Differential Effects of Lectins on the in Vitro Growth of Normal Mouse Lung Cells and Low- and High-Cancer-derived Cell Lines

M. J. Vilarem, J. Jouanneau, D. Le François, and R. Bourrillon

ABSTRACT

The comparative aspects of cell growth, i.e., \[^{3}H\]thymidine and \[^{14}C\]leucine uptake of low-cancer (P4Bis) and high-cancer (P4BisT) cell lines and of their normal counterparts, have been studied in the presence and absence of concanavalin and Robinia lectins. These lectins have similar effects on cell growth, on thymidine and leucine uptake, and on incorporation of these precursors.

The growth of normal cells is stimulated by both lectins, whereas the growth of transformed cells is inhibited. In all cases the uptake of both leucine and thymidine by cells is increased by the lectins, but the percentage of incorporation of the precursors is affected in a different manner. The percentage of thymidine incorporated by normal and transformed cells increases or decreases in direct proportion to cell growth; leucine incorporation is not affected significantly. The reversibility of these lectin effects by specific inhibitors shows that cell membranes are implicated in these phenomena. Our study with normal and transformed cells suggests that cell surface may play a role in the process of malignant transformation and that P4Bis cells are "transitory" between PB1 normal cells and P4BisT high-cancer cells.

INTRODUCTION

Studies on normal and transformed cells have suggested a relationship between the properties of cell surface and the regulation of cell growth (6-8, 11, 13).

Comparative studies on transformed cells and their normal counterparts are not easily carried out because cells may acquire different membrane properties during the transition from a strain to a line. Recent work (10) has proved that "transformation" and "malignancy" are not necessarily associated. Transformed cells but not tumorigenic cells are currently observed in vitro.

Lectins have proved to be excellent tools for the study of membrane functions (20) and have been shown to induce differential effects on normal and transformed cell growth (2, 4, 14, 19, 21).

This study describes the effects on cell growth induced in vitro by the lectins Con A and Robinia and the uptake of thymidine and leucine by normal cells and 2 spontaneously transformed cell lines which illustrate malignant cell progression.

MATERIALS AND METHODS

Culture Procedure

Cell Cultures. PB1 cells were obtained from the same pool of C57BL/6 mouse lung cells frozen in liquid nitrogen at their 4th passage in vitro. They were used to their 10th passage. P4Bis and P4BisT were permanent cell lines of the same origin (i.e., C57BL/6 mouse lung cells). The differences in their biological properties and tumor-producing capacities have been described elsewhere (10). MEM supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 units/ml) was used. Subcultivations were performed with cells dispersed by 0.25% trypsin. Five- to 8-week-old female C57BL/6J mice were supplied by the Animal Selection Center, Orléans, France.

Lectins. Grade IV Con A (Sigma Chemical Co., St. Louis, Mo.) was used at a final concentration of 3 \(\mu\)g/ml. At the pH and temperature of the experiments, the Con A lectin conformation is tetrameric; each monomer exhibits one site for a specific ligand, i.e., \(\alpha\)-methylmannoside, and had a molecular weight of 27,000 (12). Robinia lectin was prepared from Robinia pseudoacacia seeds (5) and used at a final concentration of 3 \(\mu\)g/ml; it had a molecular weight of 72,000 and a dimeric molecular form.

Dose Response of the Lectins on Growing Cells

The effects of Con A and Robinia lectins on PB1, P4Bis, and P4BisT cells grown in vitro were tested in a range of concentrations from 0.1 to 10 \(\mu\)g/ml of culture medium. Cells were trypsinized and counted after 24 hr of contact.

Cytotoxicity Tests

Tumorigenicity. Tests for tumor-producing capacity were done by s.c. inoculations of monodispersed cells in syngeneic mice.

To obtain the TD\(_{50}\), we routinely tested 3 dilutions: 100 cells, 1,000 cells, and 10,000 cells in the case of P4BisT. Five to 6 mice were inoculated with each dilution, and the TD\(_{50}\) was calculated by the method of Reed and Muench (18).

Plating Efficiency. The percentage of individual cells giving rise to colonies was evaluated as follows. Cells were

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* Attachée de Recherches INSERM.

* Attachée de Recherches CNRS.

