ISSN No. 2456-4400
Int J Med Lab Res 2020, 5(1):1-10

IJMLR International Journal of Medical Laboratory Research

RESEARCH ARTICLE

EFFECT OF VARIED PBMC CONCENTRATION AND RESTING TIME, ON INTERFERON-GAMMA(INF-Γ) ELISPOT ASSAY OUTCOME, USING *PLASMODIUM FALCIPARUM* AMA 1 SELECTED PEPTIDES

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Received: 18 January, 2020/ Revision: 07 February, 2020/ Accepted: 10 March, 2020

ABSTRACT: ELiSpot assays can be used to generate consistent results when standardized assay procedures are utilized. Some of the optimal conditions includes peripheral blood mononuclear cell (PBMC) resting and test cell concentration. INF-γ EliSpot assays were performed with PBMCs collected from three study subjects, inorder to determine the effects of varied cell concentrations and resting times on the assay outcome. PBMCs were tested at 2×10^6 cells/ml and at 3×10^6 cells/ml and for each cell concentration, resting was done for 6hrs and 12hrs. The number of spots per well which was estimated using an automated ELISpot plate reader was exported to Microsoft Excelandconverted to spots forming cells per million PBMCs. The results show that, variation in PBMC concentration and resting time, did not necessarily produce an expected linear conversion from spots counts to spots forming cells per million.

KEYWORDS: IFN-γ ELISPOT , *Plasmodium falciparum,* PBMC

INTRODUCTION:

The immune system can be monitored ex vivo, with the use of immunological assays like the Elispot assay [1], because it allows the quantification and detection of responding T cells and their secreted molecules respectively[2]. One of the methods of monitoring the immune system is by stimulation of PBMCs in IFN-γ ELISPOT assay, in order to monitor and measure detected antigen-specific T-cell responses[3]. During the assay, the effects of some cell processing techniques on PBMC viability has been investigated and specific recommendations proffered [4,5]. One of such cell preparation techniques, the duration of cell resting, is crucial as resting helps in the elimination of dead cells, ensuring accurate viable cell counts [6]. According to an earlier review, the usual PBMC resting period was overnight, although the optimal resting time had not been investigated[6]. It was

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demonstrated that overnight resting could rescue a recall response[7], by aiding in the removal of apoptotic cells[8,9]. Among different resting times (0, 2, 6 and 18 h), shorter (<2h) was found to produce a higher quality of cells in terms of viability and recovery[10]. Contrarily, a study determined that resting had no statistical significance on observed spot count, for CEF low responder PBMC[9]. However, another findings reported that the usefulness of cell resting process depends on the cell population and type of analysis[11].

In addition to the duration of cell resting, test cell concentration has been identified as a pivotal factor in assay standardization. In the identification of major factors influencing ELISpot-based monitoring of cellular responses to antigens, studies have concluded that special attention should be given to the number of cells added to ELISpot wells[12,13]. Given that the typical PBMC number frequently used for IFN-γ ELISPOT assay varies from 1×10^5 to 4×10^5 PBMC/test well [2,14,15], doubling of PBMC number tested per well, was recommended to enhance ELISPOT assay performance [9]. However, because spots counts are converted to spots per millionPBMC, normalization is expected, irrespective of the initial test concentration. When standardized assay procedures are utilized, ELiSpot assays can be used to generate consistent results [16]. Consequently, it is important to meticulously handle PBMCs and set acceptance criteria for cell viability[17, 18], as decreased cell viability infers in antigen processing due to decrease in the population and integrity of potential responder cells [7,19]. For this reason, there is need to standardize and develop a protocol defining the required concentration and resting time of PBMC in IFN-γ ELISPOT assays. This study was designed to determine the effect of change in assay conditions on the magnitude of IFN-γ Elispot assay outcome, by varying PBMC test concentration and resting time.

