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Radiolabelling Optimization of Cells with $^{51}Cr(VI)$ for Measurement of Immune Responses after Anti-Rabies Vaccinations

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Abstrak: Telah dilakukan optimasi penandaan sel dengan $^{51}Cr(VI)$ untuk penentuan respon imun setelah vaksinasi antirabies. Optimasi dilakukan karena, pada pengerjaan awal, penandaan yang didasarkan pada prosedur yang telah dipublikasi memberikan hasil penandaan dan cacah per menit per sel (CPM/sel) yang rendah. Sangat kontras dengan yang pernah dilaporkan, penandaan dalam media lengkap (media + *fetal bovin serum*, FBS) memberikan efisiensi penandaan hanya 3.7±0,7% dan CPM/sel ≤ 0.02 (persyaratan untuk *chromium release assay* ≥ 0.2 CPM/sel). CPM/sel dari sel bertanda tetap rendah walaupun diinkubasi selama lima jam. Penandaan kemudian dilakukan dalam media yang tidak mengandung FBS, atau tanpa media di mana ^{51}Cr langsung ditambahkan pada sel, untuk mengamati apakah ada kompetisi antara sel dengan FBS dalam mengikat ^{51}Cr. Efisiensi penandaan dalam media OPTI, EMEM 10, PBS, dan air, atau tanpa media memperlihatkan peningkatan yang sangat berarti. Efisiensi penandaan paling tinggi (96,3±3,9 %) dengan CPM/sel (1,7±0,07) serta kehidupan sel yang terjaga (≥ 80%) diperoleh dengan media OPTI. Efisiensi penandaan dan CPM/sel dalam media lain relatif lebih rendah, yaitu 12–26% dan 0,07–0,13. Sel vero dan sel fetus ayam, yang juga dapat digunakan untuk penentuan respon imun setelah vaksinasi antirabies, ditandai pula dengan ^{51}Cr dalam media OPTI. Penandaan dalam kondisi ini memberikan hasil yang memuaskan (efisiensi ~ 90%, dan CPM/sel > 1,5). Hasil penandaan di atas mengindikasikan adanya kompetisi antara FBS (albumin) dalam penandaan sel dengan ^{51}Cr. Sel yang ditandai dengan ^{51}Cr dalam media OPTI telah berhasil digunakan untuk penentuan respon imun setelah vaksinasi antirabies secara intradermal dan intramuskular.

Kata kunci: penandaan sel, ^{51}Cr, optimasi, *chromium release assay*, vaksin anti rabies

INTRODUCTION

CHROMIUM (^{51}Cr) release assay, first described by *Brunner et al*[1], is still considered to be a gold standard assay for the measurement of cytotoxic function[2]. Therefore, the method was chosen to be used in our comparative study of immune response after intradermal or intramuscular antirabies vaccination[3].

Chromium release assay is a very versatile method that can be applied for many purposes in the measurement of (a) the existence of antigen on a target cell, (b) the existence of a specific antibody on a target cell, (c) the existence of antigen by using modified target cells, and (d) activity assessment of effector cells such as cytotoxic T lymphocytes (T-cells) and natural killer cells[4].

The principle of chromium release assay is based upon the ability of $^{51}Cr(VI)$ ion to enter all types of life cells through the general anion channel of plasma membrane. Inside the cell, the $^{51}Cr(VI)$ ion is then reduced to $^{51}Cr(III)$ ionic state by various enzymes. This form of chromium is trapped and accumulated within the cells (cytoplasm) as $^{51}Cr(III)$ ion which is less permeable through the cell membrane than the $^{51}Cr(VI)$ ion[5]. These cells, also called as target cells, will be damaged if coming in close contact with

