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Expression of H-2 and Moloney Leukemia Virus-Determined Cell-Surface Antigens in Synchronized Cultures of a Mouse Cell Line

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ABSTRACT **A mouse cell line derived from bone marrow (JLS-V9) was infected *in vitro* with Moloney leukemia virus. After the cell-surface antigens specified by this virus appeared, cells were synchronized in mitosis by a short treatment with colcemid. The expression of H-2- and virus-determined surface antigens was monitored during one cell cycle by an indirect membrane-immunofluorescence test. The highest proportion of antigen-positive cells was found during the G1 period; the proportion dropped as the cells entered the S period, and remained low until they entered the G1 period of the next cycle. The H-2- and virus-determined surface antigens were temporally coexpressed.**

An established line of a heterozygous ($H\text{-}2^a/H\text{-}2^f$) mouse lymphoma induced by Moloney Leukemia Virus showed considerable variation in complement-dependent, antibody-mediated cytotoxic sensitivity to anti-*H-2* and anti-Moloney sera. These variations were also observed when cells were tested with the same sera in an indirect membrane-immunofluorescence test (1, 2). Experiments designed to elucidate the mechanism of this phenomenon revealed an inverse correlation between growth rate and cell volume on the one hand, and the expression of surface antigens on the other. The same relationship was subsequently observed in a bone-marrow derived mouse cell line growing in monolayer (3). Quantitative absorption studies showed that the decrease in surface antigen concentration was not merely due to a dilution of antigenic receptors on the surfaces of large cells, but to a decrease in the number of antigenic receptors per cell (1). This conclusion was substantiated in further experiments (2, 3).

The demonstration of an inverse correlation between the cell volume and surface-antigen expression led us to conclude that the expression is maximal during early interphase. Analysis of cultured mouse lymphoma cells at different times during a single growth cycle showed that the prolongation of population doubling time was mainly due to an increase in the duration of G1, while S, G2, and mitosis were altered to a minor extent (2). It seemed reasonable, therefore, to assume that surface antigens are maximally expressed during the G1 period.

In this paper, we present our findings on the expression of H-2 and Moloney-type surface antigens in a synchronized culture of JLS-V9 cells infected *in vitro* with Moloney Leukemia Virus (hereafter called JLS-V9–MLV).

Abbreviations: PBS, phosphate-buffered saline; JLS-V9–MLV cells, a cell line (derived from mouse bone marrow) infected *in vitro* with Moloney Leukemia Virus.

MATERIALS AND METHODS

Cell line

The cell line used in these experiments (JLS-V9) was obtained from Dr. Bruce Wright (Chas. Pfizer & Co., Inc., Maywood, N.J.). It originated from the bone marrow of a BALB/c ($H\text{-}2^d$) mouse strain (4). The line was maintained in our laboratory in modified minimum essential medium (Grand Island Biological Co., Grand Island, N.Y., catalogue no. F-13) supplemented with 10 or 20% (v/v) fetal calf serum and 30 units of Mycostatain (Squibb) per ml of medium. Since our interest was focused on the relationship between the expression of cell-surface antigens specified by the virus and by the *H-2* locus, respectively, cells were infected *in vitro* with virus in an experiment designed to test the susceptibility of the cell line to infection with this virus during the cell cycle (5). The surface antigens reactive with an antivirus serum, prepared in mice (see below), appeared on the infected cells about 2 weeks after infection.

