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GROWTH REGULATION OF A HUMAN MATURE B CELL LINE, B104, BY ANTI-IgM AND ANTI-IgD ANTIBODIES[1]

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An EBNA⁻ human B lymphoma cell line, B104, was established. B104 cells express IgD as well as IgM on their surface, which is thought to be a basic characteristic of mature B cells. The growth of B104 cells was inhibited by treatment with a panel of anti-IgM antibodies. Cell cycle analyses revealed that the transition of B104 cells from the G2/M to the G0/G1 phase of the cell cycle was markedly inhibited by treatment with anti-IgM antibodies. Progression of B104 cells to the M phase of the cell cycle was found to be suppressed in the presence of anti-IgM antibodies. In contrast, both the entrance of G0/G1 phase cells into the S phase and the progression of S phase cells to the G2/M phase of the cell cycle did not seem to be inhibited significantly by treatment with anti-IgM antibodies. These results indicate that the mechanism of the inhibition of growth of B104 cells by anti-IgM antibodies is blockage of the transition from the G2 to the M phase of the cell cycle. In contrast to anti-IgM antibodies, anti-IgD antibodies could not cause growth inhibition of B104 cells at all. B cell growth factors such as IL-4 and IL-6 had no effect on the inhibition of growth of B104 cells by anti-IgM antibody. IFN-α and -β, which have no B cell growth factor activity, did increase the number of cells that survived the treatment with anti-IgM antibodies. B104 is an excellent experimental model for the study of the mechanism of signal transduction through sIg as well as the functional difference between sIgM and sIgD.

Treatment with anti-IgM antibodies causes activation and DNA synthesis of mature B cells (1–6). Anti-IgD antibodies also can activate and induce DNA synthesis in resting mature B cells (3). Phosphatidylinositol turnover is promoted by treatment of B cells with anti-IgD antibodies as well as by anti-IgM antibodies (7, 8). The results indicate that sIgD has functions similar to those of sIgM in regard to the activation and the proliferation of mature B cells. However, there are reports that anti-IgM but not anti-IgD antibodies inhibit the growth and/or differentiation of mature B cells, indicating functional differences between sIgM and sIgD (5, 9–16). To date, however, little is known about the mechanism of such negative signaling to mature B cells by anti-IgM antibodies.

In this paper, we present a newly established EBNA[3] human B cell line, B104, which bears both sIgM and sIgD. B104 cells are killed by anti-IgM but not by anti-IgD antibodies. The mechanism of the inhibition of growth of B104 cells by anti-IgM antibodies is the inhibition of the transition from the G2 to the M phase of the cell cycle. The usefulness of B104 cells for the study of the mechanism of regulation of the growth of mature B cells by signals transduced through sIgM and sIgD is discussed.

MATERIALS AND METHODS

Cells. PBMC from a child with malignant lymphoma at the leukemic stage were collected by centrifugation over Ficoll-Paque (Pharmacia AB, Uppsala, Sweden) (17). PBMC were suspended in Iscove's modified Dulbecco's medium, supplemented with 15% heat-inactivated FCS, 2 mM L-glutamine, 5×10^{-5} M 2-ME, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and were delivered into a 24-well polystyrene culture plate (Falcon 3047; Becton Dickinson Labware, Lincoln Park, NJ). They were cultured in an atmosphere of 95% air and 5% CO_2, at 37°C. Cells grew in all wells, and five clones (B104-1 to -5) were established by repetitive limiting dilutions. B104-1 cells are characterized here. The phenotype and the characteristics of B104 cells have been stable for 3 years since its establishment.

Antibodies. The following mouse hybridomas were provided by American Type Culture Collection (Rockville, MD): 1410KG7 (anti-human IgG, HB43), DA4-4 (anti-human IgM, HB57), TB28-2 (anti-human κ, HB61), δTA4-1 (anti-human IgD, HB70), antibody 2.06 (anti-human Ia, HB104), and M-2E6 (anti-human IgM, HB138). mAb were purified from ascitic fluid with ammonium sulfate and DEAE column chromatography.

Purified anti-human IgG and anti-human IgD mAb were obtained from Cappel Laboratories (Cochranville, PA). The following purified mAb were obtained from Coulter Immunology (Hialeah, FL): PE-conjugated anti-B1 (CD20), anti-B2 (CD21), anti-B4 (CD19), anti-CALLA (J5) (CD10), anti-PCA1, and anti-T3 (CD3). FITC-conjugated anti-CD5 mAb was purchased from Nichirei (Tokyo, Japan). Purified anti-HLA-DR and anti-CR1 mAb were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA).

Anti-human FcεRII (CD23) mAb (H107) and anti-human IL2R (anti-Tac) mAb were generously provided by Dr. J. Yodoi (Institute for Virus Research, Kyoto University) and Dr. S. Uchiyama (First Division of Medicine, Kyoto University, Japan), respectively. Anti-

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[3] Abbreviations used in this paper: EBNA, Epstein-Barr virus-associated nuclear antigen; PE, phycoerythrin; BCGF, B cell growth factor; 5-BrdU, 5-bromodeoxyuridine; HU, hydroxyurea; PI, propidium iodide; $[Ca^{2+}]_i$, cytoplasmic free calcium ion concentration.

human FcγRI and FcγRII mAb were kindly provided by Dr. C. L. Anderson (Department of Medicine, Ohio State University, Columbus, OH). Purified anti-human κ and λ mAb were provided by Dr. M. D. Cooper (Tumor Institute, University of Alabama).

