A comparative study of colorimetric cell proliferation assays in immune cells

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Abstract Cell proliferation assays are basic and essential techniques for assessing cellular function. Various colorimetric assays, such as MTT-, WST-1-, and resazurin-based assays, are available; however, studies directly comparing the suitability of each method for immune cell proliferation are scarce. Thus, we aimed to determine the best reagent and its optimal conditions based on variables such as cell number range, stimulation dose, kinetics, and compatibility with the cell division assay using CFSE fluorescence dye which is able to directly monitor divided cells by flow cytometry. In the absence of stimulation, MTT solubilized with SDS (MTT-SDS) and resazurin appeared to accurately reflect the cell numbers in a linear fashion. On the other hand, WST-1 exhibited a higher stimulation index following strong stimulation, whereas MTT-SDS and resazurin exhibited a better sensitivity to weak stimulation. A longer duration for stimulation did not necessarily increase sensitivity. CFSE staining revealed incremental cell division in response to anti-CD3 antibody stimulation in a dose-dependent manner. The cell numbers indirectly estimated from cell division profiles were consistent with the dose–response curve in the absorbance of MTT-SDS and resazurin. The absorbance does not increase before cell division,

irrespective of T cell activation status, suggesting that these reagents reflect the cell number but not the cellular volume. Collectively, resazurin and MTT-SDS seem to be more reliable than others, and thus appear applicable in various conditions for the immune cell experiments.

Keywords MTT · WST-1 · Resazurin · T cell

Abbreviations

Ab	Antibody
CFSE	Carboxyfluorescein diacetate succinimidyl ester
FSC	Forward scatter
SSC	Side scatter
NADPH	Nicotinamide-adenine dinucleotide phosphate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
WST-1	Water-soluble tetrazolium salts or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
Resazurin	7-Hydroxy-3H-phenoxazin-3-one 10-oxide
SDS	Sodium dodecyl sulfate
PES	Phenazine ethosulfate
DMF	Dimethylformamide

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NP-40	Nonidet P-40
TCR	T-cell receptor
BCR	B-cell receptor
MHC	Major histocompatibility complex
PBS	Phosphate buffered saline
FCS	Fetal calf serum

Introduction

The human immune system functions through dendritic cell phagocytosis of pathogenic microbes followed by dendritic cell migration to a draining lymph node and the presentation of digested antigens on major histocompatibility complex (MHC) to T cells (Valitutti et al. 1995; Viola and Lanzavecchia 1996). Subsequently, T cells massively expand and undergo differentiation into effector T cells to exclude pathogens (Chen et al. 2002). The clonal expansion of T cells is the most fundamental and important process in immune responses to foreign antigens. Upon pathogen clearance, the expanded T-cell population declines by apoptosis mainly because of the exhaustion of cytokines and growth or survival factors (Koyanagi et al. 2007). The memory T cells reside in the peripheral tissues or lymph nodes and await a recall response to the same antigen in future (Sallusto et al. 1999). Thus, T cell is a key player in the immune system, and monitoring T cell expansion enables us to know magnitude and/or quality of immune responses.

Cell proliferation assays are basic experimental methods used to assess important cell functions in a wide variety of cell types. Till date, numerous methods have already been developed and utilized. In particular, the use of radioisotopes such as [³H] thymidine is very popular. Thymidine is a component of genomic DNA; thus, radioactive thymidine is incorporated into replicating DNA during cell division, thereby reflecting cell proliferation (Quent et al. 2010). This method is very simple, easy, and sensitive; however, the generation of radioactive waste restricts its use. Non-radioactive reagents, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS), water-soluble Tetrazolium salts (WST-1), and resazurin (also known as AlamarBlue), are preferred because of their lack of restrictions and are therefore commercially

available and used to assess cell viability, proliferation, and drug cytotoxicity (Riss et al. 2013).

MTT, in the form of a tetrazolium salt, is positively charged allowing it to readily penetrate cells. Within the cell, it is converted by the mitochondrial dehydrogenase into formazan. Formazan is water-insoluble; thus, it must be solubilized with acid and/or detergent prior to absorbance measurements (Mosmann 1983; Hamid et al. 2004; Twentyman and Luscombe 1987). WST-1 is a similar but negatively charged compound that is unable to penetrate into the cell. It is thus reduced outside the cell and its product, another type of formazan salt, is water-soluble (Tan and Berridge 2000). Resazurin is also water-soluble, and thus, a cell lysing process is not required (Hamid et al. 2004; Quent et al. 2010). In theory, WST-1 and resazurin can be continuously monitored over time; however, they are not suitable for it because of their cytotoxicity. All these assays are indirect methods of cell proliferation utilizing cellular enzyme activities rather than the direct estimation of proliferating cell numbers. A question therefore arises regarding the accuracy of indirect methods compared with direct ones in normal immune cells.

