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In Vitro Responses of Human Peripheral Blood Mononuclear Cells to Candidate Vaccine Based on the M2e Peptide of Influenza Virus

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Abstract: ***Due to its rapid mutation, the influenza virus A become pandemic in many countries. Particularly in Indonesia, the H5N1 cases is one of the highest with the death rate is almost 90%. An efficient vaccine to address the problem has not been discovered, and as type A influenza virus continues to mutate. The current study focuses on developing a conserved epitope-based universal influenza virus vaccine, M2e. One of them is M2e2-16-K-P25 which is the 2nd to 16th sequence M2e epitope connected to the helper T cell epitope P25 via lysine residue (K). The purpose of this study is to determine the formulation and incubation period of optimum candidate influenza virus vaccine universal epitope M2e in triggering Peripheral Blood Mononuclear Cells (PBMC) to produce the cytokines interleukin-4 (IL-4) and interferon-γ (IFN-γ). PBMC was isolated by a gradient centrifugation method with Ficoll-Hypaque 1077, then PBMC was cultured in complete growth medium and stimulated by a vaccine candidate formulation. The concentrations of IL-4 and IFN-γ were measured by the Enzyme-Linked Immunosorbent Assay (ELISA) method, and cell viability testing was performed by addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). The results showed that the concentration of 2 nmol with the addition of alhydrogel during the incubation time of 120 hours, was the formulation of vaccine candidate and the optimum incubation time in triggering PBMC to produce IFN-γ with the concentration of 328 pg / mL, but IL-4 production tended not to be provided. Cell viability test with MTT showed values that were aligned with ELISA results, which at 120 hours incubation period increased cell proliferation by 500%.***

Keywords: epitope M2e, influenza A, IFN-γ, IL-4, PBMC

1. Introduction

Influenza is a growing pandemic disease. History records that this disease began to be known in 1918 as the Spanish flu (H1N1), 1957 Asian flu (H2N2), 1968 Hong Kong flu (H3N2) and in recent years such as H7N7, H5N1, H7N9, and H9N2. Based on cumulative data from 15 countries collected by WHO from 2003 to 2013, there have been 641 cases of avian influenza. Indonesia ranks first as the country with the largest cases of bird flu, which is 197 cases and 165 people died (WHO, 2014). Subtypes on influenza viruses based on two proteins on the surface of the virus, namely Hemaglutinin (HA) and Neuraminidase (NA). It is known that there are 16 subtypes of HA and nine subtypes of NA. In its development influenza virus continues to mutate in antigen of HA and NA so it can mutate into various kinds of combination of virus types, such as H5N1, H7N3, H7N7 (CDC, 2013). Thus, a new viral strain cannot be overcome with a vaccine that has been developed for previous strains of the virus. To prevent the transmission of influenza viruses, researchers began designing and developing vaccines. The vaccine that is currently used for influenza viruses consists of inactive virus strains that have previously been bred in egg embryos. The equipment used in vaccine manufacturing is quite large and costly, so now researchers are beginning to see the possibility of producing a universal vaccine that can be used for more than one type of influenza virus, one of which is the epitope-based universal vaccine (Parida et al., 2007).

According to some previous studies the benefits of epitope-based vaccines include a high specificity in generating an immune response, high purity, large production capacity, and cost-effective production. Epitope-based vaccines can also be designed to stimulate the immune response of a conserved epitope so that it can be a universal vaccine. The vaccine is safer because it does not contain live microbes that can multiply and cause disease, and reduce the occurrence of autoimmune (Subroto et al., 2013). Efforts to develop epitope-based vaccines for influenza viruses are based on M2 proteins from influenza viruses located in the outer cell domain. Protein M2 is the proton channel necessary for viral acidification, allowing for replication. M2 contains one small ectodomain at the end of N called M2e. Unlike HA and NA also located on the surface of the virus, M2e in the form of 24 amino acids (MSLLTEVETPIRNEWGCR-CNDSSD) is conserved in all influenza viruses (Fiers et al., 2004). This M2e preservation allows the design of a universal influenza vaccine. Epitope M2e2-16 (SLLTEVETPIRNEWG) is recognized by monoclonal antibodies that inhibit the replication of influenza A viruses in model cells. Therefore, a conserved epitope-based vaccine using the M2e epitope is an alternative to stop the spread of avian influenza virus (Pejoski et al., 2010). Another epitope containing helper T cells is P25 with amino acid sequence KLIPNASLIENCT-KAEL is a fusion protein derived from viral disease in dogs (Canine distemper virus - CDV). This epitope has been widely used as a helper cell epitope, as it is easily recognized by helper T cells and activates both the innate

