Induction of CD40 Expression and Enhancement of Monoclonal Antibody Production on Murine B Cell Hybridomas by Cross-Linking of IgG Receptors

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The 55-6 murine B cell hybridoma line not constitutively expressing CD40 was treated with increasing amounts of intact anti-mouse surface immunoglobulin G antibody (anti-mIgG) either not preincubated or preincubated for 48 h with lipopolysaccharide (LPS). In vitro, cross-linking of surface immunoglobulin G (sIgG) with the whole molecule of anti-IgG antibodies induced the expression of CD69, CD40, and CD19 surface antigens on 55-6 cells. The effect of sIgG ligation was dose-dependent, and preincubation with LPS enhanced their responsiveness to anti-mIgG stimulation. The expression of these surface molecules reached the maximum value during the first part of the cell cycle, corresponding to the position of the G1 peak of the DNA distribution. Stimulation of cells with anti-mIgG did not induce changes either in the number of viable cells or in the fraction of cells undergoing proliferation (mitosis). However, preincubation of 55-6 cells with LPS for 48 h before stimulation with anti-mIgG increased both the maximum specific growth rate (μ_{max}) and the percentage of cells in the G2/M phase, in comparison with non-preincubated cells. Moreover, on cells preincubated with LPS prior to anti-mIgG treatment, specific IgG2a production rate was enhanced significantly compared to that obtained in control cultures. The correlation between the antibody production rate and the amount of IgG that is detectable on the cell surface was analyzed by flow cytometry. A good correlation between secreted and surface IgG was observed, and the results of cell cycle analyses demostrated that the 55-6 hybridoma cell line has a substantially higher sIgG content in G1 phase.

Introduction

Hybridoma cells cultures are routinely used for the production of monoclonal antibodies (mAb) in both the research and process development fronts with applications as diagnostic reagents and clinical therapeutics. The increasing demand for large quantities of monoclonal antibodies (mAbs) requires the optimization of in vitro mAb production. Several methods have been explored for mAb production. These include (a) the identification of high-producing, genetically stable clones (*1*), (b) the extension of cell survival and reduction of apoptosis, (c) the development of cell lines expressing recombinant mAb (i.e., CHO, etc.) (*2*−*4*), and (d) the use of stimulatory agents modulating the cell metabolism and, perhaps, also gene expression in favor of enhanced protein production and secretion (*5*). While these methodologies may generate higher mAb productivity, the first three are time-consuming and not suitable for screening of multiple targets. However, the addition of stimulatory agents could impact productivity rapidly, and the ease of operation would allow for concurrent screening of a number of targets. Our work is in this latter methodology. In it, we induce the expression of CD40 surface antigen and enhance specific mAb production rate through cross-linking of sIgG with anti-mIgG on a murine B cell hybridoma line.

With regard to the effect of anti-mIgG on normal B cells, several reports have demonstrated that cross-linking of (sIg)[2] receptors in B cells initiates a cascade of biochemical events that includes mobilization of intracellular ionized calcium $[Ca^{2+}]_i$ and activation of protein kinase C (PKC) and PTKs (*6*−*12*). The summation of these events reflects an early response to sIg-mediated signal transduction in B cells affecting cell viability, secretion of antibodies (*13*, *14*), and the expression of important activation molecules, including CD40, CD69, B7 molecules, and MHC class II (*15*, *16*). In this sense, it is well-established that the interaction CD40−CD40L is one of the key parameters in the activation of B lymphocytes, because it induces clonal expansion, activating the cell cycle, which is physiologically empowered by other factors derived from helper T lymphocytes, such as IL-4 and contact between members of the TNF family and TNF receptors (CD30−CD30L and BlyS/TAC) (*17*).

Since B cell hybridomas possess a component of genes derived from the normal B cells used for cell hybridization, the purpose of this work is to take advantage of this relationship to trigger in hybridomas the same metabolic routes that take place in B cells to increase proliferation and mAbs production.