* The abbreviations used are: Con A, concanavalin A; MEM, Eagle's minimum essential medium; FCS, fetal calf serum, TD\(_{50}\) dose that produces tumors in 50% of animals; TCA, trichloroacetic acid.
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trypsinized, washed once, and resuspended in MEM plus 10% FCS. They were then sedimented in conical tubes for 1 hr. The upper part of the supernatant was then removed and checked microscopically to verify the state of monodispersion of the cells and their viability as tested by the absence of color when stained with trypan blue. With the P4Bis line, 100 cells were seeded per well in Linbro multi-disposable trays (Linbro Chemical Co., New Haven, Conn.) in 2 ml of MEM plus 10% FCS. Ten wells or more were used for each kind of treated or control cells. After 8 days of incubation at 37°, in 5% CO₂ atmosphere, the cell colonies were fixed with methanol, stained with Giemsa, and counted.

Effects of Robinia and Con A Lectins on Cell Growth, and Thymidine and Leucine Incorporation

Twenty-four hr after seeding, the culture medium of PB1, P4Bis, and P4BisT cells was discarded and replaced by 0.5 ml of a mixture of lectins in culture medium without FCS, at a final concentration of 3 µg/ml. After 1 hr of incubation at 37°, 0.5 ml of medium with 20% of FCS was added. This method allowed us to set apart the possible association of lectins with the calf serum glycoproteins.

Inhibition of Lectin Effects

Con A. A specific haptenic inhibitor of Con A, the α-methylmannoside (Sigma), was used. Experiments were performed with a molar ratio of lectin inhibitor of 1/1,000 (Con A expressed as monomer with a molecular weight of 27,000; α-methylmannoside).

Robinia. There is no haptenic monosaccharide inhibitor for Robinia lectin. For inhibition of its effects, a rabbit serum anti-Robinia lectin was used at a final dilution of 1/100. For comparative control studies a normal rabbit serum was used in the same standard conditions.

Labeling of Cell Cultures

At various times of culture (3 to 72 hr), [³H]thymidine with a specific activity of 27 Ci/mmol and [¹⁴C]leucine with a specific activity of 50 Ci/mmol (Radiochemical Centre, Amersham, England) were used simultaneously, at 1 µCi of [³H]thymidine and 0.85 µCi of [¹⁴C]leucine per ml without removal of the culture medium. After 1 hr of cell labeling, the reaction was stopped by ice cooling, the supernatant culture medium was discarded, and the cells were washed 6 times in cold 0.15 M NaCl. Then 0.5 ml of trypsin and 2 ml of 10% TCA were added and left overnight at 4°. The supernatant and the precipitate, which had been washed twice, were dissolved in 10 ml of PCS scintillation fluid (Packard Instrument Co., Downers Grove, Ill.) and counted in an Intertechnique counter.

Reversibility of the Effect of the Lectins on Cell Surface

The effect of Con A and Robinia lectins could be reversed by addition of specific inhibitor to the culture medium. Cells were incubated with lectins, as described previously for 30 min at 37°. Then, 0.5 ml of a mixture of specific inhibitor (α-methylmannoside to a final molar ratio of Con A to α-methylmannoside of 1/1000, or rabbit serum anti-Robinia lectin with final dilution of 1/100) supplemented with 20% FCS was added in culture medium.

Cells were counted at different times of culture after the addition of lectins (3 to 48 hr), and the results were expressed as percentage of inhibition or stimulation versus control.

RESULTS

Dose-Response Effect of Con A and Robinia Lectins on PB, P4Bis, and P4BisT Cell Growth. Increasing doses of lectins (Con A or Robinia), from 0.1 to 10 µg/ml of culture medium, were used. Cells were counted after 24 hr of contact. The results obtained are summarized in Chart 1. Con A and Robinia lectins stimulate the growth of normal PB1 cells, but they inhibit significantly the growth of transformed P4Bis and P4BisT cells. At these concentrations no toxic effect was observed with the trypan blue test in the case of PB and P4Bis cells. However, when the concentration exceeded 5 µg/ml, a toxic effect was observed for P4BisT cells; 20% of cells were stained with trypan blue.

The maximal effect of both of these lectins on the growth of these 3 cell types was obtained by the concentration of 3 µg/ml without measurable toxic effect.

Cytotoxicity test. Inasmuch as both lectins were shown to stimulate PB cells, we did not check their possible toxicity. To test their eventual toxicity for the P4Bis and P4BisT cells, we used 2 concentrations of Con A and Robinia, 3 and 30 µg/ml.

