

Sequential assessment of cell cycle S phase in flow cytometry: a non-isotopic method to measure lymphocyte activation *in vitro*

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Abstract. Lymphocyte multiplication can be induced *in vitro* by mitogens or specific antigens, and is usually measured using isotopic methods involving tritiated thymidine. Cellular proliferation can also be analyzed by flow cytometry techniques based on cell cycle analysis through the measurement of DNA content. We applied this method to lymphocytes from 113 individuals, to evaluate lymphocyte proliferation after stimulation *in vitro* by a mitogen (phytohaemagglutinin, PHA) or a recall antigen (tetanus toxoid), using a kinetic approach with four points sequential measurements of the S and G2 phases over six days of culture. The proportion of cells in S phase after PHA stimulation was significantly higher than in controls overall and as early as on day three of the culture. Activation with a recall antigen significantly induced increasing S phase cell proportions up to day six. These data suggest that flow cytometric assessment of the S phase could be a useful alternative to isotopic methods measuring lymphocyte reactivity *in vitro*.

Keywords: Lymphocyte activation, flow cytometry, mitogens, cell cycle, phytohaemagglutinin, tetanus toxoid

1. Introduction

Proper signals of lymphocyte activation induce the proliferation of these cells, both *in vivo* and *in vitro*. The proliferation pathway used by lymphocytes is that of all nucleated cells, and involves a transition from the G0 state into the cell cycle. During the latter, several stages precede mitosis: proteins are synthesized during the G1 phase, that will later be used for the formation of new nuclear material. The DNA cell content subsequently increases during the 8 hrs of the S phase, from the euploid 2n amount to the 4n content of diploid G2 stage cells. The 2.5 to 3 hrs of the G2 phase are occupied by the duplication of centrioles and assembly of microtubules necessary for cell duplication during the sequential steps of mitosis (prophase, metaphase, anaphase and telophase) [12]. The two daughter cells resulting from the ultimate cytokinesis both have a 2n DNA content. They may return

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to the G0 phase, or eventually reenter the cell cycle. One of the developments of flow cytometry techniques has been an accurate evaluation of the amount of DNA contained in individual cells in suspension [13,22]. When this technique is applied to normal growing cells, it allows the assessment of the proportion of cells at each stage of the cell cycle. After permeabilizing the cell and nucleus membranes, fluorochromes are used to stain the DNA. Propidium iodide (PI) and ethidium bromide, which are widely used, are intercalating agents binding DNA or RNA helices [11,21]. Other stains, such as Hoechst 33342, Hoechst 33258 or DAPI are specific for adenine or thymine, while some antibiotics such as mithramycin or chromomycine A₃ bind guanines and cytidines [5,12,20].

In vitro, lymphocyte multiplication can be induced by specific antigens, that trigger monoclonal or oligoclonal proliferation of discrete specific lymphocyte subsets. Such mitogens as phytohaemagglutinin (PHA) [17], concanavalin A or pokeweed mitogen (PWM) can also be used, which will more broadly stimulate the polyclonal cell population expressing mitogen receptors [23]. Mitogen-induced proliferation provides a good indication of the functional ability of lymphocytes to multiply. Recall antigens can provide information on the sensitization state of the individual tested.

Tritiated thymidine incorporation has become the most common way to measure the lymphocyte proliferation induced by such activators *in vitro* [8], the amount of radioactivity emitted by the pelleted cells being proportional to the amount of newly synthesized DNA. However, this time-consuming and tedious method requires specific equipment and precautions as it implies the use of isotopic material. Several alternative methods have been published, using mitochondrial or DNA labels, surface markers identification or cytokine assays [9,11,14,16,26].

It has been proposed that as activated lymphocytes enter the cell cycle, lymphocyte activation could also be assessed by flow cytometry after DNA content labelling, by evaluating the presence of cycling cells following mitogenic stimulation [4]. These authors used a one time-point measurement after stimulation with mitogens. Here we report on a method developed on this principle to use a kinetic approach of *in vitro* lymphocyte activation by mitogens or antigens. In order to validate this technique, it was applied to purified peripheral blood mononuclear cells to assess the lymphocyte response to PHA and the recall antigen, tetanus toxoid. Evaluation of the percentage of cells in S phase appeared to be a reliable tool to measure the functional properties of peripheral blood lymphocytes (PBL), while the kinetic approach we used confirmed that one time-point studies may be too limited for antigen-specific assessment of lymphocyte proliferation.