MATERIALS AND METHODS :

Synthetic Peptides

Ex vivo ELISpot IFN-γ assays used commercially synthesized class 1-restricted HLA-binding 9–10mer peptides within the *Plasmodium falciparum* AMA1 which were predicted using NetMHC[20,21] and were defined according to their super type classification [22]. The choice of these HLA-binding peptides was on the basis that their predicted HLA supertypes are among the most globally prevalent HLA alleles[22]. The HLA-binding peptides were synthesized (Alpha Diagnostics Intl Inc, San Antonio, TX, USA, (>91 % purity). All peptides were originally in lyophilized states (10mg), and were diluted in500µL of DMSO and 500µL of sterile plain RPMI was added to solution to make 10mg/ml. Before use, the diluted peptides were diluted to the required concentration (20µg/ml) with RPMI 1640 with 1 % penicillin–streptomycin, 1 % l-glutamine and 10 % normal human serum.

ELISpot assay

ELISpot IFN-γ assays were performed using frozen PBMCs as previously described[14,23]. Briefly, frozen cells (two weeks cryopreservation) were rapidly thawed and washed following the standard protocol [15]. After washing, two groups of PBMCs were rested, one group for 6 hours and the other for 12 hours at 37 °C, both at 37^0C, in 5 % CO_2. For every study subject, the selected peptides and positive controls Concanavalin A (Con A, Sigma Aldrich, USA) (1.25 µg/ml and 0.625 µg/ml) and CEF (Cellular Technology Ltd, USA) (2.0 µg/ml) were used to stimulate four groups of PBMCs each. These four groups were PBMCs suspended in 10% heat-inactivated NHS in RPMI-1640 medium containing antibiotics, at: 300,000 PBMCs/100µl after 6hrs resting, 300,000 PBMCs/100µl after 12hrs resting, 200,000 PBMCs/100µl after 6hrs resting and 200,000 PBMCs/100µl after 12hrs resting. Subjects' PBMCs incubated with medium only were used as

negative controls (background). The number of IFN-γ-producing cells in the form of spots per well was subsequently estimated using an automated ELISpot plate reader (AID GmbH, Germany) and the acquired data was exported into Microsoft Excel for conversion into spot forming cells per million PBMC [14].

Statistical analysis

Actual spots forming cells per million PBMC for each stimulant was determined by deducting the number of spots forming cells per million PBMC for the background counts from the counts produced for each stimulant. Graphs were drawn using Graph Pad Prism version 7 and t-test was used to determine significant difference between responses produced by the different test cell concentration.

STUDY SITE

The study was conducted within the University of Ghana, Legon and its surrounding communities in Accra, Ghana. Legon is about 10 km north of Accra, the capital city of Ghana. It is home to the University of Ghana, and a 10 sq km area around Legon has an approximate population of 100,000.

STUDY PARTICIPANTS

Eligibility criteria for the study were the following: age 18–55 years; males, or females who were not pregnant or nursing; normal screening medical history and physical examination; haemoglobin >10 g/dL and absence of known immunodeficiency (>400 CD4 + T cells/μL). All participants generally had a normal medical history at screening and physical examination. Three subjects from a previous study(14),who met the inclusion criteria were selected. Ethical approval for this study was gotten from the Institutional Review Boards at the Noguchi Memorial Institute for Medical Research (NMIMR). Written informed consent was sought from all three study subjects who willingly agreed to be part of the study and met the inclusion criteria.

STUDY OBJECTIVES

Given that the typical PBMC number frequently used for IFN-γ ELISPOT assay varies from 1×10^5 to 4×10^5 PBMC/test well [2,14,15], for the present study, PBMC number within this test range: 2×10^5 and 3×10^5 PBMC/test well were selected. Consequently, this study was out to compare the magnitude of responses produced by these two concentrations when stimulated. Prior to stimulation, PBMCs were rested for 6 or 12 hours and the effect of resting on the magnitude of assay responses was also determined. In other words, the question to be answered in this study is, does testing at 2×10^5 and 3×10^5 PBMC/test well, after 6 and/or 12hrs restingyields significantly different magnitude of IFN-γ ELISPOT assay responses. In order to answer this question, the following study objectives were designed:

1. Compare the magnitude of IFN-γ ELISPOT assay outcome between PBMCs tested at 2×10^5 and 3×10^5 PBMC/test well after 6 and 12hrs resting.
2. Determine significant differences between percentage viability of fresh and cryopreserved; rested and non-restedPBMCs.