In the present study the 55-6 B cell hybridoma line not constitutively expressing CD40 and other surface molecules such as CD69 and CD19 was used. This work describes the effect of anti-mIgG addition in the culture medium on (i) the expression of the above-mentioned surface receptors, (ii) the

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growth kinetics, (iii) the fraction of cells undergoing proliferation, and (iv) the production of mAbs.

Materials and Methods

Cell Line and Cell Maintenance. The cell line, a mouse−mouse B cell hybridoma designated 55-6 (ATCC CRL-2156), produces IgG2a monoclonal antibodies to human immunodeficiency virus (HIV) glycoprotein 120 (gp120). The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2.1 mM L-glutamine, 100 U/mL penicillin-streptomycin, 0.125 μg/mL amphotericin B, hypoxanthine-thymidine (HT) Media Supplement (50X) Hybri-Max*Gamm and incubated at 37 °C in 5% CO_2 atmosphere. All chemicals were from Sigma-Aldrich, Inc.

Antibodies and Reagents. Goat anti-mouse IgG (whole molecule) (Sigma-Aldrich, Inc.), anti-mCD69: fluorescein isothiocyanate (FITC) (Serotec), anti-mCD40: FITC (Serotec), anti-mCD19: FITC (Serotec), affinity purified anti-mouse CD16/32 (eBioscience), and F(ab')$_2$ goat anti-mouse IgG: FITC (Serotec). LPS; *Escherichia coli*, 0111:B4, Ribonuclease A (Rase A), propidium iodide (PI), phosphate-buffered saline (PBS), PBS/albumin bovine serum (PBS/BSA), ethanol, and trypan blue, all from Sigma-Aldrich, Inc.

B Cell Hybridoma Activation. Cells (5×10^4 cells/mL) were incubated with various concentrations of anti-mIgG (0, 25, 45, 140, and 230 μg/mL) in T-175 culture flasks in the medium described above with a working volume of 70 mL for 4 days at 37 °C in 5% CO_2 atmosphere. Before activation, cells were preincubated with 0.5 μg of anti-CD16/CD32 per million cells for 10 min to reduce Fc receptor-mediated binding.

LPS-activated cells were obtained by culturing cells at a concentration of 1×10^5 cells/mL in the culture medium supplemented with 5 μg/mL LPS and incubating at 37 °C in 5% CO_2 atmosphere for 48 h. Unstimulated cells (5×10^4 cells/mL) incubated in culture medium alone and cells preincubated with LPS for 48 h prior to incubation in culture medium alone (PreLPS) for 4 days were used as control cultures.

Flow Cytometry Analysis. CD69, CD40, CD19, and sIgG were determined by direct immunofluorescence staining for flow cytometry. For these analyses, cell number was adjusted to 1×10^7 cells/mL in staining buffer. Then, 100 μL samples of this cell suspension (1×10^6 cells) were reacted with 10 μL of mAbs FITC conjugated to defined antigens for 30 min at room temperature. After two washing steps with 2 mL of PBS/BSA cells were analyzed by cytofluorometry. Shortly before cell analysis, PI (10 μg/mL) was added in order to identify dead cells.

For cell cycle analysis, 2×10^6 cells per aliquot were fixated with ice-cold ethanol (−20 °C) at a final concentration of 70% (v/v) during vortexing for 2 min. After the ethanol fixation, 1 mL of PBS were added, and the cells were centrifuged at 4,000 rpm for 5 min at room temperature and resuspended in 900 μL of PBS. RNase A was then added to a final concentration of 1 mg/mL (from a stock solution containing 10 mg/mL RNase). The cells were vortexed and incubated at 37 °C for 30 min. PI was added to this suspension at a final concentration of 0.003% (from a stock solution containing 0.05% PI). After vortexing, the cells were kept at room temperature for approximately 15 min and then analyzed in the flow cytometer.

The cellular fluorescence was measured in a Coulter Epics XL-MCL flow cytometer using System II Software for data acquisition and analysis (Coulter Corporation).

