LYMPHOMA MODELS FOR B CELL ACTIVATION AND TOLERANCE

III. Cell Cycle Dependence for Negative Signalling of WEHI-231 B Lymphoma Cells by Anti-μ

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It has become increasingly apparent that B lymphomas provide clonal models for antigen-specific lymphocytes and for the analysis of both positive and negative signalling in B cells. In addition, anti-Ig reagents have been used to mimic antigen/tolerance in their interaction with B cell receptors at a polyclonal level (1-3). We have been studying the effects of anti-Ig on a group of unique B cell lymphomas as models for either tolerogenic or immunogenic signalling (4-6). One such line, WEHI-231, has been shown (7, 8) to be readily inhibited in its growth by anti-Ig reagents. We confirmed the sensitivity of this line to growth inhibition by anti-μ and anti-κ reagents, and determined its kinetics, specificity, and site of the block in the cell cycle at the G₁/S interface (4). In this report, we have enriched WEHI-231 lymphoma cells at various points in G₁ to S and analyzed the effects of anti-μ on progression through S phase. Our data suggest that critical events occur early in G₁ and are uniquely sensitive to modulation by anti-μ. These studies now will allow sensitive molecular approaches towards an understanding of B cell signalling, as well as methods to regulate lymphoma growth per se.

Materials and Methods

Cell Lines and Their Maintenance. WEHI-231 B lymphoma cells arose in a (BALB/c X NZB) F₁ mouse after mineral oil induction (9). Clone 28, obtained from Dr. Noel Warner (Becton Dickinson Research Laboratories, Mountain View, CA), has been maintained in our laboratory for several years in DMEM supplemented with 5-10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5 x 10⁻⁵ M 2-ME (4, 5). It is grown as a single cell suspension with occasional aggregates, and is split twice weekly. As a standard test for anti-μ sensitivity, 10⁶ WEHI 231 cells were cultured in a total volume of 0.2 ml with various concentrations of goat anti-μ or other antisera for 48 h, at which time they were pulsed with 1 μCi of tritiated thymidine. 18 h later they were harvested on glass fiber filters and assayed by liquid scintillation spectrometry.

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Anti-µ. Goat anti-µ, affinity purified as described earlier (4), was used in all experiments. Identical results have been obtained with several different monoclonal anti-µ or anti-σ reagents.

Methods of Cytofluorometric Cell Cycle Analysis. Cultures of 10⁶ WEHI-231.28 in 2 ml standard medium (5 × 10⁴ cells/ml) were initiated with or without the addition of 10 µg/ml of anti-µ. At different time points thereafter, cells were harvested and washed. To analyze all samples simultaneously, certain aliquots were fixed overnight in 50% ethanol. Ethanol fixation was shown to have minimal effects on the acridine orange (AO) staining procedure (J. Cogswell and P. Keng, unpublished data). For flow-cytometric analysis, the cells were pelleted and resuspended to 10⁶ cells/ml.

DNA and RNA content for cell cycle analysis was determined by AO staining using a modification of the method of Darzynkiewicz et al. (10, 11) as previously described (12). Briefly, 0.5 ml of the cell suspension was mixed with 0.45 ml of 0.1% Triton X-100 in 0.08 M HCl plus 0.15 M NaCl (pH 2.2) for 1 min on ice. After this time, the cells were stained by the addition of 1.8 ml of chromatographically purified AO (12 µg/ml; Polysciences, Inc., Warrington, PA) in 0.126 M Na₂HPO₄, 0.037 M citric acid (pH 6.0), 0.15 M NaCl, and 1 mM sodium EDTA. This treatment denatures double-stranded RNA but not DNA. Fluorescence was analyzed immediately.