Herein, we compare the properties of MTT, WST-1, and resazurin that have been used in many high impact factor publications (Quent et al. 2010), and we aim to determine their suitability in immune response experiments using mouse spleen cells.

Materials and methods

Antibodies and reagents

MTT was purchased from Dojindo (Kumamoto, Japan) and WST-1 from Wako (Osaka, Japan). Resazurin and carboxyfluorescein diacetate succinimidyl ester (CFSE) were obtained from Sigma (St. Louis, MO, USA). Anti-mouse CD3 antibody (Ab) (145-2C11) was purchased from eBioscience (San Diego, CA, USA) and anti-mouse CD28 (37.51) from BD Biosciences (San Diego, CA, USA). RPMI-1640 medium was obtained from Wako (Osaka, Japan).

MTT, WST-1, and resazurin assays

MTT was dissolved in PBS to make a stock solution (5 mg/mL). Then, 10 µL (for SDS-containing buffer) or 20 µL (for NP-40 containing buffer) of the stock

solution was added to each well of a 96-well cell culture plate which had already contained cells in 100 μL of culture medium (final 0.45 or 0.83 $\mu\text{g/mL}$ of MTT, respectively) and incubated for 4 h at 37 °C in a CO_2 incubator. The cells converting MTT into formazan were solubilized with 100 μL of SDS-containing buffer (16 % SDS, 40 % DMF, and 1.2 % acetic acid) or 150 μL of NP-40 containing buffer (0.1 % NP-40 and 4 mM HCl), and the absorbance was read at 570 nm using a plate reader AD340 (Beckman Coulter, Brea, CA, USA). WST-1 was dissolved in phenazine ethosulfate (PES) solution (0.21 mg/mL PBS) to make a stock solution (2 mg/mL). Then, 20 μL of this stock solution was added into each well containing 100 μL of cell suspension (final 0.33 mg/mL of WST-1), and the absorbance was directly read at 450 nm. Resazurin was dissolved in PBS (stock solution, 0.15 mg/mL). Then, 20 μL of the stock solution was added into each well containing 100 μL of cell suspension (final 25 $\mu\text{g/mL}$ of resazurin), and the absorbance was directly read at 570 nm. The reference was read at 620 nm for all dyes.

Mice and cell preparation

BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained in our animal facility. The animal experiments in the present study were approved by the ethical review committee of Nippon Veterinary and Life Science University. The preparation for single spleen cell suspensions was as previously reported (Arimura et al. 2004, 2010). In brief, the spleen cells were prepared by squeezing the whole spleen with a slide glass in Hanks' solution (Life technologies, Carlsbad, CA, USA) with 2 % FCS. Then, the erythrocytes were depleted by cell lysis with Tris buffered ammonium chloride. The remaining cells were washed, transferred, and maintained in RPMI 1640 medium with 10 % FCS.

Cell number determination

To determine a cell number range, we seeded a serial number of the whole spleen cells. The cell numbers applied ranged from 5×10^4 to 1.6×10^6 cells per well, a range commonly used in immune response experiments. The cells were initially incubated for 1 h at 37 °C without any reagent. Then, MTT, WST-1, and resazurin were added and the cells were further incubated for 4 h. Then, absorbance was assessed.

Cell stimulation

For cell stimulation, the spleen cells were stimulated with soluble anti-CD3 Ab (final 0.01–1.0 $\mu\text{g/mL}$) and anti-CD28 Ab (final 0.25 $\mu\text{g/mL}$) for 1–4 days. Then, following reagent addition the cells were incubated for the last 4 h of the stimulation prior to the absorbance measurements.

Cell division assay with CFSE staining

The mouse spleen cells were stained with CFSE (final 10 μM) for 10 min at 37 °C, and more than three volumes of cold complete RPMI 1640 medium were then added to stop the staining reaction; the cells were then washed three times to remove excess CFSE. The stained spleen cells were subjected to stimulation as described above to monitor cell division. Then, the samples were fixed with 1 % paraformaldehyde in PBS and measured for CFSE fluorescence intensity using the FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) and analyzed using FlowJo software.