The following affinity-purified polyclonal antibodies were purchased from Tago Inc. (Burlingame, CA): FITC-conjugated anti-human IgA (goat), FITC-conjugated and nonconjugated anti-human IgD (goat), FITC-conjugated anti-human IgE (goat), FITC-conjugated and nonconjugated anti-human IgM (goat), FITC-conjugated anti-human IgG (goat), PE-conjugated anti-human κ and anti-human λ (goat, Fab), FITC-conjugated anti-mouse IgG (goat), and FITC-conjugated anti-mouse IgM (goat).

Human serum containing high titers of anti-EBNA antibody was a generous gift from Dr. R. Doi (First Division of Medicine, Kyoto University). FITC-conjugated anti-human C3 antibody was purchased from E-Y Laboratories Inc. With these antibodies, EBNA in an EBV-transformed human B cell line, RPMI8866, was stained clearly by using a previously described method (18).

Analysis of effects of antibodies on cell growth. Cells were suspended in RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 5×10^{-5} M 2-ME, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), at a concentration of 5×10^5 cells/ml and delivered in triplicate to a 96-well polystyrene culture plate (Falcon 3072; Becton Dickinson Labware), in a volume of 150 μl/well. After the addition of 15 μl/well medium containing or not containing antibodies, cells were incubated for planned intervals in an atmosphere of 95% air and 5% CO_2 at 37°C. Preservatives used in the preparation of antibodies were removed by repetitive dialysis. At the end of the incubation, viable cells were counted by the trypan blue dye exclusion method.

Immunofluorescent analysis. One million cells were placed in a 12 × 75-mm polystyrene round-bottomed tube (Falcon 2052; Becton Dickinson Labware). After two washings with staining solution (PBS plus 3% FCS and 0.1% sodium azide), the cells were incubated with the first antibody (usually 1 μg/tube) for 30 min at 4°C (first incubation). In the direct immunofluorescent study, FITC- or PE-conjugated antibodies were used as the first antibody. In the indirect immunofluorescent study, cells were washed twice with staining solution after the first incubation and then incubated with FITC-conjugated second antibody (usually 1 μg/tube) for 30 min at 4°C (second incubation).

After the last incubation, cells were washed twice and then resuspended in 0.5 ml of staining solution. The intensity of fluorescence was analyzed by flow cytometry (FACS 440; Becton-Dickinson Immunocytometry Systems).

Measurement of $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ in response to treatment with anti-Ig antibodies was measured by a previously described method (19–21). To load Indo-1, an indicator for $[Ca^{2+}]_i$, B104 cells were suspended in RPMI 1640 medium at a concentration of 2 to 3×10^7/ml and cultured for 30 min at 37°C in the presence of 2 μM Indo-1/acetoxymethylester (Hoechst Japan, Tokyo, Japan), a membrane-permeable form of Indo-1. After two washings, aliquots of 2 to 3×10^6 cells were resuspended in 2 ml of buffer composed of 140 mM NaCl, 3mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Tris-HEPES (pH 7.4). The intensity of fluorescence was measured by using a spectrofluorophotometer (Shimazu RF510; Shimazu Corporation, Kyoto, Japan) with excitation at 331 nm (5 nm slits) and emission at 410 nm (15-nm slits). Calibration was performed with the use of ionomycin (Hoechst Japan) and $MnCl_2$ (20).

Analysis of [^{3}H]TdR uptake. One hundred eighty microliters/well of a cell suspension of 5×10^5 cells/ml were placed into a 96-well polystyrene culture plate (Falcon 3072; Becton Dickinson Labware), in triplicate. After the addition of 20 μl/well of medium containing antibodies or reagents, they were incubated for various periods. Medium (20 μl) containing 1 μCi of [^{3}H]TdR was added to each well 3 h before harvest with an automated cell harvester (Labo Mash II; Labo Science Co. Ltd., Tokyo, Japan). The amount of incorporated [^{3}H]TdR was measured by using an automated liquid scintilation counter (Aloka LSC950; Aloka Co. Ltd., Tokyo, Japan).

Synchronization of cells in G0/G1 phase of cell cycle. Cells were suspended in RPMI 1640 medium, at a concentration of 1×10^6 cells/ml, and cultured in an atmosphere of 95% air and 5% CO_2 at 37°C for 16 h, in the presence of 0.5 mM HU (Sigma Chemical Company, St. Louis, MO). Cells were then washed three times with PBS to remove HU before being cultured with antibodies.

Cell cycle analysis. Cells (1 to 2×10^6) were put into a 17- × 120-mm polystyrene conical tube (Falcon 2095; Becton Dickinson Labware) and washed twice with PBS. Pellets of cells were resuspended in 70% ethanol that was chilled at −20°C and were kept at 4°C for longer than 4 h. The cells were then washed twice with PBS and resuspended in 1 ml of PBS. To remove RNA, 10 μl of 10 mg/ml RNase I (Sigma) were added to each tube and incubated for 30 min at 37°C. After incubation, cells were washed twice with PBS and resuspended in 1 ml of PBS. After the addition of 30 μg/tube of PI (Sigma), the intensity of fluorescence was examined by flow cytometry (FACS440; Becton Dickinson Immunocytometry Systems).

