Flow cytometry (FCM) was used to investigate antigenic expression and modulation during the cell cycle of Y-79 and WERI-Rbl tissue cultured retinoblastoma cell lines using a polyclonal anti-Y-79 antibody and fluorescein conjugated lectins. Several Y-79 resting cell populations were identified by FCM analysis of antibody binding, while only a single population with uniform antigen expression was found to exist in the synthetic and mitotic phases. WERI-Rbl cells bound antibody approximately equally in each phase of the cell cycle. Multiple cell populations with different lectin binding affinities were seen in the resting phase with FITC-concanavalin A, FITC-ricinus communis 60 and FITC-ricinus communis 120 (FITC-RCA-120). During the S-phase of the cell cycle, a higher percentage of cells bound FITC-RCA-120 and FITC wheat germ agglutinin. The relationship between antigenic expression during the cell cycle and treatment considerations in retinoblastoma is discussed. Invest Ophthalmol Vis Sci 26:1306-1309, 1985

Retinoblastoma serves as a model cancer and as an ideal tumor in which to study antigenic expression and modulation. The expression of such antigens is amenable to study during the cell cycle using flow cytometry (FCM). In this report, we used FCM to investigate antigenic expression of cell surface markers in five retinoblastomas obtained fresh at enucleation and in two tissue cultured retinoblastoma cell lines, Y-79 and WERI-Rbl, during the cell cycle using a polyclonal anti-Y-79 antibody and fluorescein isothiocyanate (FITC)-labeled lectins.

Materials and Methods. Tissues: The Y-79 and WERI-Rbl retinoblastoma cell lines have been well-described previously. Five retinoblastoma specimens were obtained immediately after enucleation. Cell clumps were broken into a single cell suspension by repeatedly pipetting the sample.

Antibody preparation: The preparation and characterization of the polyclonal anti-Y-79 antibody has been described previously. The antibody had a titer of 1:512 for Y-79 cells and 1:64 for WERI-Rbl cells by complement (C)-dependent cytotoxicity assay. The antibody did not react with peripheral blood mononuclear leukocytes at dilutions as low as 1:2.

Antibody binding to cell surface: A saturating amount of anti-Y-79 serum (twice the concentration needed for 100% cytotoxicity with C) was added to 1 million viable Y-79, WERI-Rbl or retinoblastoma cells obtained fresh at enucleation in 200 µl HBSS. After 30 min at 0°C, the cells were washed twice and resuspended in 200 µl of 1:40 diluted FITC-conjugated F(ab')2 fragment IgG goat antirabbit IgG (heavy and light chain specific) (Cappel Laboratories; Malvern, PA). The cells were washed after an additional 30 min, resuspended in PBS-EDTA and fixed with 30% ethanol. Normal rabbit serum (NRS) served as a control.

DNA and protein labeling: Retinoblastoma cells suspended in PBS-EDTA and fixed in 30% ethanol (400 µl total volume with 1 million cells) were incubated in subdued light with 20 µl of 10 µg/ml FITC for 5 min at room temperature to label cellular proteins. DNA and RNA were labeled with 100 µl of 46 µg/ml propidium iodide (PI) for 3 min. RNA was removed by the addition of 20 µl of 1 mg/ml of RNase to make the assay specific for DNA content. Fixed cells stained with FITC-conjugated antibody or FITC-lectins were treated with the PI and RNase to stain the DNA. This allowed the multiple parametric analysis of FITC-conjugated antibody or FITC-lectin binding with DNA cycle of the cell.

Lectin binding studies: FITC-Lectins (Sigma Chemical; St. Louis, MO) at 50 µg/ml were incubated with viable Y-79 or WERI-Rbl cells (5 million/ml) at 0°C for 30 min. After incubation, the cells were washed twice with chilled Hanks' balanced salt solution (HBSS), followed by phosphate-buffered saline (PBS) with 13 mM disodium EDTA (PBS-EDTA). The cells were fixed by adding sufficient 46 % ethanol to bring the final concentration to 30%. The FITC-lectins used were concanavalin A (FITC-Con A), Ricinus communis A 60 (FITC-RCA-60), Ricinus communis A (FITC-RCA-120), and wheat germ agglutinin (FITC-WGA).