The fluorescence of AO-stained cells was measured on an EPICS V flow cytometer as previously described (13). The laser excitation wavelength used was 488 nm with an intensity of 500 mW. The differential binding characteristics of AO to single- and double-stranded nucleic acids causes RNA to fluoresce red while DNA fluoresces green. These respective signals were measured by inserting a 510 nm interference barrier filter in front of the right angle collection lens with a 560-nm dichroic mirror used to split the fluorescence signal. Green fluorescence (530-560 nm) could then be observed with the addition of a 530-nm long-pass filter, and red fluorescence simultaneously monitored with a 630-nm long-pass filter. >10⁴ cells were analyzed for each histogram.

Centrifugal Elutriation. The details of the elutriation procedures for isolating synchronous cell populations from cultured cells have been reported previously (14, 15). The elution system, sterilized by autoclaving and then flushing with 70% methanol before use, was maintained at 20°C during the separation procedure; the DMEM plus 1% FCS used as elutriation fluid was also kept at 20°C. ~10⁶ exponentially growing WEHI-231 cells, suspended in 20 ml of medium, were loaded into the separation chamber at a rotor speed of 3,250 rpm and a flow rate of 30 ml/min. After loading the samples, the rotor speed was decreased in increments to 2,770 rpm, with two 40-ml fractions collected at each interval. The cell number and cell size distribution from each separated fraction were then measured with a Coulter counter and channelyzer system (model ZB1, C1000) to verify position in cell cycle. For WEHI-231 lymphoma cells, the following Coulter volumes were determined (and correlated with DNA/RNA content): G₁, 460–530 µm³; G₂ₕ, 600–650 µm³; S, 890–1,000 µm³; G₂/M, 1,150–1,250 µm³. The percentage of separated cells in G₁, S and G₂/M phase of the cell cycle was determined by flow cytometry after staining with AO or mithramycin (4, 14).

Results and Discussion

Cytofluorometric Analysis of Cell Cycle Changes Induced by Anti-µ. Based on its binding to double-stranded DNA and single-stranded RNA, AO can be used to delineate cell cycle–related changes in mammalian cells (11). We previously used AO staining to determine the site of the block by anti-µ of lymphoma growth. Those data (4) showed that a negative signal led to a block in the transition from G₁ to S, thereby leading to a reduction in thymidine incorporation. The exact point of the block is presumed to be at the G₁/S interface, since there is an increase in the frequency of cells in G₁. The current studies were designed to examine when these negative signals were received.

Abbreviations used in this paper: AO, acridine orange; PKC, protein kinase C.
Site of Negative Signalling in B Lymphoma Cells. To determine whether there was a critical stage at which time anti-μ binding led to subsequent growth inhibition, we treated WEHI-231 cells with 1 mM hydroxyurea to reduce the numbers of cells in S and to block the remaining cells at the G1/S interface. After washing, these cells then were allowed to progress through S and continue through the cycle in the presence or absence of anti-μ antibodies. As shown in Fig. 1 (top), the original hydroxyurea-treated cells showed a reduced content of cells in S and a pile-up of G1 cells, as expected. This figure also indicates that the transition through S occurs normally, if not more rapidly, in the presence of anti-μ (Fig. 1, right). Note that by 6 h (when most of the cells have progressed into S and many have entered G2/M), there are no differences between the control and experimental groups. However, by 24 h there is a clear diminution in the numbers of cells in S in the anti-μ-treated sample. These data indicate
that WEHI-231 cells are insensitive to negative signalling by anti-μ during S phase, but become sensitive at some later point, perhaps when they reenter G₁.