Quantification of Cell Number Increase and Determination of Antibody Concentration. The effect of anti-mIgG on the change in overall cell number is determined by direct cell counts following mitogenic stimulation and staining with trypan blue. For the trypan blue exclusion test 1 part of 0.4% trypan blue was mixed with 1 part cell suspension (dilution of cells) and incubated for ~3 min at room temperature. Unstained (viable) and stained (nonviable) cells were counted separately in the hemacytometer. The total number of viable cells per milliliter was obtained by multiplying the total number of viable cells by the dilution factor for trypan blue. Cells were removed by centrifugation at approximately 12,000 rpm for 10 min, and the supernatant was stored at −20 °C for the determination of antibody concentrations. IgG2a concentration was measured by the sandwich-type ELISA using goat anti-mouse IgG-coated plates and goat anti-mouse IgG peroxidase conjugate as the second antibody.

Results

Expression of CD69, CD40, and CD19 Molecules on 55-6 Cells after Treatment with Anti-MouseIgG (Whole Molecule). To examine the direct effect of anti-mIgG in the expression of these surface antigens, 55-6 cells (5×10^4 cells/mL) were incubated with various concentrations of anti-mIgG in T-175 culture flasks, and CD69, CD40, and CD19 were detected by cytofluorometry. As shown in Figure 1, the cells could be induced to express CD69, CD40, and CD19 molecules in the presence of anti-mIgG. The effect of sIgG ligation on the B cell hybridoma activation was dose-dependent: higher concentrations of anti-mIgG increased the expression of CD40 as well as the expression of CD69 and CD19 (Figure 1A).

CD69 is a very early activation antigen expressed on all lymphocytes following in vitro activation. The up-regulation of CD69 is incorporated into the assay as a positive procedure control to confirm in vitro activation. Both the percentage of viable cells and the level of expression of CD69 per viable cell (measured as the mean fluorescence intensity, MFI) decreased after a 20-h incubation. By contrast, when cells were stimulated with 230 μg/mL, the percentage of viable cells CD40+ and the percentage of viable cells CD19+ decreased after a 20-h incubation but stabilized after ~68 h. On the other hand, the levels of expression of CD40 and CD19 per viable cell decreased after a 44-h incubation and stabilized after ~68 h (Figure 1B).

Effect of Preincubation with LPS in the Induced Expression of CD69, CD40, and CD19 Surface Antigens by Intact Anti-mIgG. This experiment was undertaken to determine whether preincubation of cells with LPS enhanced their responsiveness to anti-mIgG stimulation. Cells (1×10^5 cells/mL) were incubated in T-75 culture flasks with 5 μg/mL of LPS for 48 h and were then stimulated with various concentrations of anti-mIgG as has been described above. As shown in Figure 2, cells preincubated with LPS for 48 h were more responsive to anti-mIgG stimulation. The effect of preincubation with LPS on cells was greater in the level of expression of surface antigens per cell (MFI) than in the percentage of cells that were positive for surface molecules of interest.

This difference in the expression of surface antigens between non-preincubated cells and preincubated with LPS cells was more evident when cells were stimulated with higher concentrations of anti-mIgG. The effect of preincubation with LPS declined over 48h of incubation with anti-mIgG (data not shown).

Quantification of Cell Number Increase. Radiolabeled thymidine-incorporation data provide a rough approximation of