Flow cytometry: FCM was performed on an EPICS-V (Coulter Electronics; Hialeah, FL) cell sorter. Fluorescence excitation was obtained using the 488 nm line of a 5W Innova 90 UV Argon laser (Coherent; Palo Alto, CA) at 500 to 750 mW. Information on forward angle light scatter (FALS) at 488 µm, integrated green fluorescence (IGFL), log-integrated green fluorescence (LIGFL) from 510 to 565 nm and integrated red fluorescence (IRFL or DNA) at >610 nm was collected on each cell passing through the laser beam. Cells included in the study (ie, "gated-in") were nucleated cells, as determined by DNA,
but excluded debris and large aggregates as determined by FALS. The coefficient of variation between replicates for LIGFL was usually <10%. Multiparameter and cell cycle analysis was performed by the EPICS-V computer and MDADS 86 version 2.0 software.

**Results.** Anti-Y-79 sera bound to both the Y-79 and WERI-Rb1 tissue cultured cell lines and to fresh retinoblastomas obtained at enucleation (Fig. 1). The results from five patients indicated positive fluorescence in 10, 18.6, 40.6, 45.1, and 66% of the retinoblastoma cells examined.

The percentages of Y-79 or WERI-Rb1 cells binding the anti-Y-79 antibody during each phase of the cell cycle is shown in Table 1. Y-79 cells bound the antibody predominantly in the resting G0/G1 phase (49.7%), with reduced binding in the synthetic S-phase (14.5%) and enhanced binding in the mitotic G2/M phase (35.8%). Several populations of Y-79 cells with different degrees of anti-Y-79 binding were seen in the G0/G1 resting cell population (Fig. 2), whereas when the cells entered the S-phase only one population of cells binding antibody was seen, and in the mitotic G2/M phase the other antibody binding populations of cells began to reappear. In contrast to the Y-79 cells, the WERI-Rb1 cells showed a more homogeneous distribution of antibody binding throughout the cell cycle, including the G0/G1 phase (39.1%), the S-phase (28%), and the G2-M phase (32.9%).

The percentage of Y-79 cells binding FITC-lectins during the cell cycle is shown in Table 1. All FITC-lectins bound predominantly to resting phase Y-79 cells, reduced binding in the S-phase and increased binding in the G2/M phase (Table 1). In the S-phase, Y-79 cells bound more FITC-RCA-120 (20.1%) and FITC-WGA (13.0%) than either FITC-Con A (9.4%) or FITC-RCA-60 (9.4%).

**Discussion.** The polyclonal anti-Y-79 antibody used in this study has previously been shown to bind to the Y-79 and WERI-Rb1 tissue cultured cell lines by complement-dependent cytotoxicity assay. The antibody was also useful immunohistochemically in distinguishing between a metastatic retinoblastoma and a radiation-induced sarcoma in fixed tissue sections. In this study, the antibody bound to retinoblastomas obtained fresh at enucleation as well (Fig. 1) and was useful in the study of antigenic modulation during the Y-79 and WERI-Rb1 cell cycle (Table 1).

By FCM cells can be broadly categorized as resting cells (G0/G1), ie, without measurable DNA production; as cells synthesizing DNA, ie, S-phase; and as

**Table 1. Flow cytometric analysis of retinoblastoma**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-79 + anti-Y-79 sera</td>
<td>49.7</td>
<td>14.5</td>
<td>35.8</td>
</tr>
<tr>
<td>WERI-Rb1 + anti-Y-79 sera</td>
<td>39.1</td>
<td>28.0</td>
<td>32.9</td>
</tr>
<tr>
<td>Y-79 + FITC-Con A</td>
<td>46.3</td>
<td>9.4</td>
<td>44.3</td>
</tr>
<tr>
<td>Y-79 + FITC-RCA-60</td>
<td>52.2</td>
<td>9.4</td>
<td>38.4</td>
</tr>
<tr>
<td>Y-79 + FITC-RCA-120</td>
<td>43.0</td>
<td>20.1</td>
<td>36.9</td>
</tr>
<tr>
<td>Y-79 + FITC-WGA</td>
<td>49.8</td>
<td>13.0</td>
<td>37.3</td>
</tr>
</tbody>
</table>
cells undergoing mitosis, ie, G2/M phase. The expression of cell surface antigens can vary during each of these phases of the cell cycle. Previously we showed that monoclonal antibody MAbA9-C6, which defines an epitope of retinal S-antigen, binds to the WERI-Rb1 cell line and that S-antigen was predominately expressed in the G0/G1 phase of the cell cycle. In this study using the polyclonal anti-Y-79 sera, the antigens present on the Y-79 cells recognized by the antibody were lost during the S-phase and were re-expressed during the mitotic phase (Table 1), whereas WERI-Rb1 showed a more homogeneous distribution of antigen expression throughout all phases of the cell cycle (Table 1).