Double-staining analysis of the cell cycle. Double-staining analysis of the cell cycle was performed by the method described previously (22). Briefly, S phase cells were labeled with 5-BrdU (Sigma) by preculture with 6 μg/ml 5-BrdU for 30 min at 37°C. Then, they were cultured with each reagent. After the culture, 2 to 5×10^6 cells were transferred to a 17- × 120-mm polystyrene conical tube and washed twice with PBS. Then, cells were resuspended in 2 ml of chilled (−20°C) 70% ethanol and kept overnight or longer at 4°C for fixation. Then cells were washed once with PBS and resuspended in 1 ml of PBS. After the addition of RNase I (Sigma) at a concentration of 0.1 mg/ml, cells were incubated for 30 min at 37°C, washed once with PBS, and resuspended in 2 ml of PBS. Next, 2 ml of 4 N HCl were added to the cell suspension and stirred gently. After 30 min of incubation at room temperature, the suspension was centrifuged, and the pellet was resuspended in 2 ml of 0.1 M sodium borate (pH 8.5) to neutralize the acids. Then the cells were washed once with PBS and resuspended in 50 μl of PBS containing 0.5% BSA and 0.05% Tween 20. After the addition of 20 μl/tube of FITC-conjugated anti-5-BrdU mAb (Becton Dickinson Immunocytometry Systems), the cells were incubated for 30 min at room temperature in the dark, washed twice with PBS, and resuspended in 1 ml of PBS. After the addition of 6 μg/tube of PI, the intensity of green (FITC) and red (PI) fluorescence was analyzed by flow cytometry (FACS440).

Enumeration of M phase cells. Cells were suspended in RPMI 1640 medium at a concentration of 5×10^5 cells/ml and delivered in triplicate into 96-well polystyrene culture plates (Falcon 3072; Becton Dickinson Labware), at 0.2 ml/well. After the addition of reagents, cells were cultured for 6 h in the presence of 0.2 μg/ml colcemid (Nakarai Tesque, Inc., Kyoto, Japan). Then aliquots of 0.5 to 1×10^5 cells were placed on glass slides with Cytospin 2 (Shandon Southern Products Ltd., Astmoor, England). The specimens were air dried and stained with Giemsa solution. About 500 cells/specimen were examined microscopically, and those with definite chromosomes were classified as M phase cells.

RESULTS

Surface Ag, EBNA, and chromosomes of B104 cells. The surface Ag of B104 cells are listed in Table I. The most remarkable feature of B104 cells is the coexpression of sIgM and sIgD, which is a basic characteristic of mature B cells (6, 23). The coexpression of sIgM and sIgD is valid because both can be detected consistently by various mAb and polyclonal antibodies (see *Materials and Methods*). mRNA of both μ and δ H chain were also detected by Northern blot hybridization (data not shown). Other surface Ag on B104 cells are also similar to those on normal mature B cells (24–29). Ag specific for T cells (CD3 and CD5) were not detected. B104 cells have 46 chromosomes, which are of the male type without any abnormalities. EBNA was not detected in B104 cells.

Influence of anti-Ig antibodies on cytosolic free calcium ion. The addition to B104 cells of anti-IgM antibodies caused an increase of $[Ca^{2+}]_i$ (Fig. 1, *a* and *b*). There were no evident differences in the ability to increase $[Ca^{2+}]_i$ between the two anti-IgM mAb, DA4-4 and M-2E6. Although the amount of sIgD on B104 cells is smaller than that of sIgM (Table 1), the increase in $[Ca^{2+}]_i$ caused by treatment with an anti-IgD mAb (δTA4-1) was com-

TABLE I

Surface Ag of B104 cells[a]

sIgM	++++	B4 (CD19)	+++
sIgD	++	J5 (CD10)	++
sIgG	−	CR1	+
sIgA	−	FcγR	+
sIgE	−	FcεR2 (CD23)	−
κ	++++	IL-2R	−
λ	−	T1 (CD5)	−
Ia	++++	T3 (CD3)	−
B1 (CD20)	+++	PCA1	−
B2 (CD21)	+	EBNA	−

[a] Relative intensities of fluorescence are shown.

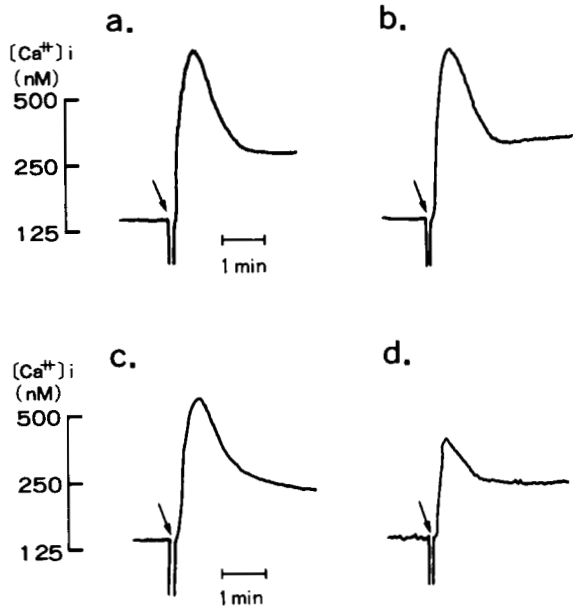

Figure 1. Increase in $[Ca^{2+}]_i$ by stimulation with anti-IgM and anti-IgD antibodies. Anti-Ig mAb were added to B104 cells (*a* to *c*) and R3 cells (*d*) at a concentration of 10 μg/ml, and the change in $[Ca^{2+}]_i$ was examined. *Arrows*, time when antibodies were added. Antibodies: DA4-4 anti-IgM antibody (*a* and *d*), M-2E6 anti-IgM antibody (*b*), and δTA4-1 anti-IgD antibody (*c*).