Estimation of the cell number from cell division number

On the basis of cell division numbers observed in the dilution assay of CFSE fluorescence intensity described above, the cell number following stimulation was calculated using the formula below:

$$\text{Current cell number} = \frac{\text{Initial cell number}}{\left(\frac{A}{2^0} + \frac{B}{2^1} + \frac{C}{2^2} + \frac{D}{2^3} + \dots\right)} \times 100 (\%)$$

A, B, C, and D indicate percentages of cells that underwent 0, 1, 2, and 3 cycles of cell division, respectively.

Results

Cell number range for MTT, WST-1, and resazurin

Initially, in order to assess the cell number capacity of each reagent, we conducted cell incorporation assays for the three substrate reagents, MTT, WST-1, and resazurin. Cells were initially incubated for 1 h without any reagent. Then, following reagent addition

the cells were further incubated for 4 h. For MTT, the cells were solubilized with SDS- or NP-40-containing buffer. For WST-1 and resazurin, the cells were directly subjected to a plate reader without cell solubilization. As shown in Fig. 1, the absorbance values of MTT solubilized with SDS (MTT-SDS) and resazurin proportionally increased to the cell number applied, whereas that of MTT solubilized with NP-40 (MTT-NP-40) showed only a marginal increase. WST-1 did not increase at all for 8×10^5 cells per well; however, there was a sudden steep increase at 1.6×10^6 cells per well in repeated experiments. MTT-SDS and resazurin collectively appeared to exactly reflect the cell numbers in a linear fashion. Therefore, 1×10^5 cells per well were used for the subsequent experiments.

Dose–response curve of MTT, WST-1, and resazurin

Next, using the mouse whole spleen cells, we compared the dose–response curve of MTT, WST-1, and resazurin with a serially diluted amount of anti-CD3 Ab ranging from 0.01 to 1.0 $\mu\text{g/mL}$ in the constant presence of 0.25 $\mu\text{g/mL}$ of anti-CD28 Ab costimulation for 2 days. Anti-CD3 and -CD28 Abs specifically bind T cell receptor (TCR)/CD3 complex and co-receptor CD28 expressed on T cells, and promote their proliferation (Riddell and Greenberg 1990; Harding et al. 1992; Arimura et al. 2002). In repeated experiments, MTT-SDS and resazurin showed a clear increase of the absorbance in response to an increased amount of anti-CD3 Ab concentration (Fig. 2). MTT-NP-40 again showed a mild increase as shown in Fig. 1. Unlike other reagents, WST-1 did not increase with anti-CD3 Ab of 0.01 $\mu\text{g/mL}$ concentration; however, it steeply increased with that of 0.1–1.0 $\mu\text{g/mL}$. These results indicate that WST-1 exhibits a higher stimulation index against strong stimulation, whereas MTT-SDS and resazurin exhibit a better sensitivity to weak stimulation.

Kinetics of responses to stimulation

Spleen cells were stimulated using anti-CD3 Ab (0.1 $\mu\text{g/mL}$) with anti-CD28 Ab (0.25 $\mu\text{g/mL}$) for 1–4 days. As indicated in Fig. 3, the highest absorbance for WST-1, MTT-NP-40, and resazurin was shown on day 2, indicating the optimal stimulation period in this

experimental setting. As for MTT-SDS, days 2 and 3 showed the highest absorbance at a comparable level. The absorbance on day 4 decreased in all reagents, indicating that the cells no longer proliferate. WST-1 exhibited very high absorbance only on day 2, indicating a markedly short reduction period. Taken collectively, a longer duration for stimulation did not necessarily increase sensitivity, and the optimal time frame for spleen T-cell receptor stimulation with anti-CD3 and -CD28 Abs was determined as 2 days.

Compatibility with CFSE dilution assay

To examine whether the absorbance indeed matches cell proliferation, the spleen cells were stained with CFSE fluorescence dye and stimulated with anti-CD3/CD28 Abs for 2–3 days. The cell division was directly monitored by CFSE fluorescence intensity dilution using a flow cytometer. In the absence of anti-CD3 Ab stimulation, one strong peak was observed, indicating that almost all cells were not divided (Fig. 4a). Upon stimulation with 0.1–1.0 $\mu\text{g/mL}$ of anti-CD3 Ab, more than half of the cells underwent 2–3 (day 2) or 2–4 (day 3) cycles of cell division (Fig. 4a, b). On the other hand, upon stimulation with 0.01 $\mu\text{g/mL}$ of anti-CD3 Ab, the cells divided to a much lesser extent. Subsequently, based on these cell division profiles, the cell number was indirectly estimated and a graph was drawn (Fig. 4c). Most importantly, a dose–response curve obtained from this estimated cell number seems consistent with an increase in the absorbance of MTT-SDS and resazurin in Fig. 2.

