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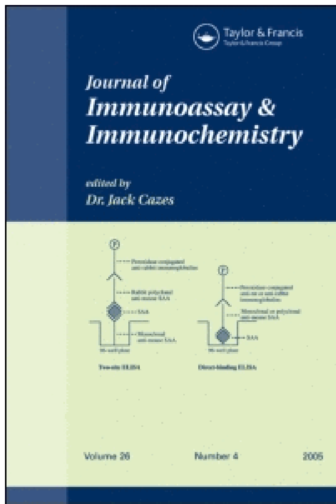

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
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Lauriane Padet[ab]; Isabelle St-Amour[a]; Eric Aubin[ab]; Dominic Paquin Proulx[ab]; Renée Bazin[ab]; Réal Lemieux[ab]
[a] Department of Research and Development, Héma-Québec, Québec, QC, Canada [b] Department of Biochemistry and Microbiology, Laval University, Québec, QC, Canada

To cite this Article Padet, Lauriane , St-Amour, Isabelle , Aubin, Eric , Proulx, Dominic Paquin , Bazin, Renée and Lemieux, Réal(2009) 'Dose-Dependent Inhibition of BrdU Detection in the Cell Proliferation ELISA by Culture Medium Proteins', Journal of Immunoassay and Immunochemistry, 30: 3, 348 — 357
To link to this Article: DOI: 10.1080/15321810903084863
URL: http://dx.doi.org/10.1080/15321810903084863

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Journal of Immunoassay and Immunochemistry®, 30: 348–357, 2009
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ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810903084863

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Dose-Dependent Inhibition of BrdU Detection in the Cell Proliferation ELISA by Culture Medium Proteins

Lauriane Padet,[1,2] Isabelle St-Amour,[1] Eric Aubin,[1,2] Dominic Paquin Proulx,[1,2] Renée Bazin,[1,2] and Réal Lemieux[1,2]

[1]Department of Research and Development, Héma-Québec, Québec, QC, Canada

[2]Department of Biochemistry and Microbiology, Laval University, Québec, QC, Canada

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Abstract: Determination of the proliferation rate of cultured mammalian cells is widely done using incorporation of 5-bromo-2-deoxyuridine into replicating DNA followed by quantitative detection in ELISA using a specific monoclonal antibody. However, we noted that the BrdU ELISA results did not correlate with viable cell counts when increasing concentrations of proteins were added to test their effects on proliferating cells. This observation suggested that proteins could interfere with BrdU incorporation or detection in the commercial BrdU ELISA used. We show here that the presence of exogenous proteins during cell fixation and DNA denaturation significantly inhibited BrdU detection presumably by coating the extracted DNA by a concentration-dependent protein film. A simple modification to the manufacturer's protocol (cell washing) permitted to avoid this interference and resulted in a significant increase of the assay sensitivity.

Keywords: 5-Bromo-2-deoxyuridine, BrdU, Cell proliferation, ELISA, Washing step

Address correspondence to Renée Bazin, PhD, Department of R&D, Héma-Québec, 1070 Avenue des Sciences-de-la-Vie, Québec (Qc), Canada, G1V 5C3. E-mail: renee.bazin@hema-quebec.qc.ca

INTRODUCTION

Determination of the proliferation rate of cultured mammalian cells is widely used in many laboratories to evaluate cell proliferation. This has traditionally been done by measuring the incorporation of tritiated thymidine into replicating DNA using scintillation counting of cells collected by filtration. Gratzner et al.[1] described a radioactivity-free alternative method in which a thymidine analog, 5-bromo-2-deoxyuridine (BrdU), is incorporated into replicating DNA followed by quantitative detection of incorporated BrdU by a specific monoclonal antibody in ELISA.[2,3] The BrdU ELISA was shown to be at least as sensitive as the radioactive method[4–7] without toxic hazards associated with the use of radiochemicals.

In subsequent years, significant improvements to the protocol assay permitted to improve the sensitivity of the BrdU ELISA and allowed this immunological assay to be done in standard solid-state ELISA format following the fixation of denatured DNA to the bottom of 96-well microplates.[5,8,9] BrdU ELISA assay kits with optimized reagents and protocols are now commercially available thus further facilitating the routine use of the method.