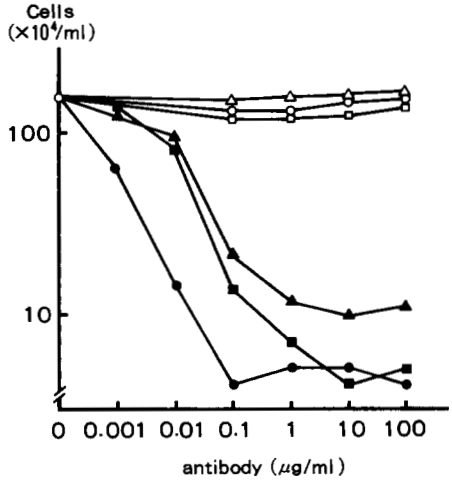

Figure 2. Inhibition of growth of B104 cells by treatment with anti-IgM antibodies. The number of viable B104 cells after 48 h of incubation with monoclonal and polyclonal antibodies is shown. ○, Control (1410KG7 anti-IgG antibody); △, δTA4-1 anti-IgD mAb; □, polyclonal anti-IgD antibody; ●, DA4-4 anti-IgM mAb; ▲, M-2E6 anti-IgM mAb; ■, polyclonal anti-IgM antibody.

parable to that caused by anti-IgM mAb (Fig. 1*c*). Treatment of R3 cells, a mutant cell line of B104 with the same phenotypic features but different responsiveness to stimulation with anti-IgM antibodies, with DA4-4 antibody also caused an increase in $[Ca^{2+}]_i$, but the peak level was considerably lower than that in B104 cells (Fig. 1*d*).

Anti-human Ia (antibody 2.06), anti-human CD10, and anti-human IgG (1410KG7) mAb did not increase $[Ca^{2+}]_i$ (data not shown).

Inhibition of growth of B104 cells by anti-IgM antibodies. Treatment of B104 cells with anti-IgM antibodies caused profound growth inhibition and cell death (Fig. 2). The potency of such negative signaling was different among the antibodies; DA4-4 antibody was the most potent, with a maximum effect at concentrations as low as 0.1 μg/ml, and M-2E6 antibody was the weakest. The $F(ab')_2$ fragment of DA4-4 antibody also inhibited the growth of B104 cells, whereas the Fab fragment of DA4-4 antibody had no effect (data not shown). Unlike anti-IgM antibodies, monoclonal as well as polyclonal anti-IgD antibodies had no effect on the growth of B104 cells (Fig. 2). When anti-IgD antibodies were added to B104 cells in combination with anti-IgM antibodies, they neither enhanced nor suppressed the growth-inhibitory effect of anti-IgM antibodies on B104 cells (data not shown). Anti-human IgG mAb (1410KG7) did not inhibit the growth of B104 cells (Fig. 2). An anti-human κ mAb (TB28-2) inhibited the growth of B104 cells, whereas anti-human Ia mAb (antibody 2.06) did not affect their growth (data not shown).

The growth of R3, a mutant cell line of B104 with the same phenotypic features as B104, was not affected by treatment with anti-IgM antibodies (Fig. 3), although the amount of sIgM decreased, as it did in B104 (data not shown).

[^{3}H]TdR uptake by B104 cells after treatment with anti-IgM antibodies. Because the inhibition of growth of immature B cells by treatment with anti-IgM antibodies has been shown to be due to inhibition of DNA synthesis (30–35), the effect of anti-IgM antibodies on the DNA synthesis by B104 cells was examined and compared with that of HU, an inhibitor of DNA synthesis.

As shown in Figure 4*a*, B104 cells stopped proliferating immediately after the addition of DA4-4 anti-IgM antibody and began to be killed 12 h after the addition of antibody. HU also caused a gradual decrease in the number of viable cells. However, many B104 cells were found to be still alive 24 h after the addition of HU, apparently more than the number of viable B104 cells cultured for 24 h in the presence of anti-IgM antibody.

[^{3}H]TdR uptake by HU-treated B104 cells dropped to the lowest level within 3 h after the addition of HU (Fig. 4, *b* and *c*). The treatment with DA4-4 anti-IgM antibody also caused a decrease in [^{3}H]TdR uptake by B104 cells. However, the rate of the decrease in [^{3}H]TdR uptake by B104 cells treated with DA4-4 anti-IgM antibody was much slower than that in HU-treated B104 cells and resembled the rate of the decrease in the number of viable cells after treatment with DA4-4 antibody (Fig. 4*b*). The level of [^{3}H]TdR uptake per viable cell in B104 cells treated with DA4-4 antibody was similar to that of non-

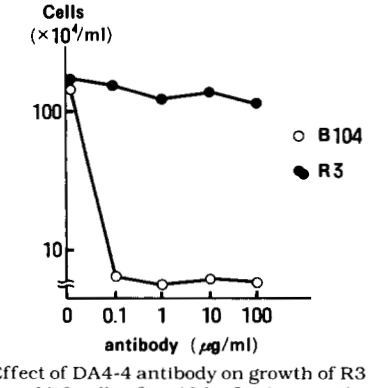

Figure 3. Effect of DA4-4 antibody on growth of R3 cells. The number of viable B104 and R3 cells after 48 h of culture with various concentrations of DA4-4 anti-IgM mAb is shown.