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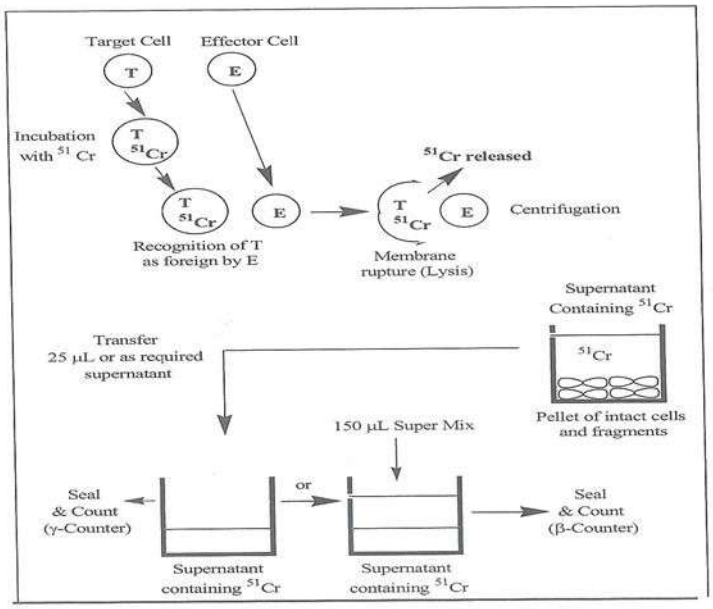
effector cells, such as life immunological responses and natural killer cells (NK). Once the membrane of $^{51}Cr(III)$-labelled target cells has been sufficiently damaged, then the $^{51}Cr(III)$ ion will be released. The $^{51}Cr(III)$ released cannot be taken back or reutilized by other life cells. The principle of chromium release assay is shown in Figure 1.



*www.perkinelmer.com/lifesciences6

Figure 1. Principle of chromium release assay.

The chromium release assay method involves a radiolabelling process of target cells with $^{51}Cr(VI)$. A certain population of the resulting ^{51}Cr radiolabelled target cells is then incubated with a certain population of test cells or effector cells such as lymphocytes T-cells or natural killer cells (NK) for three to six hours. The amount of ^{51}Cr released into the supernatant is then quantified using γ-counter or β-counter (a certain amount of Super Mix has to be added prior to its counting) for measuring the percentage of lysis caused by effector cells. In order to have reliable assay results, the radioactivity which is expressed in count per minute per cell (CPM/cell) of the $^{51}Cr(III)$ labeled target cells, has to be ≥ 0.2 with a viability level of $\geq 80\%$.

Preliminary radiolabelling work of target cells (using neuroblastoma cells) with $^{51}Cr(VI)$ carried out in our laboratory according to a published procedure produced an extremely low radiolabelling efficiency and CPM/cell ($3.7 \pm 0.7\%$ and of ≤ 0.02 respectively)[4]. This extremely low CPM/cell of ^{51}Cr labelled target cells was not useful in the chromium release assay method[7].

The purpose of this investigation is to optimize the radiolabelling of cells with ^{51}Cr (VI) in order to obtain radiolabelled target cells that satisfy the requirements for the chromium release assay. The optimization is carried out by radiolabelling of the cells in various medium and with various incubation times. The ^{51}Cr labeled target cells are then used for the measurement of immune response in patients after intradermal and intramuscular antirabies vaccinations.

MATERIALS AND METHODS

MATERIALS. Materials used in the experiment include $Na_2{}^{51}CrO_4$ (Amersham Biosciences), neuroblastoma, vero and chicken fetus cells (National Veterinary Drug Assay Laboratory), OPTI, complete medium (consists of OPTI, 5% fetal bovine serum, EMEM 10, sterile de-ionized water, phosphate buffer saline (PBS), HBSS (Hank's Balanced Salt Solution), trypsin and nonidet (Sigma). All media were sterilized prior to use, including all tubes, pipette tips etc. which were in direct contact with cells and media. Equipments used in the project include γ-counter (Nucleous), refrigerated centrifuge (Kubota), mini refrigerated centrifuge (Tommy), 5% CO_2 humidified incubator, haemocytometer (Fisher) and inverted microscope (Nikon).

METHODS. Radiolabelling of cells. There were several procedures that have to be carried out prior and after radiolabelling of cells. Prior radiolabelling, the monolayer cells had to be trypsinized to form cells suspension. Chicken fetus cells were isolated from chicken fetus. The number and viability of these cells were then measured using a haemocytometer. After radiolabelling, the radiolabelling efficiency, number and viability of cells and count per minute per cell (CPM/cell) were then measured.

