

CONSERVE EPITOPES OF INFLUENZA VIRUS INDUCE INNATE AND ADAPTIVE IMMUNE RESPONSES TO PRODUCE SPECIFIC ANTIBODY AGAINST M2E PROTEIN

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ABSTRACT

The existing vaccines against influenza are based on the generation of neutralizing antibody primarily directed against surface protein, haemagglutinin (HA) and neuraminidase (NA). However, antigenic drift and occasional shift of these two membrane glycoproteins, HA and NA, make vaccine production cumbersome and necessitate yearly revision of the vaccine seed strains by the World Health Organization. For these reasons, many investigators have often tried to look at the possibility of generating a universal vaccine useful against more than one influenza strain. The objective of research was to obtain an alternative antigen as vaccine candidate for universal flu vaccination, instead of HA and NA components. In this study, we use conserved epitope M2e which is consist of three major component such as N-terminal M2e2-24 (24 amino acids), trans-membrane (59 amino acids) and C-terminal (19 amino acids). We design two components of antigen, linier and branched structures. The antigens then formulated with aluminium hydroxide gel compared to FCA/IFA adjuvant. These vaccines were tested their immunogenicity, and the potency to mature the dendritic cells for stimulating either CD8+ T cell or antibody-mediated immune responses. The antibody titre and the maturity of dendritic cell indicated by cytokines concentration such as; IFN- γ , IL2 and IL4 were measured by ELISA test. The result of research showed that the conserved epitope of Me2 2-16 when incorporated with P25 protein from canine distemper virus (linear structure) in alhydrogel adjuvant has greater potential to produce anti-M2e antibodies than in Freund adjuvant. Alhydrogel adjuvant had a stronger effect than Freund adjuvant. Alhydrogel also stimulate the release of IL-2 and IL-4.

Keywords: Epitopes, innate and adaptive immune responses, M2e-protein, haemagglutinin (HA), neuraminidase (NA), adjuvant.

ABSTRAK

Vaksin influenza yang ada saat ini dibuat berdasarkan atas protein permukaan virus hemagglutinin (HA) dan neuramidase (NA). Akan tetapi, karena kedua membran glikoprotein HA dan NA ini mudah mutasi, secara drift dan shift, maka vaksin yang diproduksi harus selalu direvisi setiap tahun oleh Badan Kesehatan Dunia (WHO). Para peneliti berusaha mencari kemungkinan untuk membuat vaksin universal yang bisa digunakan terhadap berbagai strain virus influenza. Tujuan dari penelitian ini adalah untuk mencari antigen alternatif untuk kandidat vaksin influenza universal selain HA dan NA. Dalam studi ini, kami menggunakan epitope M2e

lestari yang mempunyai tiga komponen utama, yaitu N-terminal M2e2-24 (24 asam amino), komponen transmembran (59 asam amino) dan C-terminal (19 asam amino). Kami mendesain dua komponen antigen, yaitu struktur linier dan bercabang. Kedua antigen tersebut kemudian diformulasi dengan adjuvant aluminium hidroksida gel, dibandingkan dengan adjuvant FCA dan IFA. Vaksin-vaksin ini kemudian diuji imunogenisitasnya dan kemampuan untuk mematangkan sel dendritik yang akan menstimulasi sel CD8+ T atau respon kekebalan yang dimediasi antibodi. Adanya titer antibodi dan kematangan sel dendritik ditunjukkan oleh adanya konsentrasi sitokin seperti interferon gamma, IL-2 dan IL-4 diukur dengan metode ELISA. Hasil penelitian menunjukkan bahwa epitope lestari M2e 2-16 jika digabungkan dengan protein P25 dari CDV (struktur linier) Di dalam adjuvant alhidrogel mempunyai potensi yang besar dalam memproduksi antibody M2e dibandingkan dengan adjuvant Freund. Adjuvan alhidrogel mempunyai efek yang lebih besar dari pada adjuvant freund. Alhidrogel juga menstimulasi penengluaran sitokin IL-2 dan IL-4.

