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/tolerogen 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 G_1/S interface (4). In this report, we have enriched WEHI-231 lymphoma cells at various points in G_1 to S and analyzed the effects of anti-μ on progression through S phase. Our data suggest that critical events occur early in G_1 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 × NZB) F_1 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 5×10^{-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, 10^4 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-κ reagents.

Methods of Cytofluorometric Cell Cycle Analysis. Cultures of 10^5 WEHI-231.28 in 2 ml standard medium (5×10^4 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)[1] staining procedure (J. Cogswell and P. Keng, unpublished data). For flow-cytometric analysis, the cells were pelleted and resuspended to 10^6 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, 0.3 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 Na_2HPO_4, 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 intereference 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. $\geq 10^4$ 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 elutriation 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. $\sim 10^8$ 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): G_{1a}, 460–530 μm^3; G_{1b}, 600–650 μm^3; S, 890–1,000 μm^3; G_2/M, 1,150–1,250 μm^3. The percentage of separated cells in G_1, S and G_2/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 G_1 to S, thereby leading to a reduction in thymidine incorporation. The exact point of the block is presumed to be at the G_1/S interface, since there is an increase in the frequency of cells in G_1. The current studies were designed to examine when these negative signals were received.

[1] *Abbreviations used in this paper:* AO, acridine orange; PKC, protein kinase C.

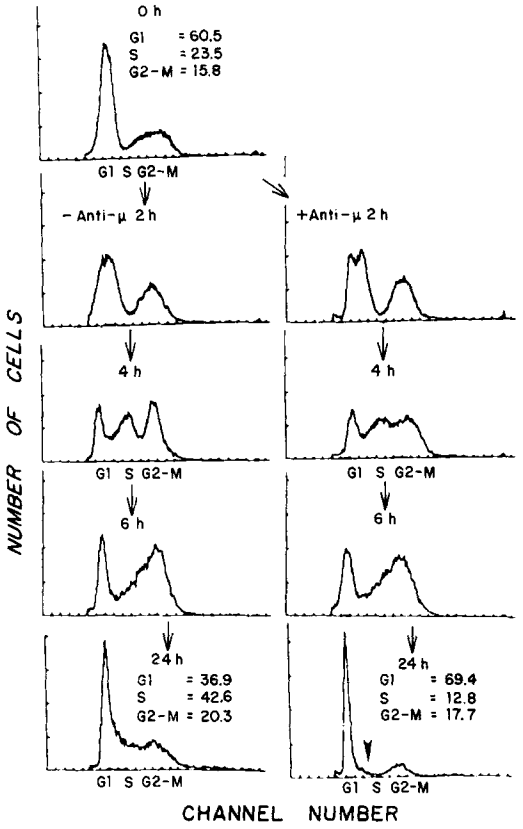

FIGURE 1. WEHI-231 lymphoma cells, treated with 1 mM hydroxyurea to kill cells in S and block remaining cells at the G_1/S interface, were cultured in the absence (*left*) or presence (*right*) of 10 μg/ml goat anti-μ for 2, 4, 6, or 24 h before AO analysis of cell cycle progression. This figure shows that WEHI-231 cells progress normally from G_1 (left peak) through S (central peak) in the presence of anti-μ for the first 6 h, but reach a sensitive stage to block this process during the next 18 h (*bottom right*). The relative positions of cells in G_1, S, and G_2/M are shown for reference on the abscissa, and the percentages in each stage at initiation and at 24 h are indicated.

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 G_1/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 G_1 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 G_2/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