and adaptive immune system to deal with pathogen attack (Jackson et al., 2004; Alphs et al., 2008).

Before application to the public, vaccine candidates should be tested for efficacy by some preclinical and clinical trials. One of the successful vaccines in protecting the pathogen attack is characterized by the ability of the vaccine to trigger the formation of cells involved in the immune system. One of them is a monocyte cell that is a mononuclear cell that is mostly found in peripheral blood. Human Peripheral Blood Mononuclear Cell (PBMC) or are the cellular part of the blood that contains all of the round-celled blood cells. PBMC consists of monocytes, T cells, B cells, natural killer cells (NK), and dendritic cells. PBMC cells contain cell types that play an important role in the immune system (Končarević et al., 2014).

In this study we used an epitope component consisting of a sustainable target epitope (M2e) and a helper T cell epitope (P25) with linear geometry (M2e 2-16-K-P25) derived from the biosynthesis process in the previous study. The epitope component with linear geometry has a T cell helper epitope P25 connected to the epitope M2e 2-16 through the lysine residue (K). The epitope component will be formulated with adjuvant aluminum hydroxide gel and tested for efficacy to see the cytokine response. Cytokines are soluble glycoproteins in the form of signals involved in the stimulation of the immune system response. In this study, we will see the responses of IL-4 and IFN-γ cytokines produced using the direct sandwich ELISA (Enzyme-Linked Immunosorbent Assay) technique. IL-4 and IFN- γ cytokine responses are selected because these two cytokines are secreted from different cells and also have various functions in the immune system. This study is expected to provide information on the optimum conditions of candidates for universal influenza vaccine, especially epitope M2e in triggering the cytokine response in question.

2. Material and Methods

2.1 Material

Materials used in this study were healthy blood samples, M2e epitopes own biosynthesis results, and as a comparison we use also from commercial (GL Biochem, Shanghai Ltd.), Phosphate Buffered Saline (PBS), Ficoll-Hypaque 1077 (Sigma Aldrich), RPMI-1640 media (Sigma Aldrich), Trypan blue (Sigma Aldrich), Gentamicin (Sigma Aldrich), Penicillin (Sigma Aldrich), L-glutamine (Sigma Aldrich), Fetal Bovine Serum (FBS), tween-20, aluminum salt hydroxide gel (Alhydrogel), sodium chloride physiological 0.9%, MTT (3- [4,5-dimethylthiazol 2-yl] -2,5-diphenyl tetrazolium bromide (Sigma Aldrich), dimethyl sulfoxide (DMSO),ELISA Ready-Set-Go kit (e-Bioscience).

2.2 Methods

Isolation Human Peripheral Blood Mononuclear Cells
Blood samples were taken from a healthy donor of ± 30 mL. Blood is accommodated in the EDTA vacutainer. Blood collection is done in the morning so that the condition of blood cells is still in good condition. Blood samples were dissolved in PBS by a ratio of 1: 1, then Ficoll-Hypaque 1077 was carefully added to form two phases. After that, centrifugation was done at 1800 rpm at 4 °C for 20 minutes. The layer between the two phases (containing PBMC) was harvested and washed twice using RPMI-1640 medium at a rate of centrifugation of 1800 rpm at 4 ° C for 5 minutes. After washing, the cell pellet was resuspended, and PBMC viability was tested with trypan blue using hemocytometer and microscope.

