 2.5 times more Wistaria agglutinin binds to EDTA-dispersed SV3T3 cells than to EDTA-dispersed 3T3 cells (unpublished).

N-Acetylgalactosamine inhibited the hemaggulination induced by both soybean agglutinin and Wistaria agglutinin. However, soybean agglutinin bound less than Wistaria agglutinin to both transformed and normal cells. In our experiments, the agglutination of transformed and normal cells by soybean agglutinin was almost similar while that by Wistaria agglutinin was very different. These results indicate that each lectin recognize its own specific carbohydrate chains on the cell surface. These results also imply that the use of various kinds of lectins with different specificities will allow wider understanding of structural changes of cell surface related to carbohydrate chains.

The authors express their thanks to Drs. M. Goto and T. Matsuoka of Kyoto Herbal Garden of this firm for the supply of Wistaria floribunda seeds.

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