TCR stimulation does not raise absorbance before cell division

Since these substrate reagents are known to be reduced by metabolic enzymes whose activity and/or expression might be upregulated by cell activation prior to cell division, we tried to examine whether TCR stimulation directly affects the absorbance before cell division. The mouse splenocytes were stained with CFSE dye, followed by the stimulation with anti-CD3 and -CD28 Abs for only 24 h. Based on CFSE intensity assessed by a flow cytometer, cells had not yet divided at this stage (Fig. 5a, lower panels), and the alteration in absorbance assessed by a plate reader was only marginal (Fig. 5b). In addition, the absorbance of cells with CFSE staining was almost same as

Fig. 1 Cell number range for MTT, WST-1, and resazurin. **a** A serial number of mouse whole spleen cells were seeded into a 96-well plate in duplicates or triplicates, and they were incubated without stimulation in the absence of any reagent for the first 1 h at 37 °C. Three different reagents were then individually added, followed by incubation for the last 4 h at

37 °C. Each absorbance value was assessed by a plate reader. **b** The figures in **a** were normalized to the respective minimal values and combined into the same graph to compare their fold change. These are representative of at least three experiments with similar results

that of cells without CFSE staining (Fig. 5b); thus, CFSE staining did not interfere with the absorbance in all reagents examined. Forward scatter (FSC) reflects cell size and side scatter (SSC) reflects internal structures such as a nucleus and granules. In contrast to the CFSE assay and absorbance measurement above, FSC markedly increased (Fig. 5a, upper panels, and 5c), indicating that T cells became larger and activated. These results indicate that the absorbance

does not increase before cell division, irrespective of cytoplasmic volume and T-cell activation status.

Discussion

In the present study, we compared three different substrate reagents MTT, WST-1, and resazurin commonly used for cell viability and cell proliferation

Fig. 2 Dose-response curve of MTT, WST-1, and resazurin. **a** Mouse spleen cells were examined for the dose-response curve of MTT, WST-1, and resazurin against a serially diluted amount of anti-CD3 Ab (0.01, 0.1, and 1.0 µg/mL) in the constant presence of anti-CD28 Ab costimulation (0.25 µg/mL)

for 2 days. **b** The figures in **a** were normalized to the respective minimal values and combined into the same graph to compare their fold change. These are representative of six experiments with similar results

assays, to determine which reagent accurately reflects the cell number and is the most superior for cell proliferation assays of primary immune cells. The critical variables for cell proliferation assays include mitogen dose, reagent concentration, cell density (cell number per well), cell culture period, incubation time following reagent addition, and so on (Twentyman and Luscombe 1987). Further, the metabolic enzyme activity should also be considered for these substrate reagents because they may fluctuate along the cell cycle.

[³H] Thymidine is limitedly incorporated into DNA of proliferating cells, and it exhibits a steep increase in radioactivity in proliferating cells as compared with quiescent cells. MTT, WST-1, and resazurin are not incorporated into DNA, and their assays are indirect methods based on a particular metabolic enzyme activity that cells possess, which is supposed to roughly reflect the cell number. For instance, the cytosolic NADPH-oxidoreductases catalyze the conversion of MTT to insoluble formazan with the color of visible

Fig. 3 Kinetics of responses to stimulation. Mouse whole spleen cells were examined for the kinetic response of MTT, WST-1, and resazurin to stimulation using anti-CD3 Ab (0.5 $\mu\text{g/mL}$) in the presence of anti-CD28 Ab (0.25 $\mu\text{g/mL}$) for 1–4 days. These are representative of two experiments with similar results

wavelength. Herein, detergents such as SDS and NP-40 led to different results; thus, the careful setting of procedures is required. In addition, resazurin may be reduced by “diaphorase” enzymes, including dihydrolipoamide dehydrogenase, quinine oxidoreductase, and flavin reductase (Zalata et al. 1998). Differences in reducing enzymes could lead to different outcomes in the cell viability test for anti-cancer chemical compounds (Hamid et al. 2004). Further, it is possible that some diaphorases are differentially regulated in expression and activity. The details remain to be explored, and further experiments are required for particularly stimulated immune cells.