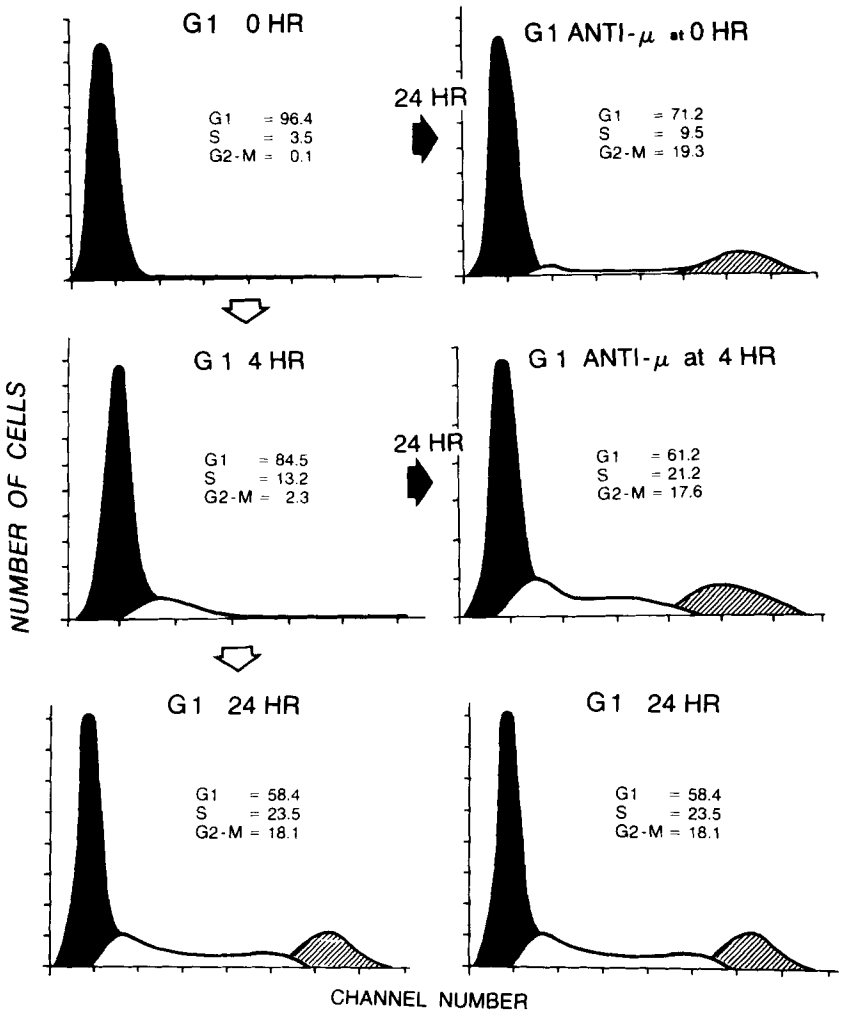

FIGURE 2. Lymphoma cells, purified by centrifugal elutriation, were cultured with 10 μg/ml anti-μ added immediately (*top*) or after a 4-h (*middle*) or 8-h delay (not shown). Analysis of cell cycle progression by AO staining was 24 h later (*right*). Only if anti-μ was added immediately upon isolation of G_1 cells was there any inhibition of cell cycle progression. The percentage of cells in G_1 (solid peak), S (white area), and G_2/M (shaded peak) are indicated in the panels.

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 G_1.

To further explore at which stage anti-μ delivers its effect, we purified G_1 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 G_1 fraction were inhibited. In fact, the anti-μ blockade was only partial (evidenced by the cells in G_2/M), perhaps due to the fact that some of the isolated cells were at a later stage in G_1 and have become insensitive to anti-μ negative signalling.

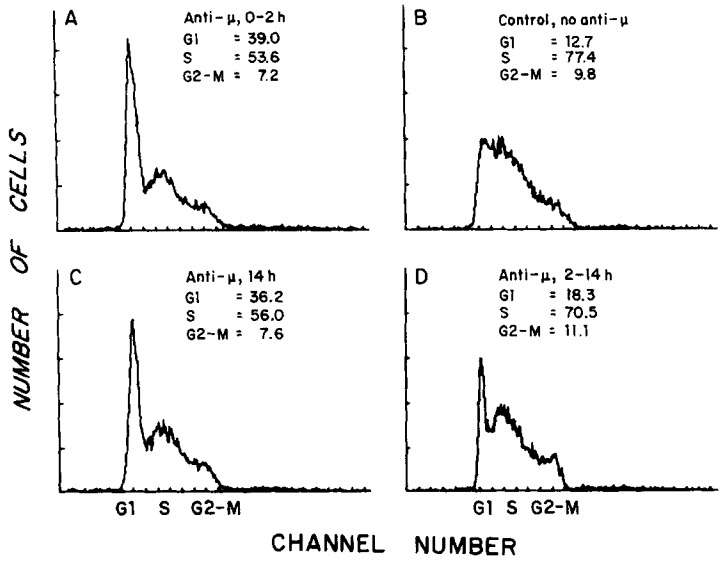

FIGURE 3. Early G_1 cells, isolated as described for Fig. 2, were cultured for 14 h in complete medium in the presence or absence of anti-μ (10 μg/ml). The cells were then labeled with AO and analyzed for progression into their first round of S. See abscissa for relative positions of cells in G_1, S, and G_2/M. The original cells (not shown) were >90% in G_1. As shown in *B*, after 14 h, 85% of the G_1 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 G_1 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 G_1 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 G_1, and may even affect the first entry into S.