Culture and Stimulation of PBMC
The isolated PBMC was diluted with a complete RPMI 1640 medium (RPMI-1640, 100 U / ml penicillin, 100 µg / ml gentamicin, L-glutamine) to a total PBMC of 1x106 cells / mL. PBMC is placed on a multi-dish plate of 100µL / well as needed. Then the culture was stimulated with a vaccine formulation of 0.5, 1, and 2 nmol concentrations with and without alhydrogel and incubated at 37 ° C for 24, 72, and 120 hours.

Determination of IFN- γ and IL-4 Concentrations by ELISA Method
Determination of IFN-γ and IL-4 in incubated cultures for 24 hours, 72 hours, and 120 hours followed the ELISA Ready-Set-Go! Manual. The ELISA NUNC Maxisorp 9018 plate is coated with 100 µL / antibody well (Ab) anti-IFN-γ in the buffer coating. After the plate was incubated overnight at 4 ° C, the plate was washed with 250 µL PBS containing 0.05% Tween-20 (wash buffer) 5 times. Furthermore, as much as 200 µL 1X Diluent Assay / ELISPOT was added, and the plate was incubated at room temperature for 1 hour. Then the plate was washed again with 250 µL wash buffer five times. A standard (human recombinant IFN-γ protein) of 100 µL is added to each appropriate well. Followed by the addition of 100 µL samples to each different well accordingly. The plates are then incubated overnight at 4 ° C. The liquid on the plate is removed and washed five times with 250 µL wash buffer. After that 100 µL detection antibody was diluted with 1X Assay Diluent / ELISPOT to each well, then the plate was incubated for 1 hour at room temperature. The liquid on the plate is removed and washed five times with 250 µL wash buffer. A 100 µL of diluted avidin-horseradish peroxidase (Avidin-HRP) enzyme in 1X Assay Diluent / ELISPOT was added to each well, and the plate was incubated for 30 min at room temperature. The liquid on the plate was removed and washed five times with 250 µL wash buffer, and then each well was soaked in wash buffer and allowed to stand for 1-2 minutes, then the liquid was removed and repeated wash with wash buffer up to 7 times. Thereafter, as much as 100 µL substrate solution of tetramethylbenzidine (TMB) was added to each well. After the plates were incubated for 15 min at room temperature, 50 µL reaction-stopping solution, H2SO4, was added to each well. The plates are measured with an ELISA reader at a wavelength of 450 nm. The same procedure was performed for the determination of the IL-4 response with a special ELISA kit Interleukin 4.

Cell viability test
Cellular viability was assayed by adding 10 µL MTT (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide) at 100 µL all cultures that had been incubated for 24, 72, 120 hours. Then incubated for 4 hours at 37 ° C. After incubation, add 250 µL DMSO then incubated again

for 1 hour under the same conditions. Then measured its absorbance at a wavelength of 630 nm.

Statistical analysis

Data were expressed in average value. The ANOVA test is used to see the difference in the effect of the treatment on the resulting response. The difference is considered significant when the p-value is less than 0.05, and the F count is greater than F table. ANOVA test was performed using SPSS software.

3. Results and Discussion

The development of epitope-based vaccines in dealing with influenza virus pandemics continues to show progress. One is the epitope M2e (2-16) -K-P25 that has been successfully designed and tested in vivo in mice with good results in inducing antibodies (Subroto et al., 2013; Mansyur, 2015). In this study, epitope efficacy test was done using PBMC induction method, to see the immune system response based on the cytokines produced. Based on its nature as a representative of the human immune system, the efficacy test using PBMC certainly helps the vaccine development process in the preclinical testing stage, so there is no need for direct vaccination to human donors that may be harmful.