SAMPLE COLLECTION

Sixty ml of venous blood was collected per subject,into heparinized tubes. PBMCs were isolated from blood by gradient centrifugation using Accuspin Histopaque-1077 cell separating tubes. Cells were washed and counted, using the trypan blue dye exclusion methods, percentage viable cells was calculated by determining the percentage of viable cells in the total number of cells counted[24]. 20 million PBMCs per vial was re-suspended in freezing medium containing 90% Fetal Calf Serum (FCS) and 10% dimethyl sulfoxide (DMSO). PBMCs which were

cryopreserved for two weeks were rapidly thawed and washed following the standard protocol[14,15].

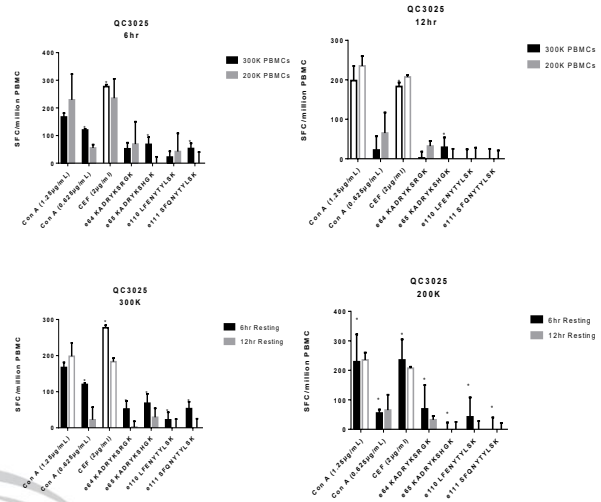
RESULTS:

Variation in PBMC concentration and resting time.

For subject 1, results on Fig 1 shows that, out of seven stimulants used to stimulate two sets of PBMC concentrations (3×10^5 and 2×10^5/test well) each, results in response to four were significantly different (t-test P value < 0.05) between the two concentrations, when cells were rested for 6hrs prior to stimulation. However when the cells were rested for 12hrs, the results in response to only two of the stimulants were significantly different (P < 0.05) between the two concentrations. Therefore, at 3×10^5 PBMC/test well, results in response to six out of seven stimulants were significantly different (P < 0.05) between cells rested for 6hrs and 12hrs. And, for cells tested at 2×10^5 PBMCs/test well, results in response to all seven stimulants were significantly different between cells rested for 6hrs and 12hrs. PBMCs subject 1 responded positively to a total of five stimulations. There was a single positive response when cells were rested for 6hrs and four positive responses when rested for 12hrs. Three of the positive responses were produced by cells tested at 3×10^5 PBMC/test well, while two were from those tested at 2×10^5 PBMCs/test well.

Table 1: Actual spots forming cells per million PBMC at varied PBMCs concentration and resting time, for Subject 1.

	6hr Resting	6hr Resting	12hr Resting	12hr Resting
Stimulants	300K PBMCs	200K PBMCs	300K PBMCs	200K PBMCs
Con A (1.25µg/mL)	167	229	198	235
Con A (0.625µg/mL)	120	55	22	65
CEF (2µg/ml)	277	235	183	207
e64 KADRYKSR GK	52	69	2	32
e65 KADRYKSH GK	68	0	29	0
e110 LFENYTYLS K	22	42	0	0
e111 SFQNYTYLS K	53	0	0	0



N/B: * Indicates significant difference, and empty bar indicates positivity.

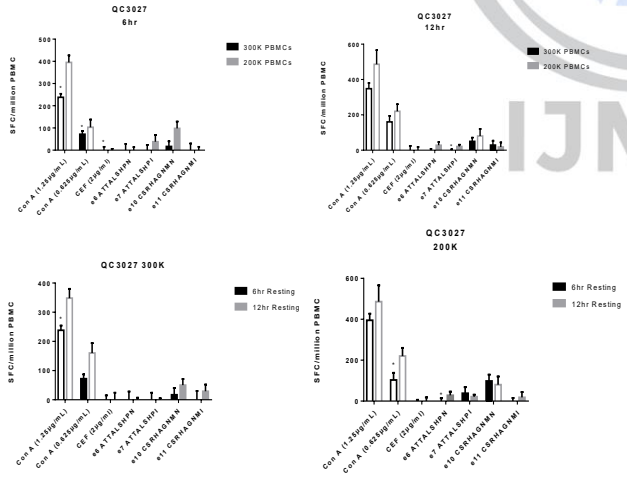
Fig 1: Assay outcome for different cell concentrations and resting times, for subject 1.