To establish this point more definitively, we examined elutriated G_1 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. 3 typify one of three similar experiments showing the progression of these purified G_1 cells into S within this time period (Fig. 3*B*). ~85% of these lymphoma cells have entered or progressed through S. This cycle movement is significantly impeded in the presence of anti-μ (Fig. 3*C*). Moreover, removal of the anti-μ after only 2 h still leads to maximal inhibition (Fig. 3*A*). Indeed, the addition of anti-μ to G_1 cells 2 h after isolation has virtually no effect on their first entry into S (Fig. 3*D*). Some of these cells presumably would have progressed in G_1 from an early, anti-μ–sensitive phase (G_{1a}), into a later, anti-μ–insensitive phase (G_{1b}).

To test this hypothesis, we isolated early (G_{1a})- and late (G_{1b})-stage G_1 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 G_2/M in the original G_{1b} fraction than in the original G_{1a} fraction (see Fig. 4, *B* and *A*, respectively), indicating that the G_{1b} cells were at a later point in the cell cycle than the G_{1a}

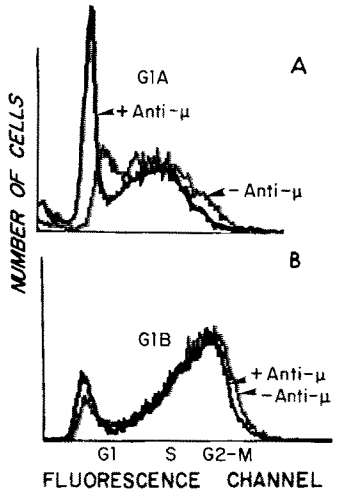

FIGURE 4. WEHI-231 lymphoma cells were separated into G_{1a} (*A*) and G_{1b} fractions (*B*) by elutriation (see Fig. 5), and allowed to progress for 16 h in the presence or absence of anti-μ (10 μg/ml). The original fractions were confirmed to be in their respective positions by Coulter volume and 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-μ (+ anti-μ, solid line; note pile-up at G_1/S as before). In contrast, G_{1b} cells (*B*) were virtually unaffected, as can be seen by the overlap of the curves ± anti-μ. 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-μ, 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-μ. 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-μ. As above, only G_{1a} cells were impeded by anti-μ.

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-μ 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-μ 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-μ–sensitive B cell lymphoma, can be inhibited in the absence of

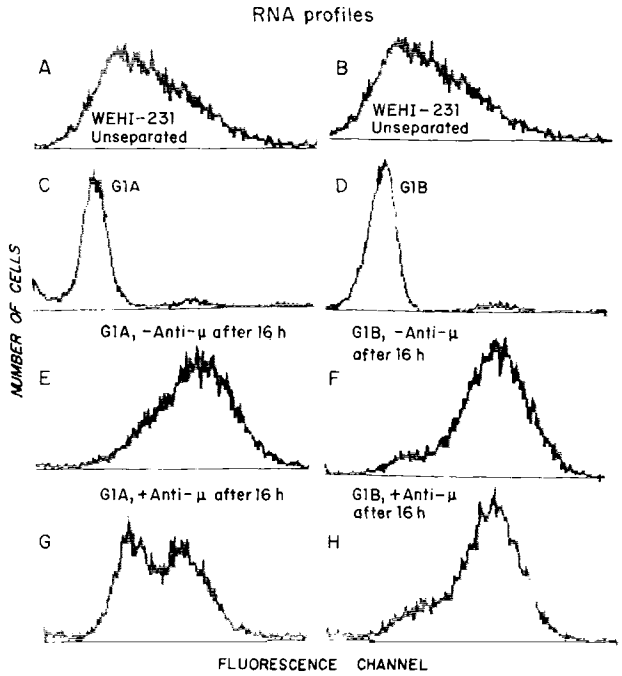

FIGURE 5. RNA profiles verifying the separation of WEHI-231 cells by centrifugal elutriation and showing cell cycle progress. The initial fractions were analyzed for Coulter volume and peaks and corresponding to G_{1a} and G_{1b}, respectively (see Materials and Methods) labelled with AO for verification 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_{1a} and G_{1b} cells (*C* and *D*), which overlap. After 16 h, both G_{1a} and G_{1b} cells have progressed into S and G_2/M (*E* and *F*; see Fig. 4 for DNA profiles). In the presence of anti-μ, the progress of G_{1a} cells is impeded (*G*), but the G_{1b} 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-μ 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-μ–resistant variants (4) for the initial responses to anti-μ 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-μ or anti-κ 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 G_1 to S phase. To further study the mechanism of growth inhibition, we have purified lymphoma cells in G_1 by centrifugal elutriation, or enriched WEHI-231 cells at the G_1/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 G_1, since delayed addition of anti-μ (to late G_1 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 G_1 cells from entering their first S phase. The nature of the relevant processes in early G_1 is discussed in terms of models of B cell activation and tolerance induction.