The immunologic detection of incorporated BrdU in denatured DNA is expected to be more sensitive to interfering agents than the measurement of radioactive thymidine by scintillation counting. In experiments designed to study the effects of therapeutic human intravenous immunoglobulins (IVIg) on the proliferation of cultured cells, we repeatedly noted that the BrdU results did not correlate with the counted cell numbers when different amounts of proteins were added to the culture medium. This observation suggested that proteins could interfere with the BrdU incorporation or immunologic detection in the commercial assay used. This effect was further studied and we obtained evidence that the addition of tested proteins to the culture medium inhibited the immunologic detection of BrdU presumably by protein coating of the extracted DNA. A simple modification to the manufacturer's protocol (cell washing) permitted to prevent this interference and further enhanced the sensitivity of the assay.

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EXPERIMENTAL

Chemicals and Reagents

RPMI 1640 culture medium and fetal bovine serum (FBS) were obtained from Gibco, (Grand Island, NY) and carboxyfluorescein (CFSE) from Invitrogen Canada Inc. (Burlington, Canada). Human serum albumin

(HSA) and IVIg (Gamunex) were obtained from Talecris Biotherapeutics (Research Triangle Park, NC). The BrdU ELISA assay Kit was purchased from Roche Diagnostics (Laval, Canada).

Preparation of Protein Additives

FBS, HSA, and IVIg were dialyzed in a Spectra/Por dialysis membrane (MWCO: 6-8000) (Spectrum laboratories, Rancho Dominguez, CA) for 4 h against 1 L of RPMI medium followed by sterile filtration through a PES membrane (0.22 μm) (Millipore corporation, Bedford, MA) and storage at 4°C. Protein concentrations were determined by spectrophotometry (NanoDrop 1000, Nanodrop Technologies Inc., Wilmington, DE).

Cell Culture

The Jurkat cell line was obtained from American Type Culture Collection (Rockville, MD) and was mycoplasma free. Cells were washed with Dulbecco's phosphate-buffered-saline (PBS) (10 mM Na/KPO_4, 136 mM NaCl, pH 7.4) supplemented with 2 g/L of glucose (PBS-Glucose). Jurkat cells were cultured in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 5% FBS and increasing dialyzed FBS concentrations as indicated. Cells were seeded at 2×10^5 cells/ml in a 24-well microplate (Corning Inc., Corning, NY) in a final volume of 1 mL/well and cultured at 37°C in a humidified atmosphere containing 10% CO_2 for 3 days. Viable cell counts were determined by trypan blue dye exclusion using an hemacytometer.

BrdU Immunoassay

The commercial BrdU ELISA was done as described in the manufacturer's protocol. Briefly, after 3 days of culture, 100 μL/well of suspended cells were seeded in triplicate in 96-well microplate followed by addition of 10 μL of the kit BrdU labelling solution. After 4 h at 37°C, microplates were centrifuged (Allegra™ X-12 Centrifuge, Beckman Coulter, Fullerton, CA) at 300 g for 10 min and the labelling medium was removed by flicking off the liquid. Cells were dried at 60°C for 60 min followed by a 30 min incubation at room temperature with 100 μL/well of the Fixation and Denaturation solution provided by the manufacturer. The plates were washed three times after addition of 100 μL/well of the peroxidase-anti-BrdU conjugate solution and incubation at room temperature for 60 min. After washing, the HRP substrate (100 μL/well) was added and the enzymatic reaction was stopped after about 20 min by addition of

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100 μL/well of H_2SO_4 (1 M). The optical densities (O.D.) were read using a microplate reader (MRX, Dynatech, Chantilly, VA) at 450 nm.

In the modified BrdU ELISA, a washing step was added just after BrdU incorporation. After 3 days of culture, the kit BrdU labelling solution (100 μL/well) was added. After 4 h at 37°C, cells were harvested, washed in 15 mL tubes (Becton Dickinson, Oakville, Canada) with PBS-Glucose and resuspended in 1 mL of RPMI 1640 medium. After washing, cells were seeded (100 μL/well) in triplicate in a 96-well microplate and centrifugated at 300 g for 10 min. The remaining steps were performed as described for the commercial BrdU ELISA assay protocol (see above).