The vaccine formulation comprises variations of peptide concentration combined with and without alhydrogel, then added 0.9% NaCl as a solvent whose concentrations correspond to human body fluids. Vaccine candidate formulations were prepared in concentrations of 0.5, 1, and 2 nmol with each peptide concentration of 18.8, 37.5 and 75.1 µg / mL, respectively. The concentration is selected based on Leggat's (2014) study which explains that the lower concentration of peptides used to produce T-cell responses, the more effective the vaccine candidate. T cell activation is naive to the effector of T cells as well as memory T cells based on peptides as antigens shown as APC via MHC by mature dendritic cells. In his research also explains, that with excessive antigen concentration can decrease the proliferation of T cells and trigger autoimmune. Activation of T cells will secrete cytokines to activate and trigger other cell functions in defense of the immune system. Subsequent variations of formulations were made with and without adjuvant, then physiological NaCl 0.9% was added until each volume reached 4 mL. The addition of these adjuvants is done to improve the immunogenicity of the low M2e epitope. Adjuvant also works in expanding the immune response and can save the dose of epitope used (Houser & Subbarao, 2015), so adjuvant can help achieve the goal of developing a universal influenza vaccine based on the increased response of the human immune system (Wiersma et al., 2015). In this study used aluminum hydroxide salt (alhydrogel). Adjuvant has been used for 70 years, due to its safety and capability. Alhydrogel absorbs and keeps peptides in vaccines and makes it last longer, so the peptide immunogenicity increases to slowly be shown by APC through the immune system response. Besides, alhydrogel also induces mild inflammation through immune cells on the infectious side but does not induce Th1 cellular immune system (Lee et al., 2014; Tetsutani & Ishii, 2012).

Induction of PBMC by epitope for producing cytokine

Blood sampling from one donor with a very good medical history, no smoking, and ideal body weight. Blood Collection was done at PMI Bandung Blood Transfusion Unit Bandung - Indonesia Branch in the morning. Blood was taken then stored in the EDTA vacutainer to maintain blood conditions to avoid coagulation. PBMC as a stimulable medium is obtained from blood isolation by a gradient centrifugation method with Ficoll-Histopaque 1077 (Sigma Aldrich) as a separating medium, in which the solution has a 1.077 g / mL density which is known to be slightly larger than that of PBMC. The centrifugation process was carried out at 1800 rpm at 4 ° C for 20 minutes. Low centrifugation speed is done to keep the blood composition from damaging. After the centrifugation process, PBMC will look like a white cloud clump between the blood plasma and the separating medium (Miyahira, 2012), because PBMC has a lower density than the separating medium and is higher than the blood plasma. PBMC is carefully pinned, then transferred to a different centrifuge tube. From 30 mL of the blood sample, obtained about 1 mL of PBMC. This stage is done to wash PBMC and remove other components such as blood plasma and separation media that may be carried along during the process of packaging. PBMC is reset with RPMI-1640 media. PBMC was piped into the plate and mixed with trypan blue as a cell dye, with hemocytometer live cell count observed and calculated using the Olympus CX 21 microscope. Trypan blue as a cell dye would mark dead cells in blue, because the cell membrane on cells that have been damaged, so that the dye trypan blue can enter and mark the dead cell. In this study found as many as 40 x 10^6 cells / mL. The obtained PBMC was diluted by adding of complete RPMI media, thus obtaining the cell.

In Figure 1- A, B, and C the following shows the variations in the concentration of vaccine formulations at different incubation times resulting in different IFN-responses. The optimum concentration at these three times was at a concentration of 0.5 nmol formulated with adjuvant alhydrogel with each IFN-γ concentration of 103 pg / mL at 24 hours incubation time, 270 pg / mL at incubation time of 72 hours and 395 pg / mL at 120 hours incubation time. At the time of concentration of the i 2 nmol vaccine without adjuvant alhydrogel, after 24 hours and 72 hours incubation resulted in a higher IFN-respon response than the vaccine with the same concentration formulated with adjuvant alhidrogel, of 94 78 pg / mL (24 h) and 240 vs. 210 pg / mL (72 hours). However, at 120 hours the difference was slightly lower with a 2 nmol concentration vaccine formulated with adjuvant alhydrogel (331 vs 350 pg / mL).