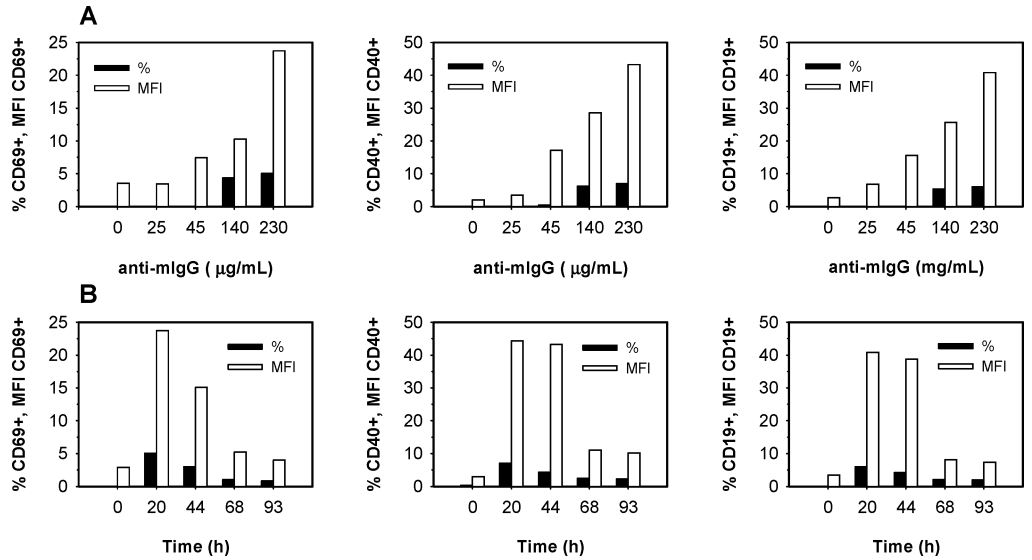

Figure 1. Intact anti-mIgG induces expression of CD69, CD40, and CD19 surface molecules. (A) Dose-dependent effects of sIgG-induced up-regulation of CD69, CD40, and CD19 by intact anti-mIgG. Cells were stimulated with increasing amounts of intact anti-mIgG. Data represent the mean fluorescence intensity (MFI) and percentage of viable cell $CD69^+$, $CD40^+$, and $CD19^+$, after 1 day of culture. (B) Time course of CD69, CD40, and CD19 expression by 55-6 cells treated with intact anti-mIgG (230 μg/mL). Similar effects were observed with lower concentrations (data not shown).

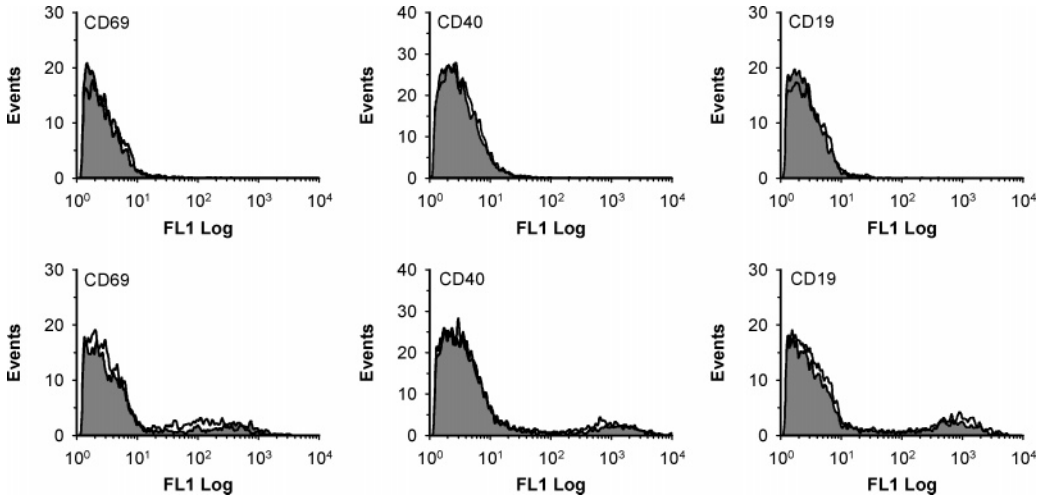

Figure 2. Effect of preincubation with LPS on the induced expression of CD69, CD40, and CD19 molecules by intact anti-mIgG. 55-6 cells (5×10^4 cells/mL), non-preincubated (shaded histograms) and preincubated (solid line histograms) with LPS for 48 h, were cultured in medium alone (top) and stimulated with 230 μg/mL intact anti-mIgG (bottom). After 1 day of culture, B cell hybridomas were analyzed by flow cytometry for surface expression of CD69, CD40, and CD19. All in vitro activation assays are representative of at least three independent experiments. Only live cells were analyzed, as dead cells were excluded by propidium iodide staining.