After 24 hr of treatment for the P4Bis cells and 48 hr of treatment for the P4BisT cells, the media of the above...
cultures were centrifuged and concentrated 10-fold before being microscopically checked for the presence of altered cells (colorable by trypan blue). In separate experiments very few cells could be detected in the 10 different kinds of supernatants. No dead cells were observed in significant proportion in any of them.

The cells were subsequently washed once with MEM with FCS, trypsinized, washed a second time with MEM/10% FCS, resuspended, and counted. Their viability was determined by trypan blue exclusion. No toxicity was found with Con A, 3 µg/ml (7% dead cells against 5% in the controls with the P4BisT cells; 1% against 0% with the P4Bis cells). Low toxicity was found with Con A, 30 µg/ml (31% dead cells with P4BisT), and no toxicity was found for P4Bis (3%). When Robinia was used at a concentration of 3 µg/ml, low toxicity was found with P4BisT (32% dead cells) and no significant toxicity was found with P4Bis (7%). When Robinia was used at a concentration of 30 µg/ml, a significant cytotoxicity was observed with P4BisT (59% dead cells) and P4Bis as well (62%).

The biological capacities of the P4Bis cells were determined by their plating efficiency (Table 1). Cells treated with the 2 concentrations of both lectins retained their potential to multiply and form colonies. Furthermore, a feeder effect was observed with the treated cells giving colonies larger in size and in number.

Because the P4BisT cells do not easily form colonies in vitro, their tumorigenicity in vivo was examined. The TD₅₀, which we obtained was the same for the treated cells as for the controls, except in the case of the cells treated with Robinia at a concentration of 30 µg/ml, where a considerable decrease of tumorigenic properties was observed. At a concentration of 3 µg/ml, neither Con A nor Robinia induced any toxic effect on both transformed cells (Table 2).

**Effect of Lectin on Cell Growth.** Cell growth of the PB1 control cells was characterized by a weak capacity for cell proliferation. On the contrary, the time course observed for P4BisT cell growth indicated a greater capacity for cell proliferation after a short period of latency, so that cell saturation density in the experimental conditions was reached more rapidly. The P4Bis cell growth was intermediate between the normal and the high-cancer cell growth (Chart 2).

After a lapse of 24 hr, Con A and Robinia lectins had different effects on the growth of normal and transformed cells; PB1 cells were stimulated after exposure to the lectins, whereas the proliferation of the transformed cells was inhibited. Therefore, it appears that Con A and Robinia lectins have similar effects on cell growth. Only PB1 cells were stimulated by these lectins, but the growth of the transformed cells, P4Bis and P4BisT, was inhibited significantly.

To prove the specific action of Con A and Robinia lectins on such growing cells, we performed the same experiments with lectins preincubated for 30 min at 37° with their specific inhibitor, as described previously. The growth curves observed are reported in Chart 2. In these conditions the lectin stimulation of PB1 cells was decreased significantly toward control growth. For P4Bis and P4BisT cells, the inhibition of cell growth by the lectins decreased, and their growth reached the control level (Chart 2).

The inhibition of Robinia by anti-Robinioa serum was specific, as shown by a control experiment with Robinia and normal rabbit serum.

**Study of Thymidine and Leucine Uptake by Cells.** The total radioactivity of the soluble (S) and precipitable (P) TCA material, expressed as dpm/cell, represents the total uptake of [³H]thymidine (Chart 3) and [¹⁴C]leucine (Chart 4).

Both thymidine and leucine uptake by PB1, P4Bis, and

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>No. of cells</th>
<th>Feeder effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated (control)</td>
<td>10².5</td>
<td></td>
</tr>
<tr>
<td>Con A-treated 3 µg/ml</td>
<td>10².7</td>
<td>+</td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>10².8</td>
<td></td>
</tr>
<tr>
<td>Robinia-treated 3 µg/ml</td>
<td>10².5</td>
<td></td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>10².6</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1**

<table>
<thead>
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<th>Feeder effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated (control)</td>
<td>21.4 ± 2.4±</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td>Con A-treated 3 µg/ml</td>
<td>29.1 ± 4.0</td>
<td>1-2</td>
<td>+</td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>29.9 ± 2.8</td>
<td>2-4</td>
<td>++</td>
</tr>
<tr>
<td>Robinia-treated 3 µg/ml</td>
<td>19.9 ± 2.4</td>
<td>1</td>
<td>=</td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>Monolayer</td>
<td>Monolayer</td>
<td>+++</td>
</tr>
</tbody>
</table>

Mean ± S.D.