The metabolic enzyme activities for MTT, WST-1, and resazurin in immune cells may alter in response to activation induced by antigens as well as to the cell cycle because immune cells increase in size following activation. Usually, in experiments for immune response to antigen or cytokines, cells need to be stimulated by mitogens through antigen receptor (TCR or BCR), particular cytokines, or growth factors. The activation status or cell size may increase metabolic enzyme activity or potentially upregulate (or downregulate) protein expression level even before cell division. To confirm such an effect, we stimulated spleen cells and examined whether or not cell stimulation raises absorbance. In Fig. 5c, the cells clearly increased in

size, showing cell activation within 24 h; however, the cells did not divide according to CFSE intensity at that time point. Importantly, the alteration in absorbance was marginal in all substrate reagents (Fig. 5b). Given that the activation status or cell size did not affect these reagents, they are reliable under circumstances of stimulation in immune cells.

CFSE dilution assays are measured by a flow cytometer and enable the exact monitoring of proliferating cells and for assessing the final outcome of cell division. On the other hand, many proliferation assays, including MTT, WST-1, and resazurin, do not necessarily reflect the final outcome. For instance, their absorbance on day 4 was low, implying very low proliferating activity (Fig. 3), while CFSE experiments reveal 3–5 cycles of cell division on day 3 (Fig. 4a, b), indicating a clear contrast between these methods. Primary cells, such as spleen lymphocytes, easily commence apoptosis following the exhaustion of cytokines or growth factors. MTT, WST-1, and resazurin seem to reveal how actively cells are proliferating within a particular time frame. Thus, the results of these absorbance assays need to be carefully interpreted. In addition, based on the percentage of each cell division on CFSE results, we estimated a tentative cell number and illustrated it on a graph (Fig. 4c). The graph indicated a gradual

Fig. 4 Monitoring of cell division with CFSE fluorescence intensity dilution assay. **a** Mouse whole spleen cells were stained with CFSE fluorescent dye and stimulated with a serially diluted amount of anti-CD3 (0.01, 0.1, and 1.0 µg/mL) and -CD28 Abs (0.25 µg/mL) for 2–3 days. The cell division was directly monitored by CFSE fluorescence intensity dilution

using a flow cytometer. Numerals above the histogram indicate cell division number. **b** The percentages in each cell division number were expressed as a graph. **c** The increased cell numbers by anti-CD3 Ab stimulation were estimated from percentages in each cell division in **b** and expressed as a graph. These are representative of five experiments with similar results

increase in the cell number by stimulation with up to 0.1 µg/mL of anti-CD3 Ab on day 2 but almost no increase by stimulation with 1.0 µg/mL of anti-CD3 Ab, suggesting saturated proliferation. This graph was similar to the dose–response curve of MTT-SDS, resazurin, and to a lesser extent of MTT-NP-40 but not of WST-1 in Fig. 3. The result revealed an advantage of the first two reagents.

As compared with MTT and resazurin, WST-1 exhibited a unique behavior. In Fig. 1, the absorbance for WST-1 did not increase despite an increase in the cell number; however, there was a sudden increase in the absorbance when the cell number was over 1.6×10^6 cells per well. This result was reproducible in repeated experiments, suggesting that cell–cell contact or at least close proximity between cells may

Fig. 5 TCR signal does not substantially raise MTT activity before cell division. Murine whole spleen cells were stained with or without CFSE fluorescent dye and stimulated with a serially diluted amount of anti-CD3 and -CD28 Abs for 16 h. **a** The cells were subjected to flow cytometry for FSC/SSC analysis and CFSE fluorescence intensity dilution assay. FSC

and SSC reflect cell size and intracellular granular structures, respectively. **b** The same cells were given MTT, WST-1, or resazurin for the last 4 h of 16 h and subjected to the absorbance assays by a plate reader. These are representative of five experiments with similar results. **c** The FSC result shown in **a** is expressed as a *graph*

be required because the absorbance increases only when cell density is highly elevated. Further, the stimulation with more than 0.1 µg/mL, but not 0.01 µg/mL of anti-CD3 Ab, induced an increase in WST-1 absorbance. The absorbance and stimulation index were the highest observed from among the reagents tested; the exact reasons for this unique behavior remain unknown. WST-1 is cell-impermeable; thus, it requires to be extracellularly reduced by plasma membrane NADPH-oxidase via superoxide, resulting in water-soluble formazan (Tan and Berridge 2000; Berridge et al. 2005). At the beginning of stimulation, this enzyme(s) might not be expressed in quiescent cells, being upregulated following stimulation. The responsible mechanism seems intriguing to unravel.

In summary, resazurin has a slightly better reproducibility than MTT-SDS as well as a high enough stimulation index for the immune cells. Further, resazurin does not require a solubilization step and is thus very convenient and easy to handle. Finally, WST-1 is not suitable for universal use; however, it manifests a merit in particular conditions.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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