the overall DNA-synthesizing capacity of a cell population but offer no information regarding the progression of cells into cell division and mitosis. Furthermore, from the results obtained from thymidine incorporation, one cannot determine whether the thymidine incorporation reflects small numbers of cells that are rapidly dividing or large numbers of cells that are slowly dividing. To adress these possibilities, the effect of anti-mIgG on the change in overall cell number was determined by direct cell counts following mitogenic stimulation and staining with trypan blue twice a day for 4 days. The number of viable cells after a period of culture divided by the number of viable cells at the initiation of culture provides the fold increase in cell number. This increase is the composite result of cell generation and cell death.

As shown in Figure 3, the maximum specific growth rate (μ_{max}) did not increase after stimulation with various concentrations of anti-mIgG (NPAIgG) compared to control culture. However, cells preincubated with LPS for 48 h before stimulation with anti-mIgG (PAIgG) showed a significantly higher μ_{max} than was obtained for both cells not preincubated with LPS (NPAIgG) and control culture.

Effects of Intact Anti-MouseIgG on B Cell Hybridomas Proliferation. To examine the direct effect of anti-mIgG on the fraction of cells undergoing proliferation, cells (2×10^6-cells) stimulated with various concentrations of intact anti-mIgG (non-preincubated and preincubated with LPS for 48 h) were fixed with 70% ethanol and stained with PI to measure (by subsequent flow cytometry) cellular DNA content.

The percentage of cells in G1, S, and G2/M phases was not increased after stimulation with various concentrations of anti-mIgG (neither non-preincubated nor cells preincubated with LPS) compared with the control culture (unstimulated cells) (data not shown). However, as shown in Figure 4, the fraction of cells undergoing proliferation at 48 and 70 h after treatment with the intact antibody was higher for cells preincubated with LPS than for non-preincubated cells.

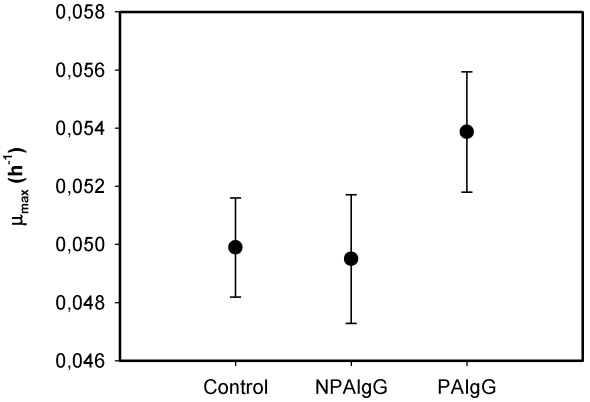

Figure 3. Cells cultured in medium with LPS 48 h before activation with intact anti-mIgG (PAIgG) show higher μ_{max} than were obtained for both control culture and cells not preincubated with LPS prior to anti-mIgG treatment (NPAIgG). The experimental points are the average values obtained in each series. A one-way ANOVA applied to data decomposed the variance into two components: a between-group component and a within-group component. The *p*-value of the *F*-test was less than 0.05, and therefore there is a statistically significant difference between the mean of μ_{max} from PAIgG to both NPAIgG and control at the 95.0% confidence level. Means and intervals around each mean are shown. The intervals displayed are based on Fisher's least significant difference (LSD) procedure.