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**Table 2**

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>No. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated (control)</td>
<td>10².5</td>
</tr>
<tr>
<td>Con A-treated 3 µg/ml</td>
<td>10².7</td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>10².8</td>
</tr>
<tr>
<td>Robinia-treated 3 µg/ml</td>
<td>10².5</td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>10².6</td>
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</tbody>
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**Chart 2.** Time course of normal and transformed cell growth control and lectin-treated cells. The initial cell concentration is 5 x 10⁶ cells/ml. The lectins (3 µg/ml) were added 24 hr after cell seeding (arrow). ——, growth of control cells; ——, growth of Con A-treated cells; ——, growth of Robinia-treated cells. The period of the experiment is conditioned by the cell saturation density. A — — A, growth of Con A-methylmannoside-treated cells. ——, growth of Robinia anti-Robinioa-treated cells. Each point is the average of 3 determinations.
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Chart 3. Time course of total thymidine uptake, TCA-precipitable and -soluble material $(I + S)$ expressed as dpm/cell. –, nontreated cells; A, Con A-treated cells; ■, Robinia-treated cells. Each point is the average of 3 determinations.

Chart 4. Time course of total leucine uptake TCA-precipitable and -soluble material $(I + S)$ expressed as dpm/cell. –, nontreated cells; A, Con A-treated cells; ■, Robinia-treated cells. Each point is the average of 3 determinations.

Chart 5. Top, time course of total thymidine incorporation per cell expressed as percentage of $[^{3}H]$thymidine incorporated into precipitable material $(I)$ to $[^{3}H]$thymidine uptake

Bottom, time course of total leucine incorporation per cell expressed as percentage of $[^{14}C]$leucine incorporated into precipitable material $(I)$ to $[^{14}C]$leucine uptake

P4BisT cells was stimulated by Con A and Robinia. This stimulation was maximal after 24 hr for PB1 cells and 30 hr for P4Bis cells. At this point there is cell-to-cell contact inhibition, so that cell activities slow down. For P4BisT cells there is no cell-to-cell contact inhibition and the uptakes of thymidine and leucine are twice the uptake of the control after 48 hr. The possibility of some toxic effect of Con A or Robinia lectins on such rapidly dividing cells was minimal, as mentioned previously.

Partial Reversibility of Lectin Membrane Cell Fixation by Specific Inhibition. Cells were incubated with Con A or

Incorporation of DNA- and Protein-labeled Precursors into TCA-precipitable Material. The total $[^{3}H]$thymidine or $[^{14}C]$leucine incorporated into the TCA-precipitable material $(I)$ may be expressed as a percentage of the total $[^{3}H]$thymidine (or leucine) taken up by the cell [insoluble $(I)$ and soluble $(S)$ radioactivity]. This ratio

$$
\% \frac{I}{I + S}
$$

may be used as an indication of the amount of thymidine incorporated into DNA for $^{3}H$ and an evaluation of leucine incorporated into cell proteins for $^{14}C$ (Chart 5).

It has been observed that the ratio of thymidine incorporation into TCA-precipitable material is increased by the 2 lectins in the case of normal PB1 cells, whereas this incorporation has been significantly decreased by the lectins in the case of the transformed cells, P4Bis and P4BisT. These effects confirm those obtained on cell growth. The ratio of $[^{14}C]$leucine incorporated into TCA-precipitable material is not significantly affected by the lectin treatment of the cells; only a weak stimulation was observed for the PB1 cells, confirming their weak total leucine uptake. It is shown that lectins facilitate leucine uptake of the transformed cells (Chart 4). Therefore, the parameter $I + S$ per cell, is increased. As the ratio $I/(I + S)$ is not changed for these transformed cells, the $[^{14}C]$leucine incorporation into TCA-precipitable material, i.e., proteins, is increased.
Robinia lectins for 30 min at 37°, as described. Specific inhibitors were subsequently added to the culture medium. After 24, 30, and 48 hr of culture, the cells were trypsinized and counted.