For subject 2, results on Fig 2 shows that out of seven stimulants used to stimulate two sets of PBMC concentrations (3×10^5 and 2×10^5/test well) each, using the t-test, results in response to three were significantly different (t-test P value < 0.05) between the two concentrations, when cells were rested for 6hrs prior to stimulation. However when the cells were rested for 12hrs, the results in response to just two of the stimulants were significantly different (P < 0.05) between the two concentrations. Therefore, at 3×10^5 PBMC/test well, results in response to one out of seven stimulants were significantly different (P < 0.05) between cells rested for 6hrs and 12hrs. And, for the cells tested at 2×10^5 PBMCs/test well, results in response to two stimulants were significantly

different between cells rested for 6hrs and12hrs. PBMCs from subject 2 responded positively to a total of eight stimulations. There were three positive responses when cells were rested for 6hrs and five positive responses when rested for 12hrs. Three of the positive responses were produced by cells tested at 3×10^5 PBMC/test well while five were from those tested at 2×10^5 PBMCs/test well.

Table 2: Actual spots forming cells per million PBMC at varied PBMCs concentration and resting time, for Subject 2.

	6hr Resting	6hr Resting	12hr Resting	12hr Resting
Stimulants	300K PBMCs	200K PBMCs	300K PBMCs	200K PBMCs
Con A-1.25µg/mL	238	395	348	486
Con A-0.625µg/mL	72	103	160	220
CEF2µg/ml	0	0	0	0
ATTALSHPN	0	0	0	28
ATTALSHPI	0	38	0	21
CSRHAGNMN	17	98	50	80
CSRHAGNMI	0	0	29	18



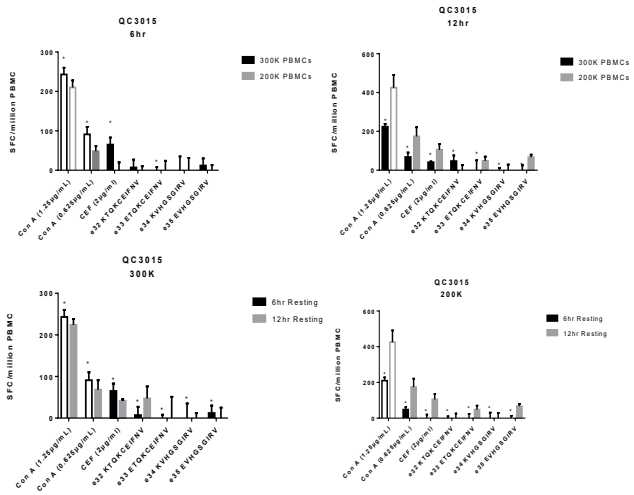
N/B: * Indicates significant difference, and empty bar indicates positivity.

Fig 2: Assay outcome at different cell concentrations and resting times, for subject 2.

For subject 3, results on Fig 2 shows that, out of seven stimulants used to stimulate two sets of PBMC concentrations (3×10^5 and 2×10^5/test well) each, results in response to four were significantly different (t-test P value < 0.05) between the two concentrations, when cells were rested for 6hrs prior to stimulation. However when the cells were rested for 12hrs, the results in response to all seven of the stimulants were significantly different (t-test P value < 0.05) between the two concentrations. However, at 3×10^5 PBMC/test well, results in response to all seven stimulants were significantly different (t-test P value < 0.05) between cells rested for 6hrs and 12hrs. And at 2×10^5 PBMCs/test well, results in response to all seven stimulants were significantly different between cells rested for 6hrs and 12hrs. PBMCs from subject 3 responded positively to a total of four stimulations. There were three positive responses when cells were rested for 6hrs and one positive response when rested for 12hrs. Two of the positive responses were produced by cells tested at 3×10^5 PBMC/test well while two were from those tested at 2×10^5 PBMCs/test well.