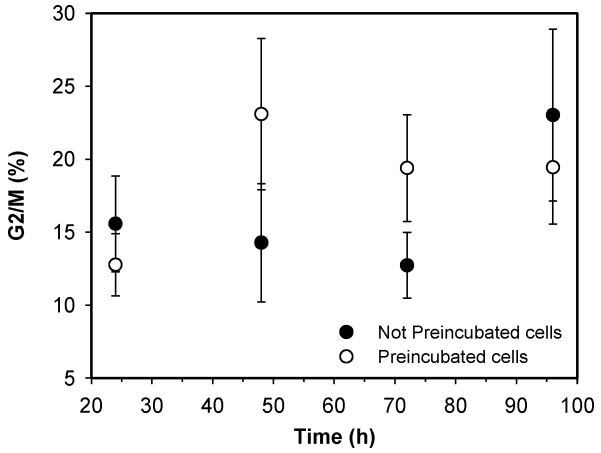

Figure 4. A fraction of the cells preincubated with LPS 48 h before stimulation with intact anti-mIgG has undergone more cell divisions than that of non-preincubated cells at 48 and 70 h of culture. The experimental points are the average values ± SD obtained for various concentrations of intact anti-mIgG in each series. Clumps of cells were eliminated displaying a cytogram of the peak versus the area of the DNA signal

Variation of Surface CD40 Content During the Cell Cycle. The values of the surface CD40 fluorescence as a function of the cell cycle position were obtained by dividing the histogramas of green fluorescence and red fluorescence into adjacent intervals along the *x*-axis of approximately equal width. For each sample, 10,000 cells for green fluorescence and 20,000 cells for red fluorescence were analyzed, and each interval contained at least 2,500 cells, except for the borders of the histograma. The mean of the green fluorescence was determined for each interval and plotted versus the mean red fluorescence of the interval. As can be seen from Figure 5, the mean of the green fluorescence reached the maximum value during the first part of the cell cycle: cells with a relative DNA content of 131 (corresponding to the position of the G1 peak of the DNA distribution) had a green fluorescence mean of about 429. After this maximum value, the mean green fluorescence decreased for cells with DNA contents corresponding to positions of the S and G2/M

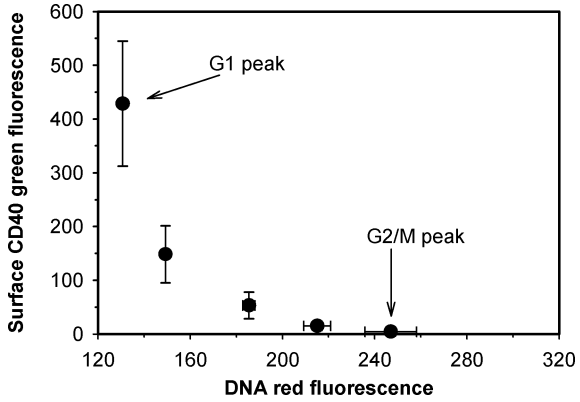

Figure 5. Evolution of the surface CD40 green fluorescence as a function of the cycle position for the samples taken after 24-h cultivation stimulated with 230 μg/mL intact anti-mIgG. The data are displayed as the mean values ± SD obtained for either non-preincubated cells or cells preincubated with LPS.

peaks of the DNA distribution. Similar results were observed for surface CD69 and CD19 green fluorescence (data not shown).

Effects of Intact Anti-MouseIgG on IgG2a Titers. To examine the direct effect of anti-mIgG on IgG2a production by hybridoma cells, the IgG2a titer was determined by sandwich-type ELISA. As shown in Figure 6, specific IgG2a production rate of cells preincubated with LPS prior to anti-mIgG treatment (PAIgG) was significantly higher than was obtained in unstimulated cells (control). Cells only preincubated with LPS (PreLPS) showed a similar production as unstimulated cells (data not shown).

On the other hand, Figure 6 shows the relationship between the specific IgG2a production rate and the cell surface staining with FITC-conjugated anti-IgG antibodies as parameter for sIgG after stimulation with various concentrations of anti-mIgG. Both the specific IgG2a production rate and the surface IgG fluorescence followed the same trend. Initially, they increased to their maximum values after ~1 day of culture. Thereafter they decreased continuously (Figure 6). The specific IgG2a secretion rates shown in Figure 6 have to be considered as apparent rates, due to the presumable capture of IgG2a by anti-mIgG in the medium.