From the graph above shows the most optimum incubation time resulting in significant IFN-respond response is at 120 hours with optimum concentration of vaccine candidate formulated with adjuvant alhydrogel concentration of 0.5 nmol as indicated by highest IFN-γ concentration. Overall, differences in IFN-respon response resulting from vaccine formulations with adjuvants and those that are not, are few. So it can be said that the Epitop M2e branched vaccine candidate is immunogenic enough to activate a specific immune response. Th1 cytokines are measured in concentration based on their absorbance compared to the

standard curve through linear regression. IFN-Standar standard used, ie at a concentration of 500; 250; 125; 62; 31 pg / mL with linear equation y = 0.000438x + 0,037693.

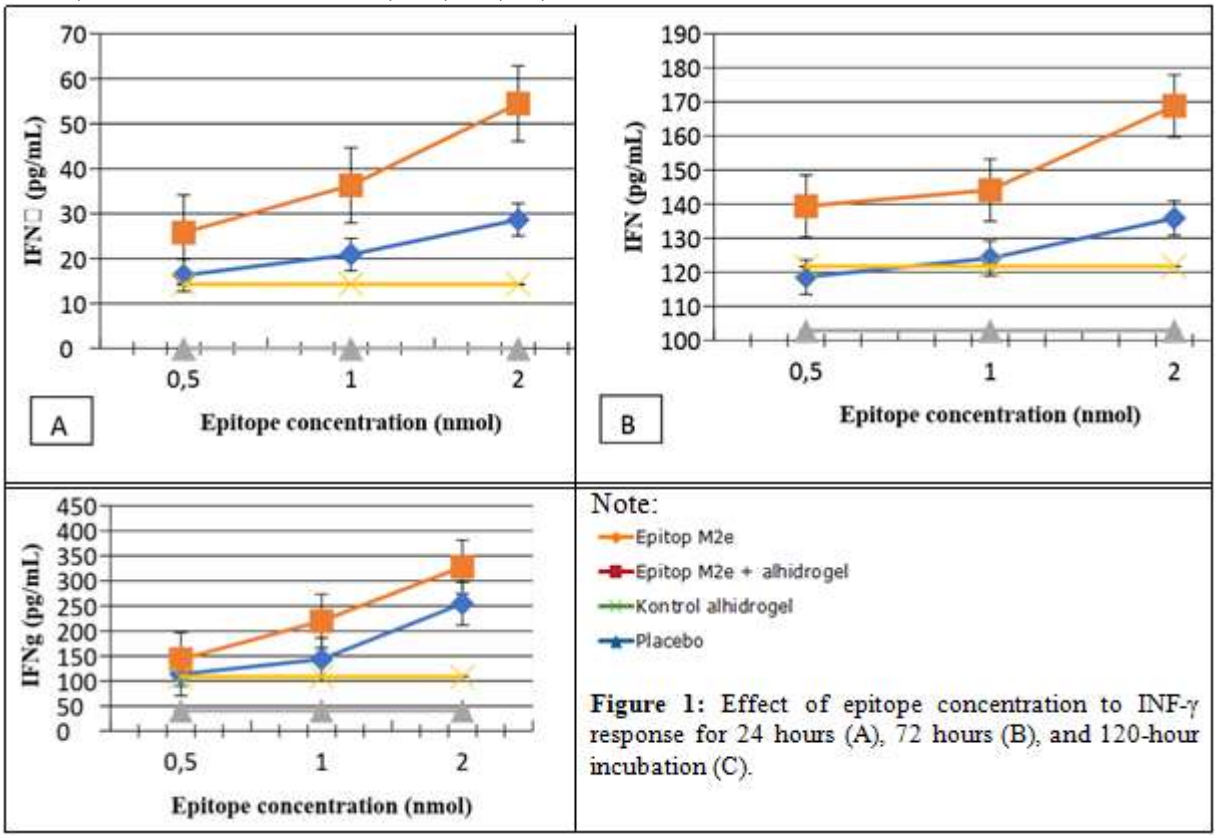
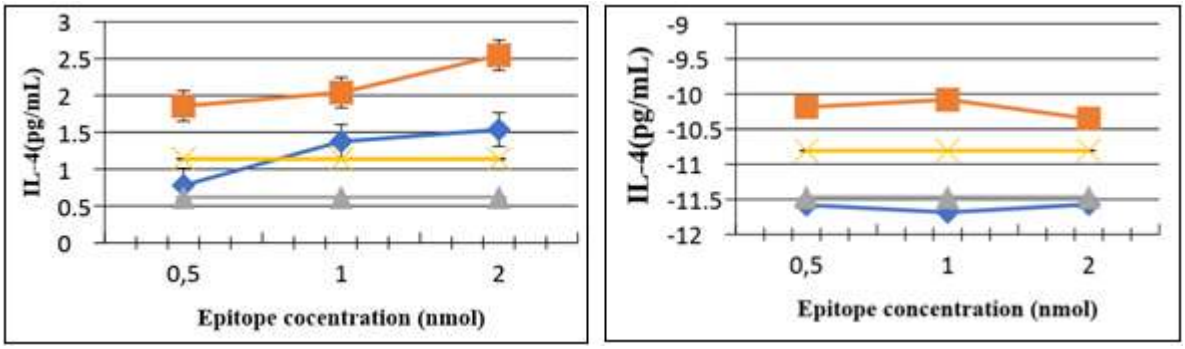