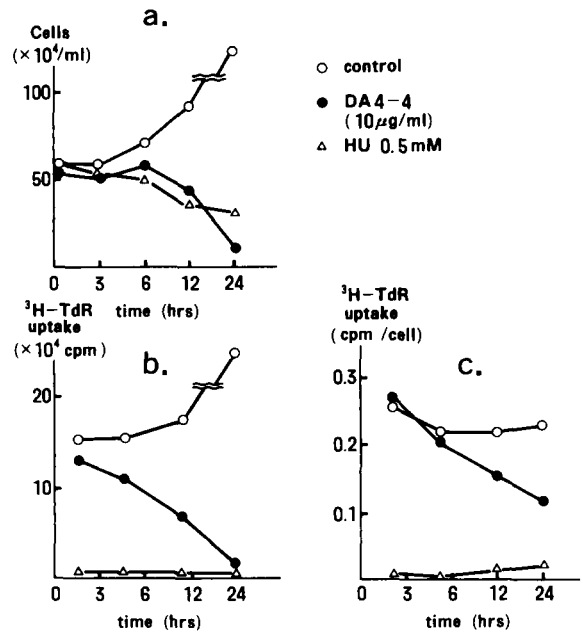

Figure 4. Effect of DA4-4 anti-IgM antibody on DNA synthesis of B104 cells. B104 cells were cultured with 10 μg/ml DA4-4 anti-IgM mAb or 0.5 mM HU. *a*, The number of viable cells; *b*, the amount of [³H]TdR uptake; *c*, [³H]TdR uptake per viable cell.

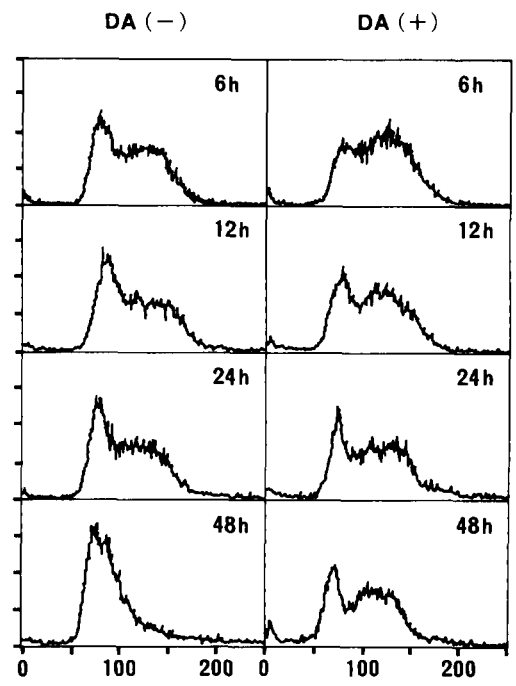

Figure 5. Effect of DA4-4 anti-IgM antibody on cell cycle progression of B104 cells. The cell cycle of B104 cells was analyzed after culture with or without 10 μg/ml DA4-4 anti-IgM mAb.

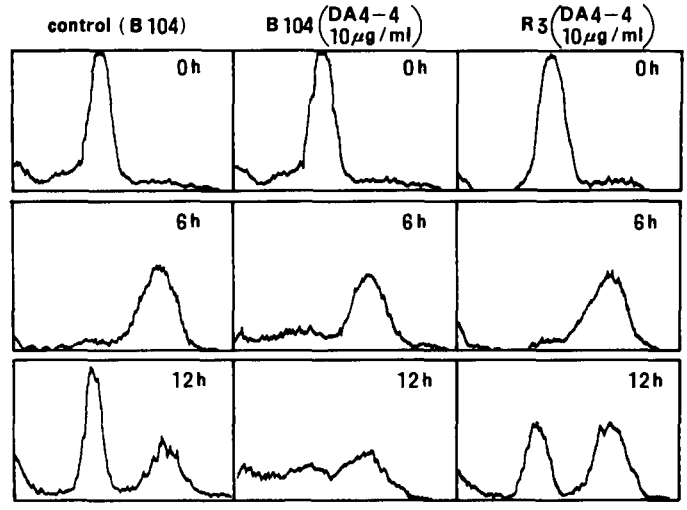

Figure 6. Cell cycle analysis of the effect of DA4-4 anti-IgM antibody on B104 cells synchronized in G0/G1 phase. B104 and R3 cells synchronized in the G0/G1 phase of the cell cycle were cultured with or without 10 μg/ml DA4-4 anti-IgM mAb.

treated cells up to 6 h after the addition of antibody (Fig. 4*c*). Although the [³H]TdR uptake per viable cell in B104 cells treated with DA4-4 antibody then began to decrease, it was still higher than that in HU-treated B104 cells even after 24 h of culture (Fig. 4*c*).