CFSE Labelling

Cells were incubated for 15 min at 37°C in presence of 2 μM CSFE followed by 2 washes with PBS-Glucose. Cells were resuspended in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 5% FBS and increasing dialyzed FBS concentrations and cultured at 37°C in a humid atmosphere containing 10% CO_2. The analysis of CSFE-labelled cells was done by flow cytometry (FACScalibur, BD Biosciences, Mississauga, Canada) after 4 h and 3 days of culture and the proliferation indexes were calculated from the CSFE dilution using the Modfit software (Verity Software House, Topsham, ME).

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RESULTS AND DISCUSSION

Effect of Increasing Concentrations of FBS in Culture Medium on Cell Proliferation

In order to determine whether increased FBS concentrations could influence cell proliferation, we cultured Jurkat cells in RPMI containing 5% FBS supplemented with increasing concentrations of dialyzed FBS (0 to 45%). Cell proliferation was determined either by cell counting, CFSE labelling or using the commercial BrdU ELISA (Fig. 1). Total cell counts and CFSE labelling yielded similar results for the different conditions tested and revealed the absence of effect of supplementary FBS added to culture medium on Jurkat cell proliferation, even at high concentrations. In contrast, the commercial BrdU ELISA resulted in a decreased BrdU signal (optical density; O.D.) normally associated with inhibition of cell proliferation. The decrease in BrdU signal was proportional to the added concentration of FBS, suggesting that FBS interfered with the BrdU ELISA either by preventing BrdU incorporation into newly synthesized DNA or BrdU detection by BrdU-specific antibodies.

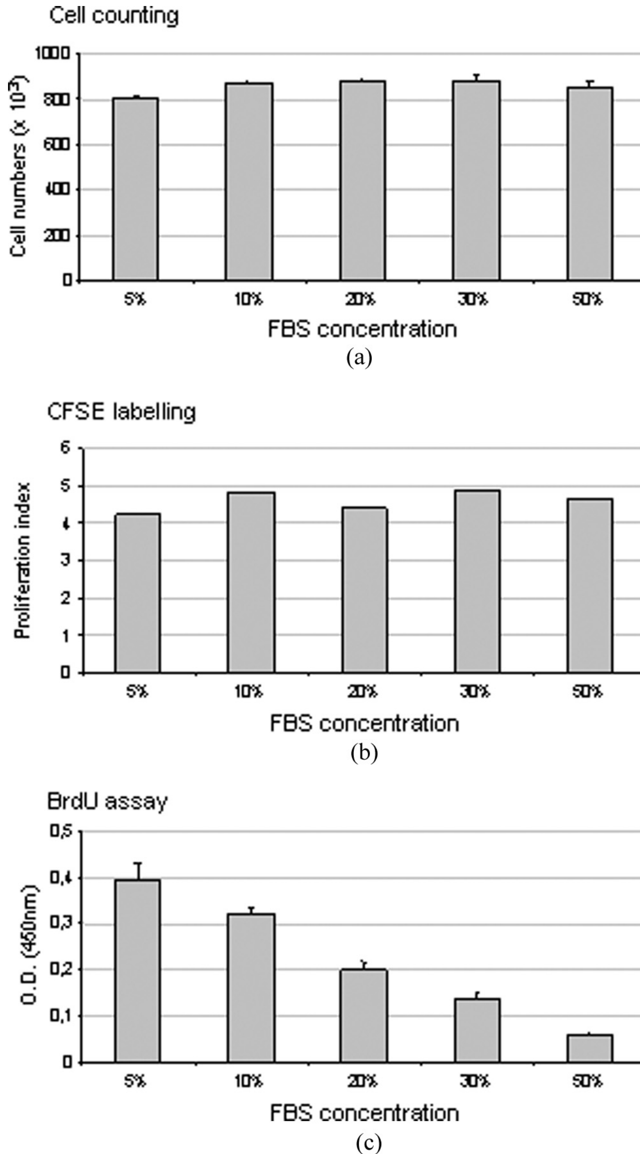

Figure 1. Evaluation of Jurkat cell proliferation in presence of increasing concentrations of FBS. Jurkat cells were cultured for 3 days in RPMI 1640 medium containing 5% FBS and supplemented with dialyzed FBS to increase protein concentration (up to 45% FBS added). Proliferation was evaluated using three different methods: (a) total cell numbers were determined by trypan blue exclusion counting on an hemacytometer; (b) proliferation indexes were evaluated by CFSE dilution using the Modfit software; (c) DNA synthesis was determined using a commercial BrdU ELISA assay kit.