Figure 1: Effect of epitope concentration to INF-γ response for 24 hours (A), 72 hours (B), and 120-hour incubation (C).

In the following Figures 2- A, B and C, shown the variations in the concentration formulation of set epitopes of M2e vaccine candidates at different incubation times resulted in different IL-4 responses. The most optimum concentrations in each of these three times were at 2 nmol vaccine candidates without adjuvant alhidrogel with IL-4 concentrations of 0.5 pg / mL at 24 hours incubation time, 0.5 nmol vaccine candidate without adjuvant with concentration IL-4 at 4 pg / mL at incubation time of 72 hours and 0.5 nmol vaccine candidate formulated with adjuvant alhidrogel resulting in an IL-4 concentration of 11 pg / mL at 120 hours incubation time. At all three times the vaccine showed different optimum doses. At 24 hours incubation, 2 nmol concentration without adjuvant resulted in the highest IL-4 response than induction of vaccine candidates formulated by adjuvant. At incubation time of 72 hours, the concentrations of 0.5 and 2 nmol vaccine candidates without adjuvant also show the same, yielding a higher IL-4 response than the vaccine formulated with adjuvants. However, at 120 hours incubation time, the formulation of the adjuvant vaccine candidate resulted in a higher IL-4 response than the vaccine candidate without adjuvant in all three concentrations. In the graph above shows the most optimum concentration of candidate vaccine at a concentration of 0.5 nmol which is formulated with adjuvant alhydrogel at an optimum time of 120 hours at 11 pg / mL. The IL-4 responses generated between vaccine candidates with and without adjuvants differ only slightly. Similarly, the IFN-respon response results. This is enough to prove that Epitop M2e vaccine candidates are quite immunogenic without added adjuvant. Overall outcome Epidop M2e vaccine candidates branched more stimulating Th1 cells by producing more IFN-daripada than Th2 cells producing IL-4. The difference is very significant (395 vs. 11 pg / mL). As is known both of these cytokines work antagonist to each other.



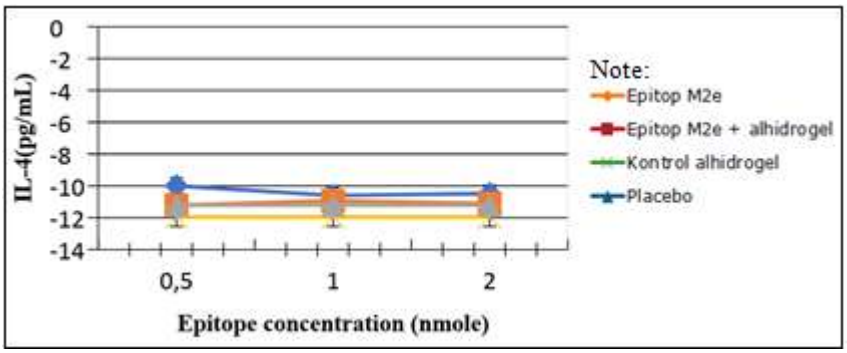


Figure 2: Effect of epitope concentration to IL-4 response for 24 hour (A), 72 hour (B), and 120 hour incubation (C)