This study revealed that, although the decrease in DNA synthesis of B104 cells caused by DA4-4 anti-IgM antibody is much slower and milder than that caused by HU, the decrease in viable cells caused by the former was much more profound than that caused by the latter. The discrepancy raises the possibility that inhibition of DNA synthesis is not a primary mechanism of the inhibition of growth of B104 cells by anti-IgM antibody.

Effect of anti-IgM antibody on cell cycle progression of B104 cells. The most remarkable change in the cell cycle of B104 cells caused by anti-IgM antibodies was the decrease in G0/G1 phase cells and the increase in S plus G2/M phase cells, which began to appear 3 h after the addition of antibody and became evident 6 h after the addition of antibody (Fig. 5). This indicates that the growth of B104 cells is inhibited in the later phases, namely from the late S to the M phase, of the cell cycle by treatment with anti-IgM antibodies. Thereafter, such a characteristic change in the cell cycle became less evident and disappeared almost completely 24 h after the addition of anti-IgM antibody. Because this corresponds to the time during which cell death of B104 cells occurred, the disappearance of the characteristic change in the cell cycle seems to be due to the removal of dead cells.

If the cell cycle progression of B104 cells is inhibited in the G0/G1 phase or the G1/S interphase of the cell cycle, an increase in G0/G1(/S) phase cells and a decrease in S/G2/M phase cells must be observed. However, such a change was not observed in B104 cells treated with anti-IgM antibody (Fig. 5).

Demonstration of the point of action of negative signaling by anti-IgM antibodies. In the absence of DA4-4 anti-IgM antibody, many B104 cells, which were synchronized initially in the G0/G1 phase of the cell cycle, appeared again in the G0/G1 phase via the G2/M phase of the cell cycle by 12 h after the removal of HU (Fig. 6). However, in the presence of anti-IgM antibody, synchronized B104 cells could not form a peak in the G0/G1 phase of the cell cycle 12 h after the removal of HU. This suggested that the transition of B104 cells from the G2/M to the G0/G1 was blocked by anti-IgM antibody.

Double-staining analysis of the cell cycle revealed that, although in the absence of DA4-4 antibody 5-BrdU-labeled B104 cells began cell division by 6 h after the initiation of culture, no labeled B104 cells could be detected in the G0/G1 phase in the presence of DA4-4 antibody (Fig. 7). Thus, it seems valid that the transition of B104 cells from the G2/M to the G0/G1 phase of the

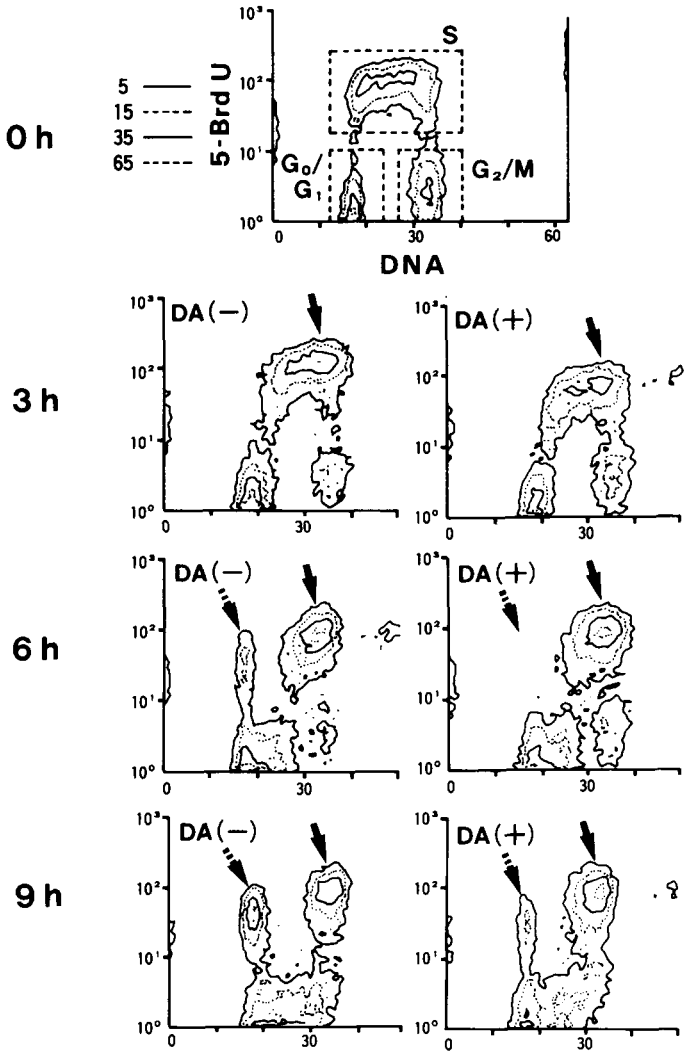

Figure 7. Fate of S phase cells after treatment with DA4-4 anti-IgM antibody. *Horizontal* and *vertical axes*, the relative amount of DNA and incorporated 5-BrdU, respectively. In the absence of DA4-4 anti-IgM antibody (*DA(−)*), most 5-BrdU-labeled S phase cells reached the G2/M phase (*6h,Da(−), solid arrow*), and some of them had already completed cell division by 6 h after the initiation of reculture (*6h,DA(−), dotted arrow*). In contrast to this, in the presence of DA4-4 antibody, all labeled cells stayed in the G2/M phase 6 h after the initiation of reculture (*6h,DA(+), solid arrow*), and no labeled cells appeared in the G0/G1 phase (*6h,DA(+), dotted arrow*). Although some labeled cells appeared in the G0/G1 phase (*9h,DA(+), dotted arrow*) 9 h after the initiation of reculture, they were many fewer than those cultured without DA4-4 antibody (*9h,DA(−), dotted arrow*).

cell cycle is blocked by anti-IgM antibody.