Table 3: Actual spots forming cells per million PBMC at varied PBMCs concentration and resting time, for Subject 3.

	6hr Resting	6hr Resting	12hr Resting	12hr Resting
Stimulants	300K PBMCs	200K PBMCs	300K PBMCs	200K PBMCs
Con A-1.25µg/mL	167	210	198	425
Con A-0.625µg/mL	120	48	22	174
CEF2µg/ml	277	0	183	105
KTQKCEIFNV	52	0	2	0
ETQKCEIFNV	68	0	29	48
KVHGSGIRV	22	0	0	0
EVHGSGIRV	53	0	0	67



* Indicates significant difference, and empty bar indicates positivity.

Fig 3: Assay outcome for different cell concentrations and resting times, for subject 3.

Effect of cryopreservation and cell resting on cell viability.

The results on Fig 4 shows that, for the three subjects, there were no siginificant differences between percentage viablity of rested and non-rested cells, irrespective of weather the cells were rested for 6hr or 12hrs. Fresh and frozen PBMCs also showed no significant variation among in their percentage viabilities.

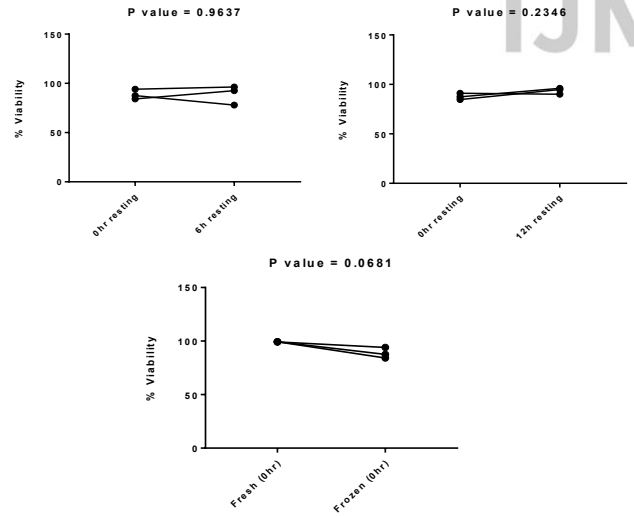


Fig 4: Comparison of % viabilty of fresh and frozen PBMCs and of PBMCs after different resting times.

DISCUSSION:

PMBCs were separated from blood samples drawn from all three recruited study subjects. The magnitude of assay outcome (number of spots/million PBMC) among these groups of cells was determined, in addition to the presence of stitistically significant differences. There were in all nine positive stimulations when the cells were tested at 2×10^5PBMC/test well, and eight positive stimulations when the cells were tested at 3×10^5 PBMC/test well. However, individual results show thatonly subject 2 had more positive stimulations at the lower concentration of 2×10^5PBMC/test well. While subject 3 had equal numbers of positive stimulations at both concentrations, subject 1 had just one positive stimulation more, at the higher concentration of 3×10^5 PBMC/test well. This shows that positive responses were slightly skwed to testing done at 2×10^5PBMC/test well. Although higher number of cells should increase the chances of contact between the stimulants and responding cells, it may have also caused the cells to pile up, resulting in a lost in linearity between the number of cells tested and the detected spots. While it is true that PBMC's concentration is crucial for sensitive detection of T-cell reponses [13], contrary to reports from Kuerten *et al*., 2012, doubling of cell concentration may not necessarily improve assay sensitivity.

Out of the seventeen positive stimulations observed from all the study subjects, ten were observed when the PBMCs were rested for 12hrs prior to stimulation and seven when rested for 6hrs. As reported by Mallone *et al*., 2011, resting had probably improved assay sensitivity by helping in the elimination of dead cells, ensuring accurate viable cell counts, However, contrary to Bourguignona *et al*., 2014, who reported that < 2hr resting produces a higher quality of cells in terms of assay sensitivity, the present study demonstrates that, 12hr resting was more beneficial. This is further supported by the negative responses

produced when both concentrations from subject 1 were each stimulated by the positive control (Con A-1.25µg/ml and Con A-0.625 µg/ml). These unexpected negative responses were observed only when the cells were rested for 6hrs. The expected positivities were restored after haven rested the cells for 12hrs (Fig 1). Resting for a longer time might have improved the quality of the cells, hence improving assay sensitivity. Quite unexpectedly, there was another negative response to the positive control (Con A-1.25µg/ml), by PBMCs from subject 3 (Fig 3). This was observed when the cells were tested at 3×10^5PBMC/test well after 12hrs resting. However, when rested for 12hrs still, but at a lower test cell concentration-2×10^5 PBMC/test well,there was a positive response to the positive control as expected. Probably, the general improvement in assay sensitivity observed at 2×10^5 cell/test well in this study, might have been responsible for this observation.