Antisera

Anti-*H-2*.3,19 serum was produced in (C57B1 × A.CA)F_1 hybrid mice by 6 to 10 subcutaneous inoculations of spleen, liver, and kidney tissues from A.SW mice at weekly intervals. Anti-*H-2*.4,8,13,31 serum was produced by the inoculation of tissues of DBA/2 strain into (C57B1 × C3H)F_1 hybrids (6). Anti-viral serum was produced in (A.BY × DBA/2)F_1 hybrid mice by repeated subcutaneous inoculations of irradiated (6000 R) syngeneic Moloney lymphoma cells; this serum contains activity against Moloney Virus-determined cell-surface antigens and lacks anti-*H-2* antibodies. Sera from several mice were pooled and stored at −20°C. In order to avoid dilution errors, all sera were diluted in phosphate-buffered saline (PBS), pH 7.2, to appropriate concentrations and stored in 30-μl samples at −20°C. The serum concentrations used in these experiments were based on data obtained in asynchronous JLS-V9–MLV cultures (3).

Indirect membrane immunofluorescence

Indirect membrane immonofluorescence on living cells was performed as described by Möller (7). Cells were suspended by treatment of the monolayer with 0.25% trypsin for 10 min at 37°C. After two washings in cold PBS, 3×10^5 cells were incubated for 20 min at 37°C with 25 μl of antiserum at the appropriate dilution. Cells were then washed twice in cold PBS and incubated for 20 min at 37°C with 25 μl of fluorescein-conjugated goat anti-mouse gamma globulin (Hyland