2. Materials and methods

2.1. Patients

Peripheral blood samples were obtained from 113 adult individuals with antecedents of skin rashes, possibly drug-induced, who were subjected to immunological investigations. The possible lymphocyte dysfunction associated to such drug adverse-effects prompted the assessment of lymphocyte reactivity *in vitro*, prior to performing a series of skin tests in search of the culprit drug. None of these patients suffered from any major immunological disease, notably from severe immune deficiency. All gave informed consent to blood sampling. Non-specific stimulation through mitogen activation was tested for all 113 of them, and 45 were also tested for recall antigen responsiveness to tetanus toxoid.

2.2. Cell suspensions

Lymphocyte suspensions were prepared from 5 ml of sterile peripheral blood samples, by density gradient centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway), after dilution of the whole blood sample with sterile RPMI 1640 medium (Sigma Chemicals, St. Louis, MO, USA). After 30 min centrifugation at 400 g, lymphocytes were harvested and washed twice in RPMI. After the second washing, the cells were resuspended at 5×10^6 cells/ml in complete medium composed of RPMI supplemented by 1% HEPES (Gibco BRL, Cergy Pontoise, France), 1% Ultrosor (Gibco BRL) and 0.5% of a mixture of antibiotics (Penicillin 10 000 U/ml, Streptomycin 10 000 $\mu\text{g/ml}$, Fungizone 25 $\mu\text{g/ml}$; Gibco BRL). Cytocentrifugates of representative samples of the cell suspensions were examined after May Grünwald Giemsa staining. This permitted us to demonstrate that the cell suspensions obtained contained between 5 and 15% of monocytes. As lymphocytes were not further purified in these preparations, it was assumed that both the small proportion of monocytes and the population of B cells would be sufficient to act as antigen-presenting cells in antigen-specific stimulation.

2.3. Mitogen and antigen preparations

PHA was purchased from Sigma, and used at a concentration of 1 $\mu\text{g/ml}$ in sterile complete RPMI. This optimal dilution was determined after a series of preliminary experiments with PHA diluted sequentially between 10 $\mu\text{g/ml}$ and 0.01 $\mu\text{g/ml}$. Tetanus toxoid was used as is in the Tetavax[®] vaccine (Merieux, Lyons, France) preparation currently used in France. The content of a Tetavax[®] syringe was diluted in sterile complete medium yielding a final concentration of 0.8 UI/ml. The optimal Tetavax[®] dilution had also been experimentally determined.

2.4. In vitro activation of lymphocytes

Four wells culture plates (Nunc Maxisorp, Roskilde, Denmark) were used for cell culture. Five hundred microliters of the cell suspension (i.e., 5×10^5 lymphocytes) were placed in three of the wells. Then 500 μl of respectively complete RPMI, PHA solution or Tetavax[®] solution were added to the wells. The plates were identified, closed, and placed for 6 days in a 37°C incubator with a monitored water saturated and 6% CO₂ atmosphere (Sanyo, Osaka, Japan).

One hundred microliters aliquots from each cell suspension were harvested under sterile conditions on days 3, 4, 5 and 6 of the culture.

2.5. DNA analysis

The cells of each harvested aliquote were stained for DNA quantitation using a DNA-Prep (Coulter, Leukocyte Preparation, Hialeah, FL, USA), which distributes controlled volumes of first a permeabilizing solution, then a solution of PI and RNase. After distribution of the reagents, the cells were allowed to stain for at least 15 min before being processed in flow cytometry.