According to Mcelhaney (2011), Th's response to influenza virus plays a key role in humoral and CTL immune responses to influenza vaccination. T helper type 1 (Th1) effectively stimulates the production of IFN-γ, and CTL memory, while Th2 stimulates the antibody response and the production of IL-10 and IL-4, which suppresses the Th1 response. Anti-influenza antibodies are stimulated by Th1 and Th2 for each, producing IgG2a and IgG1 to respond to influenza vaccination. For vaccine development, Th and CTL can recognize viral peptides through MHC II and MHC I, resulting in effective antigen presentation. Peptides derived from live viral replication are effectively presented on both MHC I and MHC II. In effective viral vaccines are effectively turned off at MHC II but are less effective at MHC I so it generally stimulates weak CTL responses (Rock & Shen, 2005) that rely on prior primary responses of natural infections. Human studies have confirmed that CTL recruitment to infected lung tissue depends on the production of Th1 cytokines and thus a shift from the production of Th1 to Th2 cytokines. Thus, influenza vaccines that can produce a shift towards Th1 responses and more effectively stimulate both the production of antibodies and CTL memory, will increase protection against influenza virus. It should also be noted that the delivery of viral antigens to MHC should be effective as well as both MHC I and MHC II. Th1 cytokine is IFN-γ which is in charge of the expression of both. Thus, the Epitop M2e vaccine candidate can be used effectively as an influenza vaccine candidate. Adjuvant alhidrogel used according to Pelendran and Ahmed (2011), adjuvant aluminum activates the innate immune system, activation of dendritic cells and also activates Th2 which will produce an antibody response. This is contrary to research conducted. In the adjuvant control itself more stimulates Th1 cells than Th2 cells. Seen at optimum time of 120 hours on adjuvant diol to produce IFN-γ equal to 357 pg / mL while IL-4 concentration yielded only 8 pg / mL.

Figure 3 shows the results of cell viability test with MTT test (3- (4,5-dimethylazol-2-yl) -2,5-diphenyltetrazolium bromide) is a colorimetric method, wherein this MTT reagent is a tetrazolium salt which can be broken down by the succinate system tetrazolium reductase present in the cell respiration pathway in the active mitochondria of living cells form the purple formazan crystals. The addition of a stopper reagent, such as DMSO, will dissolve the formazan crystals which are then read using the ELISA reader. The intensity of the purple color that is formed is proportional to the number of living cells. If the intensity of the purple color is greater, then the number of living cells is increasing. PBMC proliferation based on cell viability test results with MTT method showed results in line with ELISA results. The graph shows (Fig.3) an increase in the percentage of living cells after induction with various vaccine formulations at different incubation times. With significant cell proliferation occurring in peptide-induced cells at a concentration of 0.5 nmol with the addition of alhidrogel at 120 hours incubation time.

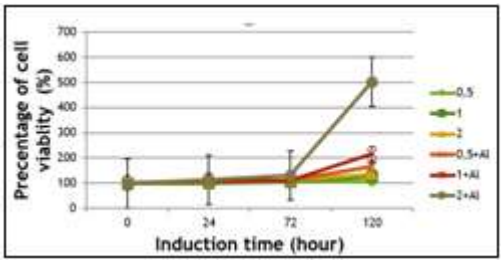


Figure 3: Proliferation of PBMC cells after induction by epitope vaccine

4. Conclusion

Candidates for universal influenza virus vaccine through efficacy testing on PBMC may trigger IFN-© cytokine production, but IL-4 production tends not to be produced. The 2 nmol vaccine formulation with the addition of alhydrogel and incubation time for 120 hours is the optimum condition in triggering PBMC to produce 328 pg / mL of IFN- γ cytokine.

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