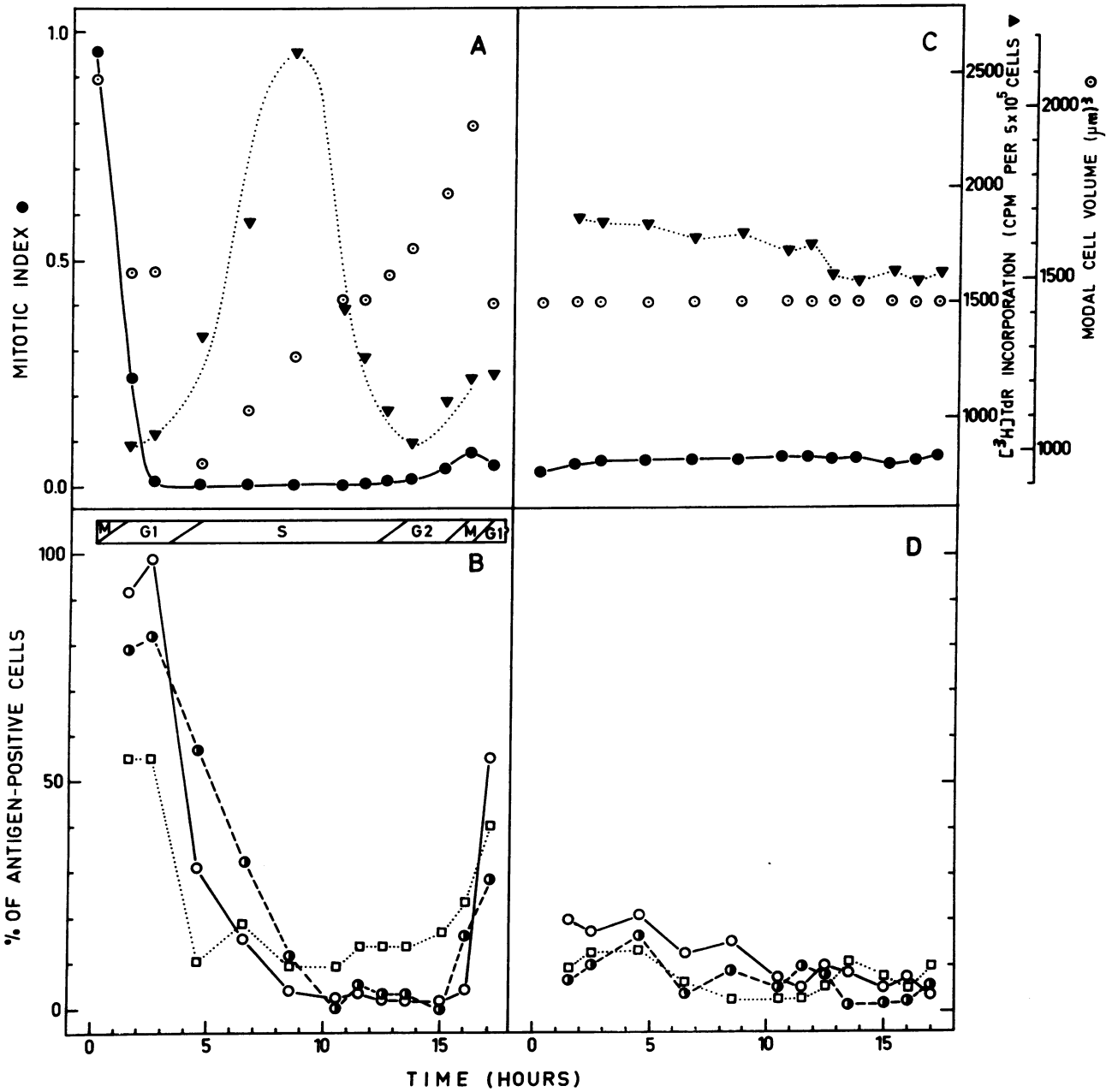

FIG. 1. Events during synchronous (*A* and *B*) and asynchronous (*C* and *D*) growth of JLS-V9–MLV cells. Cells were arrested in mitosis by treatment of a subconfluent monolayer with 0.04 μg/ml colcemid for 4 hr, resuspended in fresh, prewarmed medium supplemented with 10% fetal calf serum, and distributed in replicate 30-ml Falcon plastic flasks. Control asynchronous JLS-V9–MLV cells were trypsinized during the logarithmic phase of growth, resuspended in fresh medium, and distributed in 30-ml Falcon flasks. At the indicated times, monolayers of synchronized and asynchronous cultures were detached with 0.25% trypsin and the cell volume, the mitotic index, the incorporation of [³H]T (*A* and *C*), and the proportion of antigen-positive cells (*B* and *D*) were determined. Three sera were used: anti-*H-2*.3,19 in dilution 1:10 (○); anti-*H-2*.4,8,13,31 in dilution 1:20 (◑); and anti-Moloney serum in dilution 1:5 (□).

Laboratories, Los Angeles, Calif.) diluted 1:15 in PBS. Stained cells were examined under an ultraviolet dark-field microscope; those with sectorial and circular immunofluorescence patterns (2) were scored as positive. 200 cells were counted in each sample.

Cell count and cell volume

Medium from a monolayer culture was transferred into a chilled tube and 1 ml of 0.25% trypsin was added to each flask. Cultures were incubated for 10 min at 37°C, conditions sufficient for the detachment of cells. The original culture volume was restored by adding 4 ml of cold PBS; this was mixed with 5 ml of the culture medium that had been harvested from the same flask and kept in a chilled tube. The number of cells in this suspension was determined in Coulter Counter Model B. An electronic particle size distribution plotter (Coulter Electronics, Hialeah, Fla.), attached to a Coulter Counter, was used for the determination of cell volume.