Under these conditions the observed reversibility of the effects of the lectins was maximal after 30 hr of culture. It was in the order of 100% for PB1 cells, 30 to 40% for P4Bis cells, and 60% for P4BisT cells (Chart 6). This experiment shows that lectins must be bound to the cell membrane and that specific inhibitors are able to release them, at least partially.

**DISCUSSION**

Morphological and biochemical differences between normal and transformed cells have been reported previously (4, 6–8, 11, 13). Three types of cells of the same origin (C57BL/6 mouse lung cells) but of different degrees of malignancy, the PB1 diploid normal cells and 2 spontaneously transformed permanent lines, P4Bis and P4BisT, provide a very suitable model for such work. Their biological properties and tumor-producing capacity have been described elsewhere (10). Our results on the growth of PB1, P4Bis, and P4BisT cells indicate that the doubling time decreases with the increase in the tumorigenic potential of the cells; it is greater than 48 hr for normal PB1 cells but about 30 and 12 hr for the low-cancer PB4Bis and high-cancer P4BisT cells, respectively.

Lectins are useful tools for the study of the biochemical properties of cell surface, because they induce various effects on cell agglutinability (17, 19) and on cell growth of normal and transformed cells (1, 2, 7, 9, 16). These effects involve a lectin-cell surface binding. In order to investigate the eventual differences in membrane properties of normal and derived transformed cells, the effects of Con A and Robinia lectins were studied on cell growth and uptake of DNA and protein precursors of such cells.

P4Bis and P4BisT cells were inhibited by Con A and Robinia. Toxicity tests on these cells showed that neither Con A nor Robinia was toxic at the given concentration. Moreover, they induced a feeder effect on the proliferation of the treated transformed cells. Con A and Robinia lectins affect normal and transformed cells in a different manner. The capacity of PB1 cells to proliferate is increased by the presence of these lectins, whereas the important proliferative capacities of the low- and high-cancer cells are decreased significantly.

The effect of lectins on [3H]thymidine, TCA-insoluble incorporation per cell, was parallel to that observed on cell growth. An increase in the thymidine incorporation of normal cells (diploid-cell, 40 chromosomes) was observed, whereas in the transformed cells, the lectins induced a decrease of thymidine incorporation. This effect was lower for P4BisT cells than for P4Bis cells, and the fact that the high-cancer cells possess 62 chromosomes and the low-cancer cells possess 90 chromosomes (10) could be associated with this phenomenon. This loss of DNA could explain the observed differences in DNA thymidine incorporation in these low- and high-cancer cell lines.

The percentage of [14C]leucine incorporated into TCA material was not significantly changed by Con A and Robinia lectins and appeared to be similar for the normal and transformed cells.

Thymidine and leucine uptake was increased by the lectins for the 3 types of cells. It appeared to be dependent on the contact of the cells with other cells and was maximal during the proliferative phase. We also observed a decrease in cell uptake by PB1 and P4Bis cells when they reached cell density saturation, whereas the lectins increased P4BisT cell uptake on a large scale. The observed effect was not of a toxic nature.

It is of special interest that the described effects of both lectins on normal and transformed cells could be partially reversed by specific inhibitors. Moreover, the complex inhibitor-lectin, i.e., Con A-a-methylmannoside or Robinia anti-Robinia immunglobulin, cannot induce these effects. The observed reversibility is not total for different possible reasons. On the one hand, some molecules of lectin could have penetrated into the cell (15) during the 30 min of incubation and are inaccessible to their specific inhibitors; on the other hand, it has been reported that the Con A affinity for a cellular system is 10² higher than its affinity for a specific ligand, i.e., a-methylmannoside (19). This experiment on the lectin reversibility effect suggested that the binding of lectins to the cell surface is a prerequisite for their action (inhibition or activation) on growing cells.

Our study on normal murine cells and spontaneously transformed cell lines in the presence of lectins suggests that the cell surface may play a role in the regulation of growth of such cells. They may also offer an additional approach for the exploration of the possible role of the cell surface during the process of malignant transformation.
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fact, P4Bis cells appeared to be “transitory” between the PB1 and P4BisT cells as indicated by their tumorigenic and social behavior on one hand (10) and their in vitro proliferation and thymidine or leucine uptake on the other.

The molecular basis for these differential effects of lectins on normal and transformed cells is currently under investigation.

ACKNOWLEDGMENTS

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