When B104 cells were cultured for 6 h in the presence of colcemid, 12.6% of the cells were located in the M phase of the cell cycle (Table II). In the presence of anti-IgM antibodies, the percentage of M phase cells decreased to about 2% (Table II).

All these results strongly suggest that the inhibition of the growth of B104 cells by anti-IgM antibodies is due to blockage of the transition from the G2 to the M phase of the cell cycle.

Effect of cytokines on the cytotoxity of anti-IgM antibodies. Most of the currently available human cytokines were examined for their ability to counteract the negative signaling of anti-IgM antibodies (Table III). IFN-α and IFN-β were found to increase the number of surviving cells, even at concentrations as low as 10 U/ml, and

TABLE II

Effect of DA4-4 anti-IgM antibody on progression of B104 cells into the M phase[a]

Antibody (μg/ml)[b]	Colcemid (0.2 μg/ml)	M Phase Cells (%)
None	−	3.0 ± 0.6
None	+	12.6 ± 2.6
DA4-4, 100.0	+	1.4 ± 0.6
DA4-4, 10.0	+	2.6 ± 0.4
DA4-4, 1.0	+	2.6 ± 1.2
DA4-4, 0.1	+	2.0 ± 1.0
DA4-4, 0.01	+	6.8 ± 1.8
M-2E6, 10.0	+	2.4 ± 0.8
δTA4-1, 10.0	+	12.8 ± 1.8
1410KG7, 10.0	+	10.0 ± 1.2

[a] B104 cells were cultured with the indicated reagents for 6 h. Then the percentages of M phase cells were examined and presented as the mean ± 1 SD of triplicate samples.

[b] DA4-4 and M-2E6, anti-human IgM mAb; δTA4-1, anti-human IgD mAb; 1410KG7, anti-human IgG mAb.

TABLE III

List of human cytokines examined in this study

Cytokine[a]	Donor	Cytokine	Donor
rIL-1α	Otsuka[b]	rIFN-β	Kyowa Hakko[i]
rIL-1β	Otsuka[b]	rIFN-γ	Takeda[c]
rIL-1	Takeda[c]	rTNF-α	Genzyme[j]
rIL-3	Amersham[d]	TGF-β	R&D Systems[k]
rIL-4	Dr. K. Arai[e]	rGM-CSF	Sumitomo[l]
	Ono[f]	rG-CSF	Chugai[m]
rIL-5	Suntory[g]	rM-CSF	Otsuka[b]
rIL-6	Drs. T. Hirano and T. Kishimoto[h]	PAF	Bachem[n]
		B104 supernatant	
rIFN-α	Takeda[c]		

[a] TGF, transforming growth factor; PAF, platelet-activating factor.
[b] Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan.
[c] Takeda Chemical Industries, Ltd., Tokyo, Japan.
[d] Amersham International plc, Aylesbury, England.
[e] DNAX Research Institute of Molecular and Cellular Biology, Inc., CA.
[f] Ono Pharmaceutical Co., Ltd., Osaka, Japan.
[g] Suntory Co., Ltd., Osaka, Japan.
[h] Institute for Molecular and Cellular Immunology, Osaka University, Osaka, Japan.
[i] Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan.
[j] Genzyme Corporation, Boston, MA.
[k] R&D Systems, Inc., MN.
[l] Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan.
[m] Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
[n] Bachem Fein Chemkauen AG., Bubendorf, Switzerland.

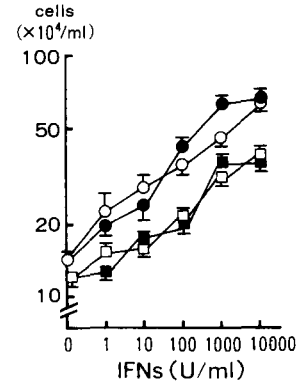

Figure 8. Effect of IFN-α/β on the inhibition of growth of B104 cells by anti-IgM antibodies. The number of living cells after 48 h of culture with 10 μg/ml anti-IgM antibodies is presented as the mean ± 1 SD of triplicate samples. ○, M-2E6 anti-IgM antibody plus IFN-α; ●, M-2E6 anti-IgM antibody plus IFN-β; □, DA4-4 anti-IgM antibody plus IFN-α; ■, DA4-4 anti-IgM antibody plus IFN-β.

this effect increased with increasing concentrations (Fig. 8). Although treatment with anti-IgM antibodies decreased the percentage of M phase cells, the addition of IFN-α/β reduced the degree of decrease (Fig. 9). The increase in M phase cells caused by the addition of IFN-α/