To further explore at which stage anti-μ delivers its effect, we purified G₁ cells by centrifugal elutriation to obtain a population that was >96% pure. These cells were incubated with anti-μ either immediately or after a 4- or 8-h delay. All cells were stained with AO the next morning. The data in Fig. 2 indicate that only those cells that were exposed to anti-μ immediately upon isolation of the G₁ fraction were inhibited. In fact, the anti-μ blockade was only partial (evidenced by the cells in G₂/M), perhaps due to the fact that some of the isolated cells were at a later stage in G₁ and have become insensitive to anti-μ negative signalling.
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FIGURE 3. Early G1 cells, isolated as described for Fig. 2, were cultured for 14 h in complete medium in the presence or absence of anti-μ (10 μg/ml). The cells were then labeled with AO and analyzed for progression into their first round of S. See abscissa for relative positions of cells in G1, S, and G2/M. The original cells (not shown) were >90% in G1. As shown in B, after 14 h, 85% of the G1 cells had entered S. In contrast, there was a significant reduction of lymphoma cells progressing into S if exposed to anti-μ immediately (C), but not after a 2-h delay (D). Moreover, exposure to anti-μ during the first 2 h of G1 led to maximal inhibition of cell cycle progress (A). These data (and those shown in Fig. 2) show that interaction with anti-μ during early G1 prevents the entry of these cells into S.

A 4-h delay before addition of anti-μ prevented the growth inhibition (Fig. 2, middle), suggesting the critical events occur quite early in G1, and may even affect the first entry into S.

To establish this point more definitively, we examined elutriated G1 WEHI-231 B lymphoma cells over a 12–16-h period after isolation, i.e., as they entered their first S phase, in the presence or absence of anti-μ. The data in Fig. 5 typify one of three similar experiments showing the progression of these purified G1 cells into S within this time period (Fig. 3B). ~85% of these lymphoma cells have entered or progressed through S. This cycle movement is significantly impeded in the presence of anti-μ (Fig. 3C). Moreover, removal of the anti-μ after only 2 h still leads to maximal inhibition (Fig. 3A). Indeed, the addition of anti-μ to G1 cells 2 h after isolation has virtually no effect on their first entry into S (Fig. 3D).

Some of these cells presumably would have progressed in G1 from an early, anti-μ-sensitive phase (G1a), into a later, anti-μ-insensitive phase (G1b).

To test this hypothesis, we isolated early (G1a)- and late (G1b)-stage G1 cells by centrifugal elutriation (13, 14), and tested their sensitivity to anti-μ (Fig. 4). Their position in the cell cycle was verified by Coulter volume analysis and by AO staining immediately after elutriation and 16 h later (see Fig. 5). At this later time, there was a much higher percentage of cells in G2/M in the original G1b fraction than in the original G1a fraction (see Fig. 4, B and A, respectively), indicating that the G1b cells were at a later point in the cell cycle than the G1a.
FIGURE 4. WEHI-231 lymphoma cells were separated into $G_{1a}$ (A) and $G_{1b}$ fractions (B) by elutriation (see Fig. 5), and allowed to progress for 16 h in the presence or absence of anti-$\mu$ (10 $\mu$g/ml). The original fractions were confirmed to be in their respective positions by Coulter volume and AO analysis of RNA content (see Fig. 5). As shown in A, $G_{1a}$ lymphoma cells progressed into S but were significantly inhibited by anti-$\mu$ (+ anti-$\mu$, solid line; note pile-up at $G_{1a}$/S as before). In contrast, $G_{1b}$ cells (B) were virtually unaffected, as can be seen by the overlap of the curves ± anti-$\mu$. The relative positions of $G_{1}$, S, and $G_{2}/M$ are shown on the abscissa.

cells at the time of isolation (this assumes equal rates of progression for the two fractions). The data in Fig. 4 show that $G_{1a}$ cells (A) are inhibited by anti-$\mu$, whereas the $G_{1b}$ cells were not (B). These data confirmed that, as cells progressed from $G_{1a}$ into $G_{1b}$, they lost their sensitivity to negative signalling by anti-$\mu$. Fig. 5 verifies the purity of the $G_{1a}$ and $G_{1b}$ cells in terms of RNA content and shows the cell cycle progress of these fractions in the absence or presence of anti-$\mu$. As above, only $G_{1a}$ cells were impeded by anti-$\mu$.