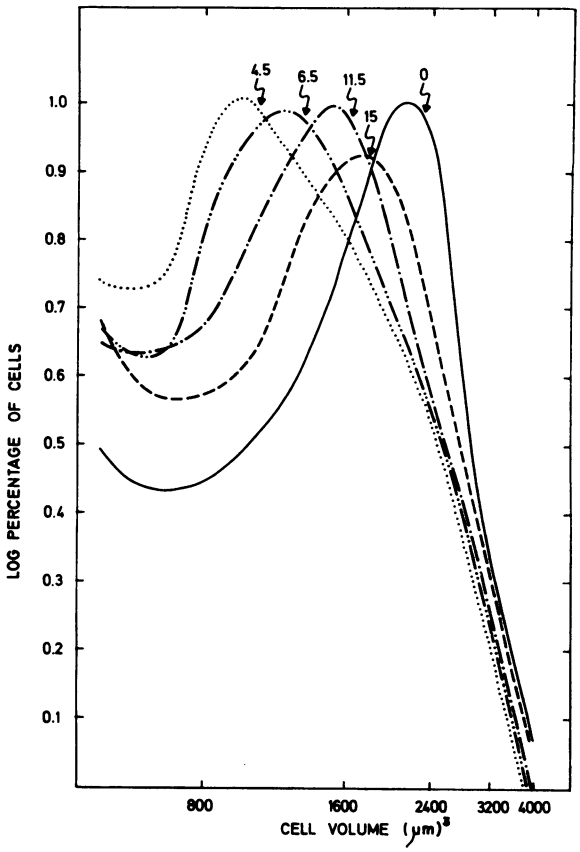

FIG. 2. Volume distribution spectra (log-log scale) of synchronized JLS-V9-MLV cells at indicated times after release from colcemid blockage.

Synchronization of cells

JLS-V9-MLV cells were synchronized in mitosis by treatment of a subconfluent monolayer with 0.04 μg/ml of colcemid for 4 hr and subsequent harvesting of mitotic cells by gentle shaking (8). Suspensions of mitotic cells were centrifuged for 8 min at 800 rpm (International Centrifuge, model PR-J) and resuspended in fresh, prewarmed medium supplemented with 10% fetal calf serum. Cell suspensions, containing 6.5×10^4 cells/ml, were then distributed in sterile, disposable (30 ml, 25-cm² Falcon) plastic tissue-culture flasks in 5-ml amounts per flask. Control cultures were derived from JLS-V9-MLV cells in the logarithmic phase of growth. Suspensions containing 1×10^5 cells/ml were seeded in identical Falcon flasks in 5-ml volumes.

[³H]Thymidine incorporation

At intervals after release from colcemid, a 30-min pulse of tritiated thymidine ([³H]T; Radiochemical Center, Amersham, Buckinghamshire; 0.20 μCi per ml medium; 23 Ci/mmol) was given. Thereafter, the culture medium was discarded, the monolayer was washed with 4 ml of PBS, and cells were detached with 0.25% trypsin (10 min at 37°C). 10 ml of cold PBS was added to each flask, and the cell suspension was transferred to chilled plastic tubes. Cells were pelleted in the International Centrifuge (6 min at 2000 rpm) at 0°C. The cell pellet was washed once in cold PBS and precipitated with 10% trichloracetic acid. The precipitate was washed twice with 4 ml of 10% trichloracetic acid, dissolved in Hyamine, and the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer.

Mitotic index

A part of the cell suspension used for the determination of cell number and volume was sedimented in the International Centrifuge for 4 min at 800 rpm. The cell cytoplasm was disrupted with 1% sodium citrate and cell nuclei were washed in 2–3 ml of Clarke's fixative (glacial acetic acid–methanol 1:3). Smears were prepared on clean microscope slides and stained with Giemsa solution. One thousand nuclei were counted on each slide, and the mitotic index was calculated. A loss of mitotic figures is inherent to this method (2); therefore the mitotic index is generally lower than the real fraction of cells in mitosis.

RESULTS

When mitotic cells were resuspended in fresh, warm medium supplemented with 10% serum, about 80% of the cells divided within 1.5 hr and the rest within the next hour (Fig. 1). Modal cell volume, however, dropped to a value approximately half of that of the mitotic cells about 2 hr later. This might be explained by incomplete separation of pairs of daughter cells, which were then registered by the particle size distribution plotter as large cells. Modal cell volume rose during interphase in an approximately linear fashion, and almost reached the value of the colcemid-blocked cells at a time which corresponded to the peak of the second mitotic wave. Fig. 2 shows the volume distribution spectra of JLS-V9-MLV cells at four representative time points during synchronous growth.

The incorporation of [³H]T started between 3 and 4 hr after release from colcemid blockage, and reached a maximum about 4 hr later.