Trypsination of monolayer cells. Aliquot of 1% trypsin solution was added into the monolayer cells. The cells were then incubated in a 5% CO_2 humidified incubator for 15 minutes. A 50 ml of fresh washing solution was added. The mixture was carefully mixed then followed by centrifugation at 600 g for 5 minutes. The supernatant was discarded and one ml of fresh medium was added. The number and viability of cells were then determined.

Determination of number and viability of cells. The number and viability of cells were

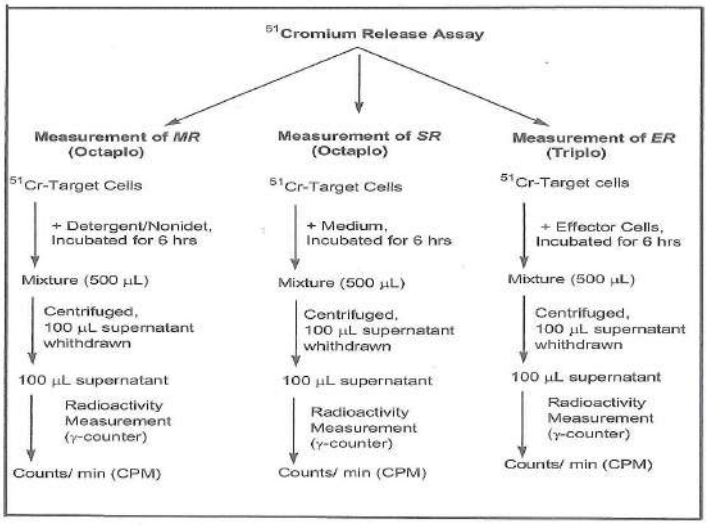
determined using a haemocytometer. A 100 µl of cells suspension was added to 900 µL of 5% trypan blue solution and carefully mixed. The solution (50 µL) was pipetted into the haemocytometer. The number and viability of cells were then determined under an inverted microscope.

General procedure for radiolabelling of cells. The suspended cells were transferred into a 15 ml polypropylene tube and then centrifuged at 200 g for five minutes. The supernatant was discarded and 500 µl of fresh medium of choice was then added (there was no medium added for radiolabelling of pelleted cell). An aliquot of $Na_2{}^{51}CrO_4$ (200 µCi of ^{51}Cr) was then added. The mixture was gently mixed and incubated at 37°C for one to five hours in a 5% CO_2 humidified incubator with frequent shaking (every 15 minutes). The washing solution (14 ml) was then added. The mixture was gently shaken before centrifugation at 200 g for five minutes. The supernatant was again discarded. Washing was repeated until the cell radioactivity was stable. The cells were then suspended in one ml of the assay medium. The radiolabelling efficiency, the CPM/cell, the number and viability of radiolabelled cells were then determined. The radiolabelling optimization was carried out by radiolabelling of cells in various media and during various incubation times.

Determination of radiolabelling efficiency and CPM/cell. The radioactivity expressed as count per minute per cell was measured by using a γ-counter. The radiolabelling efficiency was determined from the total count per minute of cells divided by the radioactivity of added $Na_2{}^{51}CrO_4$ solution and multiplied by 100%. The CPM/cell was calculated by dividing the total count per minute of cells with the total number of cells.

Isolation of lymphocyte. Blood samples, which were withdrawn from patients, were transferred into heparinized tubes. Aliquot of PBS (5-9 ml) was then added to each tube. The mixture was shaken before carefully pouring to the surface of Ficol solution (3 ml) and centrifuged at 600 g for 30 minutes at 4°C. The suspension of lymphocyte, that can be recognized as a white ring between red blood cell and plasma was then pipetted out. Three fold volumes of HBSS solution were then added to the suspension. The mixture was carefully shaken before centrifugation at 600 g for 10 minutes. The lymphocyte washing was repeated before re-suspending in a RPMI solution (1 ml). The concentration and viability of lymphocyte were then determined.

Assay design. The chromium release assay involved four to six hours co-incubation of effector cells (E) with ^{51}Cr-labelled target cells (T) at various effector cell (E:T) ratios. The ratio between effector cells and ^{51}Cr-radiolabelled target cells were 100:1, 50:1, 30:1, 15:1 and 5:1 depending on the effectiveness of effector cells. The level of ^{51}Cr radioactivity (as count per minute) released from target cell into the supernatants was then measured. The basic protocol of chromium release assay for the determination of percentage of cells lysis caused by T-cells is shown in Scheme 1.