A specific protocol devised for DNA content assessment was used on an XL flow cytometer (Coulter Corporation, Hialeah, FL, USA) after checking the cytometer laser alignment with calibrated beads (DNA Check, Coulter) in order to obtain the smallest peak coefficient variation (CV) while measuring DNA fluorescence on a linear scale. The cells were gated according to the surface and peak of the fluorescence signal, in order to avoid taking doublets into account. The histogram of linear fluorescence distribution was stored and later analyzed using polynomial analysis derived from the Dean and Jett

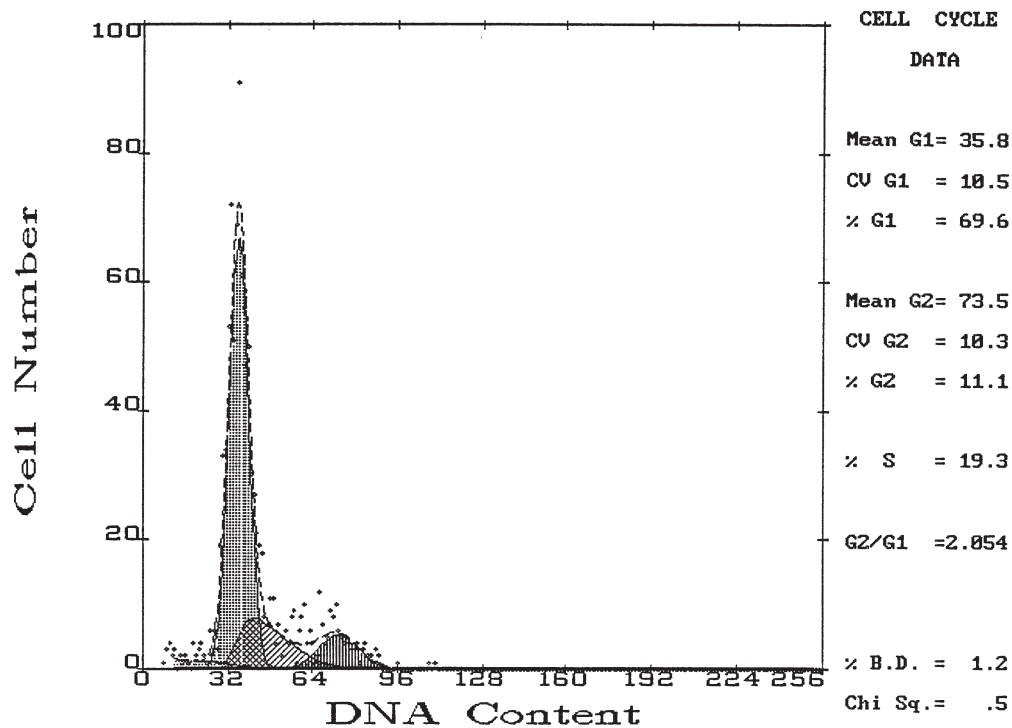


Fig. 1. Typical cell cycle analysis histogram obtained using the M-Cycle software after data acquisition of the flow cytometer.

model [6] with the specific software MultiCycle AV (Phoenix Flow Systems, San Diego, CA, USA; Fig. 1). This permitted the assessment of the proportions of lymphocytes and lymphoblasts in G0/G1, S and G2+M phases at each time point of the stimulation. The proportions of cells in S and G2+M phases, respectively, were collected as percentages of the gated population of singulets. A forward scatter/side scatter biparametric scattergram, that was not used for gating purposes, allowed us to verify the appearance of larger lymphoblasts in the course of cell activation.

2.6. Statistical analysis

Three factor analysis of variance was used to test the statistical significance of the kinetics of cell activation over time comparing control, PHA and Tetavax[®] wells. Two factor analysis of variance was then used for PHA and Tetavax[®] separately. Statistical analysis was performed using A.P. Stats (Micro-6, Nancy, France).

3. Results

3.1. S phase kinetics

The mean percentages (\pm SE) of cells in the S phase at the four time points tested for all the samples assessed, as well as the response ranges, are summarized in Table 1.

A small proportion of S phase cells was observed in control wells. There was no significant variation of this proportion over time, as shown by two factor variance analysis ($p = 0.17$). However,

Table 1
Sequential assessment of the percentage of cells in the S phase among peripheral blood mononuclear cells cultured for 3, 4, 5 or 6 days with medium alone, PHA or Tetavax[®]