The changes of the aforementioned parameters allowed an assessment of the onset and duration of cell-cycle periods: G1 period lasted about 3 hr; S, 9 hr; G2, 3 hr; and mitosis, 1–1.5 hr.

The highest proportion of cells showed positive immunofluorescence staining with all three sera during the time interval that coincided with the G1 period (Fig. 1*B*). As cells started to enter the S period, the percentage of immunofluorescence-positive cells dropped abruptly and remained at a low level until the cells divided again.

In control JLS-V9-MLV cultures derived from an asynchronous culture in the logarithmic phase of growth, the incorporation of [³H]T, expressed per 5×10^5 cells, and the percentage of immunofluorescence-positive cells were rather constant. Modal cell volume did not change during the observation period, and the mitotic index varied between 0.03 and 0.05 (Fig. 1 *C* and *D*). However, when cells from a confluent monolayer were used as a control, a certain degree of synchrony was observed after distribution in fresh medium. Incorporation of [³H]T started in these cultures 2–6 hr after resuspension, depending on the age of culture. In these cultures, also, a higher percentage of antigen-positive cells was observed during the presynthetic period.

DISCUSSION

The degree of expression of surface antigens on cultured mouse cells varies considerably during a single growth cycle (1–3). When antigenic expression was related to growth rate,

and cell-cycle kinetics during one growth cycle were analyzed, it was assumed that the surface antigens of two mouse cell lines (YCAB and JLS-V9), specified by the *H-2* locus and Moloney Leukemia Virus, were maximally expressed during the G1 period of the cell cycle (see *Introduction*).

In this study we were able to confirm this assumption by the use of synchronized JLS-V9 cells infected *in vitro* with leukemia virus. Monitoring the expression of *H-2* and virus-determined cell-surface antigens by indirect membrane immunofluorescence during synchronous growth, we found the highest percentage of antigen-positive cells during the G1 period.

Kuhns synchronized HeLa cells by a single treatment with thymidine; he found that the expression of "H" blood-group activity was maximal during the mitotic wave that followed release from thymidine blockage (9). His results are not necessarily in contradiction with ours, since antigen-positive cells found during a prolonged mitotic wave in Kuhns' experiment might have belonged to the G1 fraction.

A comparison of the behavior of cell-surface antigens and the production by cultured mammalian cells of some differentiated-cell products *in vitro* (2) favors the view that variations in the expression of surface antigens are the result of sequential repression and derepression of genes specifying the synthesis of these antigens. A striking resemblance to the expression of surface antigens was seen in the variations in activity of some enzymes in cultured cells during a single growth cycle (11, 12). Further support for the hypothesis of restricted periods of expression of genes specifying the synthesis of surface antigens comes from studies on immunoglobulin production in established cell lines (12, 13).

It is possible, but less probable, that a substance is produced, during certain periods of the cell's life cycle, that coats antigenic receptors on the cell surface and prevents their detection by the immunological methods used. This possibility has been largely excluded by our experiments in which the fate of fluorescein-labeled antigens on the surface of living cells was followed under different culture conditions. When such cells were incubated at 37°C in ordinary culture medium, only 10% of the cells retained the label after 24 hr. However, 90% of the cells were still labeled if incubated for the same time period at 0°C, and 70% were labeled if incubated at 24°C. When the cells were incubated at 37°C at different concentrations, a higher percentage of the cells retained the fluorescent label if incubated at high cell concentrations (unpublished results). Since even pinocytosed fluorescent material is regularly visible by fluorescent microscopy, it seems unlikely that a "masking" substance is responsible for the observed variations in antigen expression.

A remarkable feature of the virus-determined cell-surface antigens on *in vitro* infected JLS-V9 cells (3) and on cultured mouse lymphoma cells (1, 2) is that they are temporally coexpressed with the antigens specified by the *H-2* locus. This suggests that a common regulatory mechanism is responsible for the expression of both types of antigens. Further studies are needed to ascertain the significance of this observation.

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