Scheme 1. The chromium release assay basic protocol.

Note:
1. ER: ^{51}Cr released caused by the presence of effector cells.
2. SR: ^{51}Cr released spontaneously without the presence of effector cells.
3. MR: ^{51}Cr released by severely damaged cells due to the presence of a detergent or an excessive amount of effector cells.

The ^{51}Cr-radiolabelled target cells were diluted to obtain an approximate of 20,000 CPM in 50µl (~ 500 cells/µl). The ^{51}Cr-radiolabelled target cells (10,000 to 20,000 cells) were transferred into three series of micro tubes. The first series (eight tubes) represented a control for the measurement of maximum release (MR), *i.e.* total ^{51}Cr released into the supernatant by totally disrupted cells due to the presence of detergent (such as Nonidet or Triton) or excessive amount of effector cells.

To each of these tubes was added an aliquot amount of Triton or Nonidet to give 1% final

concentration of Triton or Nonidet in 500 µl. The medium was then added to each of the tubes to give a final volume of 500 µl. The second series (eight tubes) represented a control for the measurement of spontaneous release, *i.e.* ^{51}Cr released into the supernatant by intact ^{51}Cr-radiolabelled target cells (without the presence of detergent or effector cells). To each of these tubes was added an aliquot amount of medium to give a final volume of 500 µl. The third tube series was for the measurement of ^{51}Cr released by target cells which was caused by cell disruption due to the presence of effector cells. The number of tubes in this series depends on the number of samples which will be analyzed, and also depends on the number of E:T ratio will be used. For example, if there are three samples to be analyzed, and the number of E:T ratio to be used are 100:1, 50:1, and 25:1, then 27 tubes will be required (three tubes for each sample and for each E:T ratio). To each of these tubes was added a certain amount of lymphocytes depending on the E:T ratio. The medium was then added to produce a final volume of 500 µl. In order to encourage binding between test cells and target cells, the tubes were centrifuged at 200 g for five minutes. The tubes were then incubated for six hours in a 5% CO_2 humidified incubator, followed by centrifuging at 600 g for 10 minutes. The supernatant (100 µl) was then pipetted from each tube and transferred to a counting tube. Radioactivity in each tube was then measured in a γ-counter for one minute.

The percentage of cell-lysis of a known population of target cells caused by a known population of effector cells T-cells was calculated to according Equation 1.

$$\% \text{ Lysis} = \frac{(\text{Mean ER} - \text{Mean SR})}{\text{Mean R} - \text{Mean SR}} \times 100\% \quad \text{Eq. 1}$$

RESULTS AND DISCUSSIONS

Three types of cells, neuroblastoma, vero and chicken fetus cells were used in this work. A preliminary radiolabelling work using neuroblastoma cells, which was carried out according to a published procedure (in complete medium: OPTI + fetal bovine serum), produced a very low radiolabelling efficiency (3.7 ±0.7%). This in turn produced a very low CPM/cell of ≤ 0.02. The CPM/cell of this radiolabelled cells was extremely low when compared to the chromium release assay requirement (> 0.2).

An effort to increase the radiolabelling efficiency was carried out by increasing the incubation time up to five hours. The radiolabelling efficiency and the CPM/cell are shown in Table 1.

Table 1. Radiolabelling efficiency of neoroblastoma cell with ^{51}Cr(VI) in complete medium*.

No	Incubation Time (Hr)	Radiolabeling Efficiency (± SD)	CPM/Cell
1	2	3.1 ± 1.0%	≤ 0.02
1	3	4.1 ± 1.0%	≤ 0.02
2	5	3.6 ± 0.9%	≤ 0.02

*Complete medium (OPTI + 5% fetal bovine serum).