	D3 mean % (\pm SE) [range]	D4 mean % (\pm SE) [range]	D5 mean % (\pm SE) [range]	D6 mean % (\pm SE) [range]
Control ($n = 113$)	1.63 (\pm 0.16) [0–8.20]	1.99 (\pm 0.14) [0–8.80]	2.24 (\pm 0.18) [0–11.20]	2.31 (\pm 0.22) [0–8.30]
PHA ($n = 113$)	13.05 (\pm 1.01) [1.80–42.90]	10.44 (\pm 0.83) [1.30–49.10]	9.48 (\pm 0.88) [1.10–35.90]	7.07 (\pm 0.59) [1.70–29.10]
Tetavax ($n = 45$)	3.57 (\pm 0.58) [0–15.30]	4.07 (\pm 0.38) [0.10–10.80]	3.98 (\pm 0.41) [1.00–10.50]	4.11 (\pm 0.47) [0.80–10.70]

examination of individual cases showed noticeable increases of the proportion of cells in S phase, at least at one time point, in 23 cases interpreted as a possible specific reactivity to one of the medium components, i.e., antibiotics, or as ongoing specific APC-mediated stimulation initiated *in vivo*.

In 10 cases, no significant increase of the proportion of S phase cells was detectable at any time after PHA stimulation, suggesting poor reactivity of the cells possibly related to anergy. For the 103 reactive patients (Fig. 2), maximum stimulation was noted on day 3 with, as a mean, 14% (\pm 1.04) of the cells in the S phase. Two-factor analysis of variance confirmed the statistical significance of this increase ($F = 13.2$ (3/56); $p < 0.0001$). Day-to-day comparisons using a protected T test showed significant differences between D3 and D4 ($t = 2.71$), D3 and D5 ($t = 4.01$) and D3 and D6 ($t = 6.14$).

Tetavax[®] stimulation always was more moderate than PHA activation, but induced an overall continuing increase in the proportion of S phase cells, suggesting secondary activation of newly generated daughter cells. Statistical analysis of this increase using two-factor analysis of variance showed a significant difference ($F = 3.39$ (3/18); $p = 0.024$). Day-to-day comparisons showed significant differences between D3 and D4 ($t = 2.4$), D3 and D5 ($t = 2.85$) and D3 and D6 ($t = 2.51$).

Three-factor analysis of variance showed a highly significant difference between the three culture conditions ($F = 10.6$ (6/408); $p < 0.0001$).

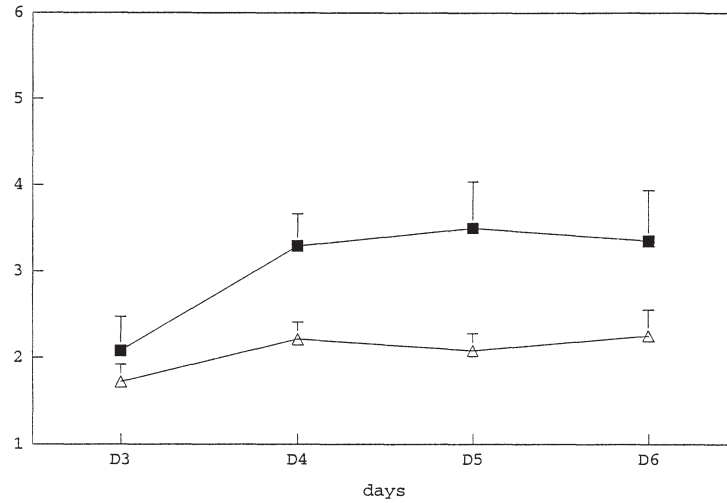
Examination of individual profiles showed variability both in the intensity of the response and in its kinetics, as exemplified on Fig. 3, comparing characteristic Tetavax[®] activation curves from three different patients. Proliferation profiles differing from those of the same cells cultured with medium alone were observed in 78% of the cases. The difference was usually at least two-fold at the peak time point.

3.2. G2+M phase kinetics

These data are summarized in Table 2.

There were no G2+M cells in the control wells at any time, while PHA stimulated lymphocytes contained a maximum of 3% of G2+M cells on day 3. There was a very small proportion of such cells after Tetavax[®] stimulation, nevertheless higher than that from control wells, especially on day 3. Three-factor analysis of variance indicated a statistical significance of the differences between the three culture conditions ($F = 2.24$ (6/405); $p = 0.039$).