Moreover, the values of the surface IgG fluorescence as a function of the cell cycle position were obtained as described above for CD40 molecules. The experimental data demostrated that surface IgG fluorescence was highest during the G1 cell cycle phase (Figure 7).

Discussion

The experiments described in this study demonstrate the effects of intact anti-mIgG in both the expression of CD69, CD40, and CD19 surface antigens and mAb production rate on the B cell hybridoma line 55-6. These hybrids do not express these molecules constitutively on the cell membrane, as was observed by flow cytofluorometry analyses after staining the cells with mAbs FITC conjugated to defined antigens.

As shown in Figure 1, 55-6 cells could be induced to express CD69, CD40, and CD19 surface molecules after treatment with anti-mIgG, and this effect was increased when cells were preincubated with LPS for 48 h before treatment with the intact antibody (Figure 2). On the other hand, anti-mIgG could not produce significant changes either in growth kinetics or in the fraction of cells undergoing proliferation with respect to cells not stimulated (control culture). In contrast, preincubation of 55-6 cells with LPS for 48 h prior to anti-mIgG treatment had

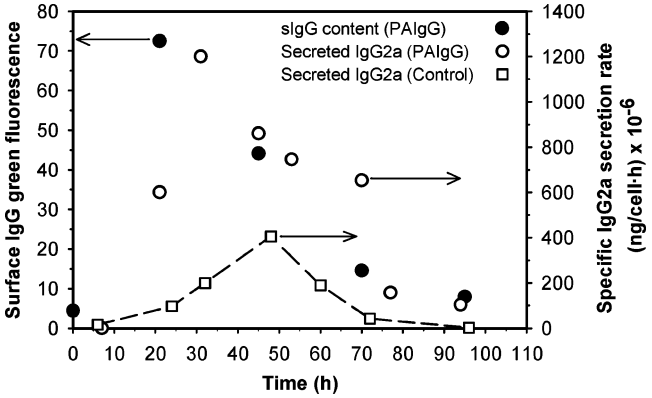

Figure 6. Evolution of the specific IgG2a secretion rate and the mean of the surface IgG green fluorescence during the culture of 55-6 cells. The relationship between surface IgG and secreted IgG2a was analyzed for B cell hybridomas stimulated with higher concentrations of anti-mIgG. Represented data correspond to the values obtained on 55-6 cells preincubated with LPS and activated with 230 μg/mL of anti-mIgG (PAIgG). This positive correlation was observed for the remainder of the concentrations. For surface IgG green fluorescence dead cells were excluded by propidium iodide staining. For comparison purposes, specific IgG2a secretion rate of unstimulated cells (control culture) is shown. Values are shown as the average for triplicate cultures ± SD.

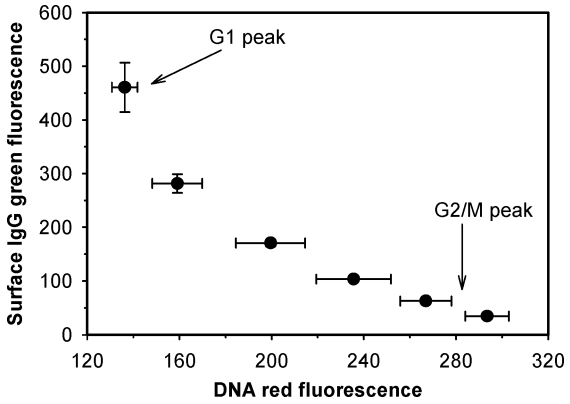

Figure 7. Variation of the surface IgG fluorescence during the cell cycle for the samples taken at 24 h in cultures stimulated with 230 μg/mL intact anti-mIgG. The relative DNA content of 137 corresponds to the G1 peak and 267 to the G2/M peak. Data are displayed as mean ± SD of triplicate cultures.

a positive effect in both the μ_{max} and proliferation in comparison with the values obtained for cells not preincubated with LPS (Figure 3 and 4). The specific IgG2a production rate on cells preincubated with LPS prior to anti-mIgG treatment showed significantly higher values than were obtained in control cultures (Figure 6).