As shown in Table 1, there is no improvement in the radiolabelling efficiency and the CPM/cell even when the incubation times are prolonged up to five hours. This indicates the unsuitability of the radiolabelling condition since life cells have been known to uptake readily Cr(VI) through their general ion channel. Although there are only three components involved in the radiolabelling process (cells, ^{51}Cr(VI) and media), any one of them is not suitable for radiolabelling process, so a poor radiolabelling efficiency would be expected. In this work, the media was suspected to be the cause of low radiolabelling of ^{51}Cr(VI) to target cells, since the two other components had fulfilled the required specification (cell viability of ≥ 80% and ^{51}Cr(VI) specific activity of 250-900 mCi/mg).

The media used in this preliminary work was a complete media, which consisted of OPTI and 5% fetal bovine serum (FBS). The presence of FBS might affect the radiolabelling process of cells with ^{51}Cr(VI). In order to avoid the influence of FBS, the radiolabelling process was carried out in a FBS-free medium. Radiolabelling without medium, where ^{51}Cr(VI) was directly added to the cell pellets, was also carried out. The efficiency of these radiolabelling procedures is shown in Table 2.

As shown in Table 2, the radiolabelling efficiency of cells with ^{51}Cr(VI) in a FBS-free medium and the radiolabelling without media (pelleted cells only)

Table 2. Radiolabelling efficiency of neoroblastom cells with ^{51}Cr(VI) in free fetal bovine serum medium*.

	Medium	Labeling Efficiency (± SD)	Viability (%)	CPM/Cell (± SD)
1	Sterile H_2O	11.4 ± 2.8	? 80	0.10 ± 0.3
2	Pellet	26.4 ± 1.8	? 80	0.13 ± 0.04
3	EMEM 10	12.5 ± 2.1	? 80	0.17 ± 0.03
4	PBS	15.2 ± 2.8	? 80	0.19 ± 0.03
5	OPTI	96.3 ± 3.9	? 80	1.70 ± 0.07

*One hour incubation time.

with only one hour incubation were remarkably improved. The highest radiolabelling efficiency of 96.3±3.9% and CPM/cell of 1.7±0.07 with cell viability of ≥ 80%, was produced in an OPTI media. Radiolabelling in other medium (EMEM 10, PBS and sterile water) or without any medium (pelleted cells only), however, produced a much lower radiolabeling efficiency of 12–26% and CPM/cell of 0.07–0.13.

Based upon the above results it can be concluded that the fetal bovine serum had significantly influenced the radiolabelling of cells with $^{51}Cr(VI)$. Fetal bovine serum like any other serum consisted of many components, such as macromolecules (albumin, antibodies and globulin, enzymes, peptides, hormones) and organic compounds (amino acids, glucose and fatty acids). One or some of these components, particularly such macromolecules as albumin, antibodies and/or globulin might compete with target cells in taking up the $^{51}Cr(VI)$ during radiolabelling process. The *in vitro* and *in vivo* study carried out by Merritt et al. and Anghileri reported that serum albumin was able to strongly binding of $Cr(VI)$[8,9]. This competitive binding phenomenon may explain the low radiolabeling efficiency of cells when the process was carried out in a medium containing serum albumin.

Vero and chicken fetus cells that have been considered to be suitable for the chromium release assay were also labeled with $^{51}Cr(VI)$ in an OPTI medium. The radiolabelling efficiency and the CPM/cell for both cells were found to be very satisfactory (≥ 90% and ≥ 1.5 respectively).

The neuroblastoma and vero cells which were radiolabelled in OPTI medium had successfully been used in the chromium release assay for the measurement of immune response in patients after intradermal (ID as well as intramuscular (IM) antirabies vaccinations. Two hundred and forty samples taken from 60 subjects after intradermal and intramuscular vaccinations had been analyzed. Full results and details of this study shall be reported in due course.

CONCLUSIONS

The chromium release assay had been acknowledged to be a gold standard for assay of cytotoxic function. The assay required ^{51}Cr radiolabelled target cells with a certain specification in order to produce a reliable assay result. Our preliminary work using a previously reported procedure for radiolabelling of target cells with $^{51}Cr(VI)$ in a complete medium, produced both very low labelling efficiency and CPM/cell. The best radiolabelling efficiency (96.3 ±3.9%) with high CPM/cell (1.70±0.07) of target cells was obtained in OPTI medium, a medium with no fetal bovine serum. This radiolabelling condition was found to be a reliable condition when applied to other types of cells.

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