Percentage of cells in S phase



Percentage of cells in S phase after PHA stimulation

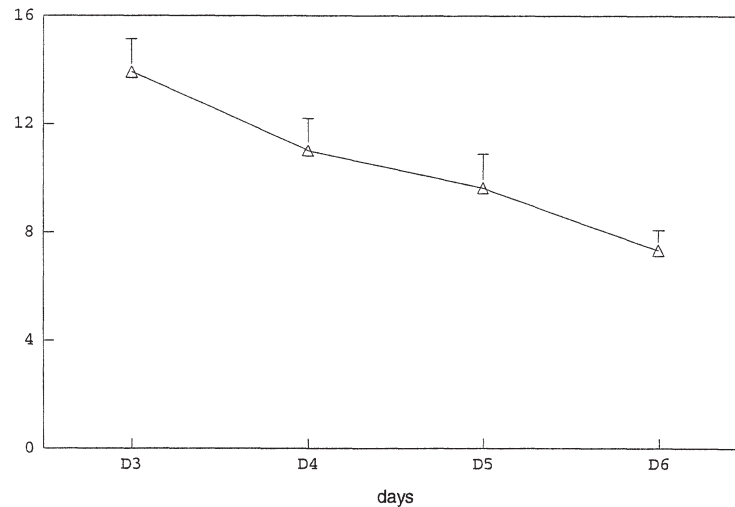


Fig. 2. Mean percentages (topped by S.E.) of peripheral blood mononuclear cells in S phase, assessed in flow cytometry on sequential aliquotes obtained after 3, 4, 5 and 6 days of culture in medium alone (\triangle : control) or with Tetavax[®] (\blacksquare) (top panel) or with PHA (\triangle) (lower panel). These curves only take into account the population of 103 non-anergic patients who showed a significant proliferative response to PHA.

4. Discussion

This study establishes the feasibility of sequential cell cycle analysis as a non-isotopic alternative to appreciate specific and non-specific lymphocyte activation *in vitro*.

Previous studies have suggested the use of whole blood samples to measure lymphocyte proliferation, both with tritiated thymidine [3] or after PI staining [4]. Our method rather uses purified PBL, since such suspensions are enriched in both lymphocytes and antigen-presenting cells (monocytes and B cells) and devoid of the short-lived polymorphonuclear releasing potentially deleterious compounds over the culture duration [18].

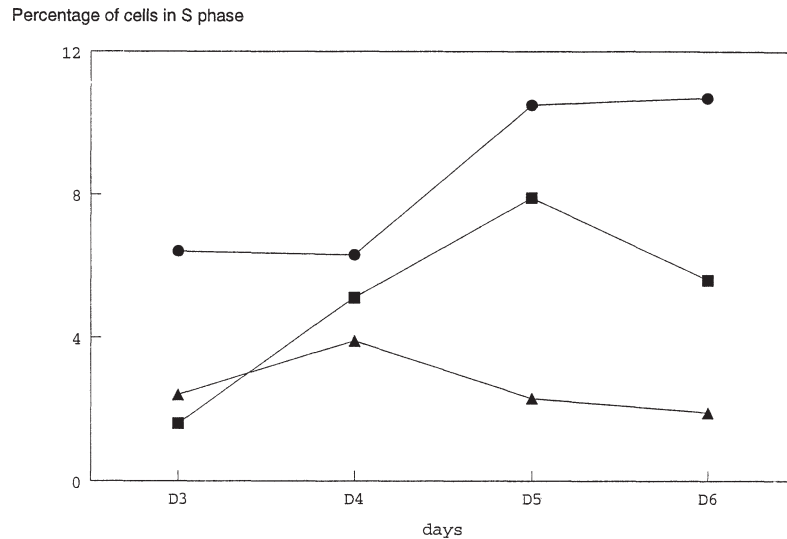


Fig. 3. Tetavax[®] proliferative response profiles of peripheral blood mononuclear cells from three individual patients, assessed by flow cytometric evaluation of the percentage of cells in the S phase on days 3, 4, 5 and 6 of culture. The kinetics are clearly different, maximal stimulation occurs at different time points, and might go unnoticed if using a single time point evaluation.