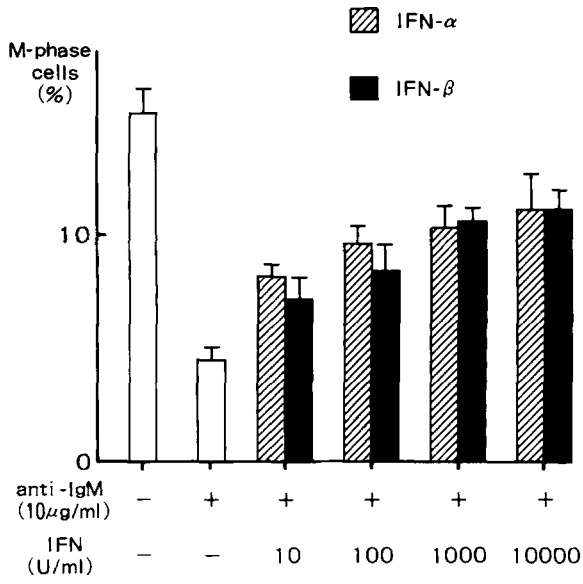

Figure 9. Increase by IFN-α/β of percentage of M phase cells in B104 cells stimulated with anti-IgM antibody. The percentage of M phase cells was determined after 6 h of culture of B104 cells with the indicated reagents and is presented as the mean ± 1 SD of triplicate samples.

β could not be due to the promotion of growth of B104 cells, because IFN-α/β suppressed the growth of B104 cells (data not shown). These results indicate that IFN-α/β counteract specifically the negative signaling of anti-IgM antibodies, namely the blockage of proliferation in the G2 phase of the cell cycle.

DISCUSSION

The inhibition of growth by treatment with anti-IgM antibody is a remarkable characteristic of B104 cells. Activation and proliferation are the most common responses of resting mature B cells to stimulation with anti-IgM antibodies (1–6). However, it is also known that anti-IgM antibodies inhibit the growth and/or differentiation of mature B cells (5, 9–16). Thus, sIgM is likely to deliver in some situations a negative signal to mature B cells. However, our knowledge about the mechanism of negative signaling by anti-IgM antibodies to mature B cells is very incomplete. Because some investigators reported that DNA synthesis by B lineage cells with phenotypic characteristics of mature B cells was inhibited by treatment with anti-IgM antibodies (36–38), the inhibition of DNA synthesis might be a mechanism of negative signaling in normal mature B cells.

We have shown in this paper, by a precise analysis of the cell cycle, that the inhibition of growth of B104 cells by treatment with anti-IgM antibody may be due to blockage of the transition from the G2 to the M phase of the cell cycle. Such a mode of inhibition of the growth of human mature B cells has not yet been noted. In murine mature B cells, however, a restriction point for proliferation has actually been demonstrated in the G2 phase of the cell cycle (39, 40), although it was not noted whether the growth inhibition at this restriction point is due to stimulation by anti-IgM antibodies. Thus, some, if not all, aspects of negative signaling by anti-IgM antibodies to normal mature B cells might be explained by such a mode of growth inhibition.

Although the amount of sIgD on B104 cells is smaller than that of sIgM, treatment of B104 cells with anti-IgD antibodies caused as much increase in $[Ca^{2+}]_i$ as did the treatment with anti-IgM antibodies (Fig. 1). This indicates the functional competence of sIgD on B104 cells to transduce signals, which is not surprising, because the cross-linkage of only a small percentage of sIg is enough to transmit maximum signals to increase $[Ca^{2+}]_i$ to normal mature B cells (41). However, treatment with anti-IgD antibodies could not inhibit the growth of B104 cells, even though the antibodies were used at a concentration of as high as 100 μg/ml. The results suggest that sIgD constitutionally lacks the ability to transduce signals to cause cell death of B104 cells. B104, the first established human B cell line coexpressing sIgM and sIgD with apparently competent function, may be of value in elucidating the differences in the functions of sIgM and sIgD.

A murine lymphoma cell line, WEHI231, has been used as an experimental model for the inhibition of growth of immature B cells by anti-IgM antibodies (30–33). The mechanism of inhibition of the growth of WEHI231 cells is considered to be inhibition of DNA synthesis (34, 35). Such growth inhibition was counteracted by co-stimulation with LPS, a polyclonal B cell mitogen (42). Moreover, the growth inhibition was reduced by a T cell factor with BCGF activity (43). Unlike WEHI231 cells, the inhibition of growth of B104 cells by stimulation with anti-IgM antibodies could not be reversed, even if they were co-stimulated with *Staphylococcus aureus* Cowan I, a polyclonal human B cell mitogen (data not shown), or anti-human IgD antibodies. Recombinant BCGF, such as IL-4 and IL-6, as well as B104 supernatant, which has a BCGF activity in B104 cells, also failed to decrease the inhibition of growth of B104 cells by anti-IgM antibodies. Thus, BCGF activities seem to be unrelated to regulation of proliferation in the G2 phase of the cell cycle.

Unexpectedly, the inhibition of the growth of B104 cells by stimulation with anti-IgM antibodies was relieved by IFN-α and -β, well known antiviral and antitumor agents. To date, the effect of IFN-α/β on Ig synthesis is still controversial. Both suppression (44–47) and enhancement (48–50) of Ig synthesis by IFN-α/β have been reported. It has been demonstrated that IFN-α/β act on both B and T cells and that the effect of IFN-α/β on Ig synthesis varies with factors such as the concentration of IFN-α/β, the time of addition of IFN-α/β, and the duration of exposure to IFN-α/β. However, much remains to be studied about the mechanism of the action of IFN-α/β on Ig synthesis. The activity of IFN-α/β in counteracting the negative signaling of anti-IgM antibodies, which was first revealed in this study, may provide a new basis for the study of this problem.

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