Lectins specifically bind to carbohydrate groups at the cell surface. In this study, differences in cell cycle expression were found between FITC-Con A (mannose- and glucose-specific), FITC-RCA-60 (N-acetyl-galactosamine-specific), FITC-RCA-120 (galactose-specific), and FITC-WGA (N-acetyl-glucosamine-specific). The binding sites recognized by FITC-RCA-120 and FITC-WGA showed less modulation during the cell cycle in contrast to the other lectins studied (Table 1).

The demonstration of antigenic modulation during the Y-79 and WERI-Rb1 cell cycle, as reported in this study, may have future implications for the treatment of retinoblastoma. For example, a therapeutically efficacious monoclonal antibody for retinoblastoma may be difficult to prepare because of differences in antigenic expression during different phases of the cell cycle. For example, a cytotoxic antibody directed at antigens expressed during the resting G0/G1 phase would not be effective at eradicating tumor cells during S and G2/M phases of the cell cycle if the cells did not express the antigen during these phases. To overcome these difficulties, a panel of monoclonal antibodies, each with specificities for antigens expressed at different times during the cell cycle, could be used. Investigators should be aware of the modulation of cell surface antigens in future studies concerning the treatment of retinoblastoma.

Key words: antigen modulation, cell cycle analysis, flow-cytometry, lectin binding, retinoblastoma

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Effects of Cholinergic Drugs and 4-Aminopyridine on Cat Ciliary Muscle Contractility

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The effects of some cholinergic agents and 4-aminopyridine (4-AP) on neurally mediated contractions of in vitro cat ciliary muscle preparations were studied. The contractile response to trains of stimuli was enhanced by eserine and completely blocked by tetrodotoxin or atropine. Low concentrations of carbachol did not modify muscle resting tension but clearly attenuated contractile response to electrical stimuli, while higher concentrations increased the resting tension leading to contracture which did not respond to further stimulation. 4-AP is known to be a potassium-channel blocking drug that increases neurotransmitter release at nerve terminals during the action potential. This substance exhibited a dose-related potentiation of the evoked ciliary muscle contractions without changing resting tension. The eventual reducing effect of 4-AP on the accommodative convergence/accommodation ratio (AC/A) is discussed in relation to its potential clinical application in certain strabismus patients. Invest Ophthalmol Vis Sci 26:1309–1313, 1985

The fundamental role played by the ciliary muscle in the visual accommodative process is well known. Most experimental studies concerning mechanical activity of isolated preparations of this muscle have measured the changes induced by different substances on its basal tension in the absence of electrical stimulation. We have considered that a useful tool to study the physiology and pharmacology of accommodation would be in vitro neuromuscular preparations of ciliary muscle in which contraction is elicited by electrical stimulation. The function of this smooth muscle is mainly due to the activity of cholinergic mediators, and the contribution of adrenergic transmitters has not been fully established although it has been demonstrated that catecholamines inhibit contractile response.1

Aminopyridine compounds, particularly 4-aminopyridine (4-AP) have received special attention in recent years because of their facilitatory actions upon neurotransmitter release. This substance has been clinically employed as an anticholinesterase agent and in some diseases where neuromuscular transmission is impaired. For instance, it has proven to be effective in the treatment of myasthenia gravis, botulism and other pathologic conditions of the motor endplate. These effects of aminopyridines have been ascribed to blockade of potassium channels at the nerve membrane.2

The purpose of the present experiments was to test the actions of some cholinergic drugs and 4-AP on the electrically induced contractions of the cat ciliary muscle. Our results showed that carbachol diminished the evoked contraction of this muscle while raising its resting tension, and that 4-AP enhanced the evoked response of the preparation without modifying the resting tension.

Materials and Methods. Healthy adult cats were anesthetized with 35 mg/kg pentobarbital sodium ip and then killed by an air injection into the femoral vein. Procedures were carried out conforming to the ARVO Resolution on the Use of Animals in Research. Both eyes were immediately enucleated and placed in oxygenated Krebs solution. Meridional strips 5 mm in width were cut from the optic nerve to the center of the cornea and then the iris and retina were