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Effect of Modifying FBS Concentration After Cell Culture and BrdU Incorporation

Jurkat cells were cultured in RPMI supplemented with 20% FBS for 3 days. After BrdU incorporation, FBS (to increase protein contents to 40% serum equivalent) or RPMI (to dilute protein contents to 13.3%) was added at the cultures to evaluate whether FBS prevented BrdU detection. As shown in Fig. 2, addition of FBS considerably reduced BrdU detection by at least 10-fold whereas addition of RPMI increased O.D. by almost 2-fold. Because BrdU incorporation was equivalent in all conditions since it was done prior to addition or dilution of proteins, we concluded that proteins interfered with BrdU detection by anti-BrdU antibodies.

Effect of Addition of Washing Steps After BrdU Incorporation

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Jurkat cells were cultured as described in Fig. 1 and after BrdU incorporation, the commercial BrdU ELISA assay protocol was modified to add one or two washing steps prior to cell drying to confirm that proteins interfered with the immunological detection of incorporated BrdU (Fig. 3). These results clearly showed that the O.D. obtained in the BrdU ELISA were much higher with a single washing step (about 2 to 6-fold for normal FBS concentrations (5 to 20% FBS) and up to 20-fold at 50% FBS). The second washing step did not permit to increase further the

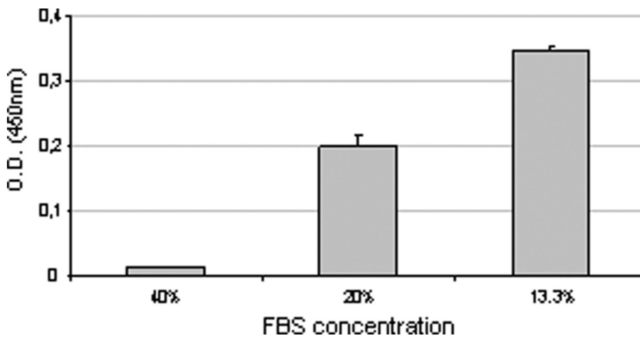

Figure 2. Variation of protein concentration following BrdU incorporation impaired BrdU detection. Jurkat cells were cultured in RPMI supplemented with 20% FBS and BrdU was added for the last 4 h of culture. Following incorporation, protein contents was increased to 40% by adding 40 μl of FBS, left unchanged (addition of 40 μl of RPMI containing 20% FBS) or reduced to 13.3% by adding 40 μl of RPMI without FBS. The cells were then heat-treated and fixed, and the presence of BrdU was detected with anti-BrdU antibodies.

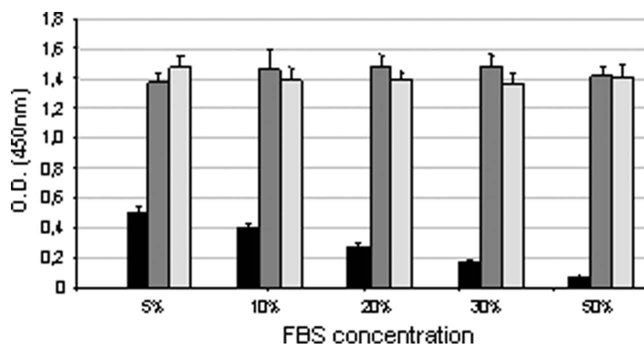

Figure 3. Addition of a single washing step after BrdU incorporation significantly increased BrdU detection. Jurkat cells were cultured for 3 days in RPMI supplemented with 5 to 50% FBS (as in Fig. 1). After a 4 h BrdU incorporation, cells were: not washed (black bars), washed once and resuspended in RPMI only (dark grey bars), washed twice and resuspended in RPMI only (light grey bars). Cells were then heat-treated and fixed, and the presence of BrdU was detected with anti-BrdU antibodies.