Table 2
Sequential assessment of the percentage of cells in G2+M among peripheral blood mononuclear cells cultured for 3, 4, 5 or 6 days with medium alone, PHA or Tetavax[®]

	D3 mean % (\pm S.E.)	D4 mean % (\pm S.E.)	D5 mean % (\pm S.E.)	D6 mean % (\pm S.E.)
Control ($n = 113$)	0.05 (\pm 0.02)	0.04 (\pm 0.01)	0.07 (\pm 0.02)	0.09 (\pm 0.02)
PHA ($n = 113$)	2.71 (\pm 0.32)	2.37 (\pm 0.27)	1.93 (\pm 0.21)	1.86 (\pm 0.22)
Tetavax ($n = 45$)	0.32 (\pm 0.10)	0.24 (\pm 0.07)	0.17 (\pm 0.05)	0.25 (\pm 0.09)

The method we developed provides, for stimulation with PHA or a recall antigen, comparable data as previously reported by other authors, which suggests that it could be an interesting alternative to classical isotopic techniques. Indeed, Fried et al. [11] showed, after growing the human hematopoietic cell line SK-L7, that the fractions of cells in the different phases of the cell cycle assessed after PI staining were in good agreement with those obtained from autoradiography after pulse labelling with tritiated thymidine. The kinetics we observed also compares well with data from Ferlini et al. [9] who evaluated the kinetics of lymphocyte activation on days 1, 2, 4 and 7 through mitochondrial uptake of rhodamine 123. These authors stressed the fact that their method allowed the identification of lymphocyte subsets, which can also be performed in multiple-colour flow cytometry using our method. Other alternatives, such as cytokine production *in vitro* which has been demonstrated to be induced by mitogens as well as antigens [16,26], have not always been correlated to PHA stimulation [7], and, as tritiated thymidine incorporation, do not allow the identification of cell subsets. Other authors such as Maino et al. [14], have proposed the use of membrane activation markers such as CD69, to assess lymphocyte responsiveness in short-term assays. They demonstrated that the expression of this surface antigen was correlated to the incorporation of tritiated thymidine. Our data, however, indicate that activation kinetics may vary between individuals, and stress the interest of sequential assessment of each patient's responsiveness profile.

As could have been expected, owing to the polyclonal stimulation it generates, PHA activation yielded the maximal signal observed on day 3, with highly significant numbers of S and G2+M cells at this time point. This is consistent with data from isotopic techniques indicating that 72 hrs is the optimal incubation time to demonstrate PHA stimulation [2,10]. Little is known of the expression of mitogen receptors by newly generated daughter lymphocytes, but our data suggest that there is no further stimulation of these cells by the remaining PHA, or that all has been bound to responding lymphocytes. Interestingly, about 9% of the patients tested failed to respond to PHA. This proportion is similar to what has been reported by Mathews et al. [15] in an isotopic study.

Conversely, the slower but sustained activation induced by Tetavax[®] suggests that the small subset of T cells expressing a specific TCR recognized a properly presented antigen both as resting or G1 peripheral cells and as newly generated daughter cells with the same TCR. This ongoing activation can best be seen by such sequential assessments by the curve of cumulative stimulation. The higher levels of S phase cells on day 6 are consistent with the classical 7 day culture used for isotopic tests allowing antigen-specific stimulation of T cells *in vitro* [1,15], but individual profile analysis demonstrated that this might not always be the optimal assessment time.

Both for mitogen and antigen activation, assessment of the proportion of cells in S phase appeared to be a better indicator than the measurement of cells in G2+M. Analysis of individual data indicates that the global kinetics reported here may vary by one or two days. These individual variations could be interpreted as depending on each subject's immunological status and on the efficiency of antigen presenting cells. Such variations are less apparent when whole cultured suspensions are supplemented with thymidine and tested at only one time point, and poor responses may be erroneously suspected if the addition of thymidine occurs at a time when cell growth either stalls or only begins. The kinetic approach chosen here permits the observation of such individual patterns, even in the expected good response to a potent recall antigen, used as a vaccine, i.e., administered several times, with adjuvant, to the subjects. This approach might be even more useful to reveal the kinetics of cell activation when assessing responsiveness to drugs liable to yield cutaneous hypersensitivity in minute amounts.

In conclusion, this report indicates that flow cytometric assessment of cell cycle phases in cultured lymphocytes is an efficient alternative to isotopic methods which can be applied not only to mitogen stimulation but also to specific antigens identifying discrete sensitised subsets. This suggests that this method might also be easy to apply to various recall or vaccination antigens in order to verify the presence of sensitised cells and/or vaccination efficacy [24,25]. This method could also be useful for *in vitro* non-invasive delayed type hypersensitivity tests [15,19].

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