In comparing responses to each stimulant at different test cell concentrations and resting times, there were deviations from the expected linear relation, contrary to expectations,after conversion of spots counts to spot forming cells per million PBMC. Inline with another findings, variation in cell number has been reported to deviate from the expected linear relation upon conversion of spots counts to spot forming cells per million PBMC [12]. These deviations varied with the study subjects in this current study.For example,for subject 2, responses to only one of the seven stimulants was significantly different (P value < 0.05) between test cell concentrations at 2×10^5 PBMC/test well and 3×10^5 PBMC/test well, when cells were rested for 12hrs. However, when cells were rested for 6hrs, responses to three of the stimulants were significantly different (Fig 2), further indicating that assay sensitivity was improved when cells were rested for 12hrs. This trend was the same for subject 1, with two significantly different responses when the cells were rested for 12hrs and four significantly different responses, when the cells were rested for 6hrs. Thus for subjects 1 and 2, 12hrs resting proves to be more beneficial in improving the expected linear relation between the two concentrations. However for subject 3, there was a deviation from this trend, as responses to all seven stimulations were sigificantly different between the two concentrations when the cells were rested for 12hrs. When rested for 6hrs, responses there weresignificant difference to four of the stimulants. More positive responses were also observed in this subject whenPBMCs were rather rested for 6hrs. This supports the fact that in some individuals, there are deviations from the expected linear relation upon conversion of spots counts to spot forming cells per million PBMC[12]. Similarly, Wang *et al*., 2016 reported that usefulness of cell resting process depends on the cell population. Consequently, peculiarities among different cell populations seem to be a contributing factor during optimisation of test cell concentration and resting time in IFN-γ EliSpot assay.

Inorder to obtain best results, the viability of the cells should not be compromised by the set criteria. As earlier reported by Lenders *et al.,* 2010, cell viability infers in antigen processing due to decrease in the population of potential responder cells. However, because the PBMCs were notcryopreserved for a long time the percentage viability of cells were neither affected by cropreservation nor resting. Therefore, in line with findings from Owena *et al*., 2007 and Bourguignona *et al*., 2014 decrease in cell viability may only be affected by long term cropreservation. Because there were no significant difference between percentage viablity of cells rested at the different times, the concentration of PBMCs is crucial in determining assay outcome.

CONCLUSION:

The general findings from this study suggest that, with variation in test cell concentration and resting time, there was deviation from the expected linear conversion from spots counts to spots forming cells/million. Although the linear conversion from

spots counts to spots forming cells/million is expected in EliSpot assays, this study has demonstrated that PBMC resting, test number and peculiarities of cell population contributes to this deviation. Consequently standard optimization assays are recommended for optimal PBMC resting time and test cell concentration determination, for each batch of cells, especially when frozen assays are considered. Further experiments designed to include both frozen and fresh assays, with cells from additional study subjects, are underway.

Data Availability and Funding Statement

I acknowledge the Bill and Melinda Gates Foundations for sponsoring my postdoctoral fellowship program (current research findings was part of the study), at the Noguchi Memorial Institute for Medical Research. All the data for this study can be gotten from the authors, on request. This study was funded by the authors.

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Cite of article: Nfor Omarine Nlinwe. effect of varied pbmc concentration and resting time, on interferon-gamma(inf-γ) elispot assay outcome, using *plasmodium falciparum*ama 1 selected peptides. Int. J. Med. Lab. Res. 2020, 5(1):1-10

CONFLICT OF INTEREST: Authors declared no conflict of interest

SOURCE OF FINANCIAL SUPPORT: Nil