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References

1. Raff, M. C., J. J. T. Owen, M. D. Cooper, A. R. Lawton, M. Megson, and W. E. Gathings. 1975. Differences in susceptibility of mature and immature mouse B lymphocytes to anti-immunoglobulin–induced immunoglobulin suppression in vitro. *J. Exp. Med.* 142:1052.
2. Parker, D. 1980. Induction and suppression of polyclonal antibody responses by anti-Ig reagents and antigen-nonspecific helper factors. *Immunol. Rev.* 52:115.
3. Ralph, P. 1979. Functional subsets of murine and human B lymphocyte cell lines. *Immunol. Rev.*, 48:107.
4. Scott, D. W., J. Tuttle, D. Livnat, W. Haynes, J. Cogswell, and P. Keng. 1985. Lymphoma models for B-cell activation and tolerance. II. Growth inhibition by anti-μ of WEHI-231 and the selection and properties of resistant mutants. *Cell. Immunol.* 93:124.
5. Scott, D. W., M. Piper, P. S. Pillai, W. Haynes, and M. Ling. 1983. Regulation of growth and differentiation in B cell colonies and tumors. *In* Regulation of the Immune Response. P. Ogra and D. Jacobs, editors. S. Karger, Basel, Switzerland. 57.
6. Scott, D. W., P. S. Pillai, J. Chace, D. Livnat, and P. Keng. 1985. Regulation of specific B cell responses. *In* Proc. 16th Ann. Meet. Scand. Soc. Immunol. H. Valdimarsson, editor. In press.
7. Boyd, A., J. Goding, and J. Schrader. 1981. The regulation of growth and differentiation of a murine B cell lymphoma. *J. Immunol.* 126:2466.
8. deFranco, A., M. Davis, and W. E. Paul. 1982. WEHI-231 as a tumor model for tolerance induction in immature lymphocytes. *In* B and T Cell Tumors E. Vitetta and C. Fox, editors. Academic Press, New York. 445.
9. Lanier, L. L., and N. L. Warner. 1981. Cell cycle related heterogeneity of Ia antigen expression on a murine B lymphoma cell line: Analysis by flow cytometry. *J. Immunol.* 126:626.
10. Darzynkiewicz, Z., D. Evenson, L. Staino-Coico, T. Sharpless, and M. R. Melamed. 1979. Relationship between RNA content and progression of lymphocytes through S phase of cell cycle. *Proc. Natl. Acad. Sci. USA.* 76:358.
11. Darzynkiewicz, Z., T. Sharpless, L. Staino-Coico, and M. R. Melamed. 1980. Subcom-

partments of the G_1 phase of cell cycle detected by flow cytometry. *Proc. Natl. Acad. Sci. USA.* 77:6696.

12. Bauer, K. D., and L. A. Dethlefsen. 1980. Total cellular RNA content; correlation between flow cytometry and ultraviolet spectroscopy. *J. Histochem. Cytochem.*, 28:493.
13. Bauer, K. D., P. C. Keng, and R. M. Sutherland. 1982. Isolation of quiescent cells from multicellular tumor spheroids using centrifugal elutriation. *Cancer Res.* 42:72.
14. Keng, P. C., C. K. Li, and K. T. Wheeler. 1980. Characterization of the separation properties of the Beckman elutriation system. *Cell Biophys.* 3:41.
15. Keng, P. C., C. K. Li, and K. T. Wheeler. 1980. Synchronization of 9L rat brain tumor cells by centrifugal elutriation. *Cell Biophys.* 2:191.
16. Hawrylowicz, C., and G. G. B. Klaus. 1984. Effects of tumour promoter phorbol myristate acetate on mouse lymphocytes: selective inhibition of B cell activation by mitogens and antigens. *Immunology.* 51:327.
17. Rosoff, P. M., and L. C. Lewis. 1985. Lipopolysaccharide and phorbol esters induce differentiation but have opposite effects on phosophatidylinositol turnover and Ca^{2+} mobilization in 70Z/3 pre-B lymphocytes. *J. Biol. Chem.* 260:9209.
18. McCormack, J. E., V. H. Pepe, R. B. Kent, M. Dean, A. Marshak-Rothstein, and G. E. Sonenshein. 1984. Specific regulation of c-*myc* oncogene expression in a murine B-cell lymphoma. *Proc. Natl. Acad. Sci. USA.* 81:5546.