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BrdU signal and was, therefore, not necessary. When proliferation of Jurkat cells in culture medium containing increasing amounts of FBS was evaluated with the BrdU ELISA that included a washing step, the results obtained were similar to those obtained previously with cell counting and CFSE labelling, and permitted to conclude that Jurkat cell proliferation was not affected by increased FBS concentrations. Previous studies using a similar BrdU ELISA reported the use a washing step with PBS or RPMI after BrdU incorporation. Magaud et al.[5] suggested that this washing step permitted to reduce the protein concentration of the medium. However, the positive impact of protein dilution on the results of BrdU ELISA was never demonstrated. In the present study, we showed that addition of a single washing step prior to cell drying resulted in a significant performance improvement since it increased the sensitivity (O.D./cell number) of the BrdU ELISA. The observations done in the present study on Jurkat cells were confirmed using cell types such as the DB cell line and human PBMC (data not shown).

Since 1982, several studies reported modifications to DNA denaturation method to improve the sensitivity of BrdU ELISA. Thermal denaturation, as used in the present study, is not universally applicable for all types of cells[10] and, thus, other denaturation procedures have been proposed. For example, it was demonstrated that proteolytic enzyme (pepsin) digestion simultaneous to acid denaturation allowed reduction of background binding and increased sensitivity.[11,12] Furthermore, Muir et al.[9] reported that trypsin treatment increased BrdU-DNA

immunolabelling. These observations showing that protein digestion improved BrdU detection are in agreement with the results of the present study.

Effect of a Very Low FBS Concentration on BrdU Detection

Jurkat cells were cultured in RPMI supplemented with 5% FBS and after BrdU incorporation, cells were washed and resuspended in RPMI 1640 medium supplemented with increasing FBS concentrations (0 to 5%) in order to evaluate if very low protein concentrations could interfere with BrdU detection. Results showed that BrdU detection was decreased at least 2-fold between 0% and 5% FBS concentrations (O.D. of 1.40 and 0.58, respectively). The decrease in BrdU signal was proportional to FBS concentration and could already be observed at 1% FBS (data not shown). Therefore, although inhibition was more pronounced at high FBS concentrations, FBS concentrations currently used for cell culture (5 to 20%) were also inhibitory. Addition of a washing step to remove proteins prior to cell drying was thus necessary to improve the performance of the commercial BrdU ELISA, even for cells cultured at low FBS concentrations.

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Effect of HSA and IVIg Addition on BrdU Detection

In order to emphasize that the phenomenon of inhibition of BrdU detection described above was not restricted to FBS, Jurkat cells were cultured in RPMI supplemented with 5% FBS and after BrdU incorporation, cells were washed and resuspended in RPMI 1640 medium with or without various proteins (FBS, HSA or IVIg) at a final concentration of 20 mg/ml. Results showed that the O.D. obtained in presence of HSA or IVIg were significantly reduced as previously observed for FBS (data not shown), confirming the general inhibitory effect of proteins on BrdU detection.

CONCLUSIONS

The mechanism by which proteins interfere with BrdU detection is still speculative but is likely to be due to inhibition of access of BrdU-specific antibodies to DNA following formation of a protein coat on the surface of denatured cells. The findings presented in this work applied to both adherent and suspended cell cultures. When large numbers of conditions need to be tested, cells can be directly labelled with BrdU and washed in 96-well microplates. While washing of adherent cells in microplates is

easily done, efficient washing of non-adherent cells by centrifugation of culture microplates is more tricky due to the limited volume of washing solution per well. However satisfactory results were obtained by doing at least two successive washes [5] instead of the single step used for cells washed in 15 ml tubes as described in the present study.

ABBREVIATIONS

BrdU, 5-bromo-2-deoxyuridine; IVIg, intravenous immunoglobulins; HSA, human serum albumin; CFSE, carboxyfluorescein.

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Received November 10, 2008
Accepted February 8, 2009
Manuscript 3325

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