Therefore we propose that as these lymphoma cells enter $G_{1}$, certain critical events occur that can be modulated by crosslinking of their surface immunoglobulin receptors. The identity of these events is unknown at present, but certain signalling molecules may be implicated. For example, other studies (5, 6, 8) have established that WEHI-231 B lymphoma cells can be rendered resistant to the effects of anti-$\mu$ by the cocultivation in the presence of LPS. Indeed, the effects of LPS could be mimicked by exposing WEHI-231 cells to phorbol esters (6). Interestingly, others (16) have recently shown that LPS and phorbol esters both cause a rise in the levels of protein kinase C (PKC) activity in B cells, although phorbol esters may cause a much more prolonged increase. The mechanisms by which activation of PKC can modulate the growth of WEHI-231 and lead to a relative protection against the negative signalling by anti-$\mu$ are currently unknown. Since phorbol esters may modulate calcium flux (17), and since PKC is a Ca++-dependent enzyme, alternative pathways of calcium mobilization and PKC activation may be involved. However, we currently believe that Ca++ influx is independent of the negative signalling process, since the growth of CH31, another anti-$\mu$-sensitive B cell lymphoma, can be inhibited in the absence of
RNA profiles verifying the separation of WEHI-231 cells by centrifugal elutriation and showing cell cycle progress. The initial fractions were analyzed for Coulter volume and peaks and corresponding to $G_{ia}$ and $G_{ib}$, respectively (see Materials and Methods) labelled with AO for verification of RNA content (see C and D, below). The broad RNA content of unseparated WEHI-231 lymphoma cells is shown in the top panels (A and B), and is easily distinguished from that of purified $G_{ia}$ and $G_{ib}$ cells (C and D), which overlap. After 16 h, both $G_{ia}$ and $G_{ib}$ cells have progressed into S and G2/M (E and F; see Fig. 4 for DNA profiles). In the presence of anti-$\mu$, the progress of $G_{ia}$ cells is impeded (G), but the $G_{ib}$ cells complete the transition normally (H).

Ca++ influx (C. Pennell, J. Whitin, S. Dillon, R. Snyderman, and D. Scott, manuscript in preparation).

Interestingly, recent data (18) suggest that unseparated WEHI-231 lymphoma cells show an early rise and then a dramatic drop in $c$-myc message 4 h after anti-$\mu$ treatment. Thus, even earlier changes in a particular gene (putatively concerned with cell growth) can be perceived in nonsynchronized lymphoma cells. It will be worthwhile to examine WEHI-231 and its anti-$\mu$-resistant variants (4) for the initial responses to anti-$\mu$ in terms of calcium mobilization and PKC activation at various stages of the cell cycle. These studies should help pinpoint the exact mechanism of growth inhibition of these lymphomas, and to apply it, by analogy, to the specific regulation of normal B lymphocytes.

Summary

WEHI-231 B lymphoma cells have proven to be a useful model for the regulation of growth of normal B cells by anti-Ig reagents. We previously reported that the growth of these lymphoma cells is inhibited by heterologous or monoclonal anti-$\mu$ or anti-$\kappa$ reagents. Such cells cease to incorporate thymidine
within 24–48 h of exposure to anti-Ig reagents, but are not adversely affected by antibodies directed at either class I or class II histocompatibility antigens. In fact, cell cycle analysis revealed that anti-μ causes a block in the transition of these cells from G1 to S phase. To further study the mechanism of growth inhibition, we have purified lymphoma cells in G1 by centrifugal elutriation, or enriched WEHI-231 cells at the G1/S interface by treatment with hydroxyurea, and followed their progression through the cell cycle in the presence or absence of anti-μ. Our data show that WEHI-231 B lymphoma cells receive a negative signal early in G1, since delayed addition of anti-μ (to late G1 cells) leads to no alteration in cell cycle progression at 24 h, and exposure to anti-μ during S does not alter progress through DNA synthesis and mitosis. Moreover, exposure to anti-μ for only 2 h prevents purified G1 cells from entering their first S phase. The nature of the relevant processes in early G1 is discussed in terms of models of B cell activation and tolerance induction.

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