These results suggest that the studied B cell hybridoma may possess some component of genes from normal B cells that can be induced to express when stimulated with anti-mIgG. In addition, preincubation of cells with LPS prior to anti-mIgG treatment enhanced their responsiveness to anti-mIgG stimulation. Cell activation was caused mainly by the presence of anti-mIgG in the culture medium, since cells preincubated with LPS but not stimulated with anti-mIgG (PreLPS) enhanced significantly neither the μ_{max} nor specific IgG2a production rate with respect to unstimulated cells (control).

The expression of CD69, CD40, and CD19 surface molecules reached the maximum value during the first part of the cell cycle, corresponding to the position of the G1 peak of the DNA distribution (Figure 5). The progress through the cell cycle may influence the ability of a cell to synthesize individual proteins, such as cell surface proteins, that are not directly involved in cycle events (*18*). The knowledge of whether CD40 expression reflects cell-cycle-dependent gene expression is an essential element, because it may allow a significantly higher expression of CD40 by control of cell cycle events.

Previous experiments with hybridoma cell cultures have indicated that the average cellular antibody content associated with the cell surface, as detected with immunofluorescent techniques, is correlated with the average specific rate of antibody secretion of the hybridoma culture (*19*−*22*). Moreover, it is believed that antibody synthesis and secretion is modulated throughout the cell cycle (*18*, *21*, *23*−*27*). Figure 6 clearly shows a good correlation between secreted and sIgG for 55-6 cells stimulated with anti-mIgG.

Determination of the pattern of the surface IgG fluorescence as a function of cell cycle position can then indicate the pattern of the specific antibody secretion rate as a function of the cell cycle position. The experimental data of the surface IgG fluorescence as a function of the cell cycle position demostrated that surface IgG fluorescence was highest during the G1 cell cycle phase and were in agreement with the predicted secretion pattern during the cell cycle (Figure 7).

Although this study focused on 55-6 cell line, the protocols may find applicability with other hybridomas not constitutively expressing CD40 or CD19. Moreover, analysis of CD40−CD40L interactions to increase cell proliferation and mAbs productivity can be applicabled to other hybridoma cell lines since previous studies on B lymphocytes and hybridoma cells have shown that CD40−CD40L interactions play a critical role in B cell activation, proliferation, and differentiation into Ig-secreting plasma cells (*28*−*32*).

Conclusions

On the basis of studies discussed above, the following specific observations can be made:

1. Intact anti-mIgG enhances CD69, CD40, and CD19 expression on 55-6 cells. The effect of sIgG ligation on hybridoma activation is dose-dependent and increases when cells are preincubated with LPS for 48 h prior to anti-mIgG treatment.

2. Preincubation of cells with LPS prior to anti-mIgG treatment enhances significantly both the μ_{max} and the percentage of cells undergoing proliferation in comparison with non-preincubated cells. These cells show a specific IgG2a production rate significantly higher than that obtained in control cultures.

4. The expression of CD69, CD40, and CD19 reaches the maximum value during the first part of the cell cycle, corresponding to the position of the G1 peak of the DNA distribution.

5. A positive correlation exists between the antibody production rate and the amount of IgG that is detectable on the cell surface.

6. Surface IgG fluorescence is highest during G1 cell cycle phase, and thus cells probably have a higher specific antibody secretion rate at this point of the cell cycle.

In view of the presented results and in the search for ways to obtain increased production of monoclonal antibodies (IgG2a), B cell hybridomas should be synchronized in G1 phase before stimulation with anti-mIgG. Fluorescence-activated cell sorting will be very useful for the isolation of cell variants with higher CD40 and sIgG expression ability.

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