Kata kunci: Epitop, respon imun bawaan dan adaptif, M2E-protein, hemagglutinin (HA), neuraminidase (NA), adjuvant.

INTRODUCTION

The world history recorded six times pandemic flu caused by influenza A virus. The worst case pandemic flu happened in 1918 in Spain, fifty million people were killed. WHO always monitored the movement of influenza A virus every year. The effort seemingly successful to control the situation, there is no pandemic flu since 1977 until now. However, the mankind still worry as the epidemic influenza still happened in several countries like Hongkong, and Mexico in 2009 and almost 50 countries has influenza H5N1 outbreak since 2000-2006. The worst case of influenza H5N1 infection during this period was in Indonesia. The mortality rate was 84% compare with the world mortality rate case which is only 46%. Vaccination was a turning point in the war between microbes and humans, vaccines represent the most cost-effective life-saving device in history. The failure to develop influenza vaccines against global pandemics will bring to a great disaster for humanity (WHO, 2012).

Research during the past decade has identified a fundamental role for the innate immune system in sensing

vaccines and adjuvants and in programming protective immune responses. The innate immune system can sense microbes through pattern-recognition receptors (PRRs), such as the Toll-like receptors (TLRs), which are expressed by various cells, including dendritic cells (DCs). In addition to TLRs, other types of PRRs, including the C-type lectin-like receptors and the cytosolic Nod-like receptors, sense a broad range of microbial stimuli, and the cytosolic RIG-I-like receptors sense viral nucleic acids (Reed et al., 2008). Dendritic cell (DCs) take up antigen, generate peptide epitopes from it, and then load these epitopes into molecules that are encoded by the major histocompatibility complex (MHC). After export to the cell surface, MHC molecule-epitope complexes are presented to T cell, leading to their activation. Activated CD4+ helper T (Th) cells are now able to deliver signals to DCs, enabling them to activate naïve CD8+ T cell more efficiently and also improve the CD8+ cell's ability to assume memory cell status, providing the ability to clear pathogens when subsequently encountered. Activated Th cell can also interact directly with B cells, providing them with signal that controls differentiation, expansion, and shaping of the antibody iso type that they secrete (Alberts et al., 2008).

Epitope has a high specificity in eliciting immune responses. Despite several potential advantages, the poor immunogenicity of epitope in the absence of adjuvant. And the adjuvant system suitable for human use has limited number approved by WHO. Many experimental adjuvants provide danger signals to DCs by engagement of one or more Toll-like receptors (TLRs) (Jackson et al., 2004). In this study we have designed a simple synthetic vaccine structure composed of a Th epitope from conserved M2e protein as a target epitope joined with P25 as T helper epitope, M2e 2-16-K-P25 and branched structure M2e 2-10-K-(P25)M2e 11-24 representing two Th epitopes from M2e 11-24 and P25 and B epitope from M2e 2-10. The antigen was formulated with aluminium hydroxide gel in comparison with Freund's complete adjuvant, targeting DC maturation, and induction antibody or cytotoxic T lymphocyte (CTL) responses.

Alhydrogel adjuvant was chosen in this study since this adjuvant has been approved by WHO and was used for long time without any adverse reactions.

To be more effective and enhance the solubility of peptide/epitope afforded by placement of the lysine (K) molecule between the epitopes to create the linear and a branched structure, makes them highly attractive for the development of vaccines for human and animals. Dendritic cells (DCs) are instrumental in the initiation of the innate immune response because of their ability to take up antigens, to transport antigens to lymph nodes and to activate naive antigen-specific T cells (adaptive immune response) (Alberts et al., 2008). Effective stimulation of T cells requires appropriate signals from DCs. The first signal is provided by processed antigenic peptides bound to MHC molecules recognized by the T cell receptors, and the second signal by the binding of co-stimulatory molecules to their ligands on T cells. Presentation of signal 1 in

the absence of signal 2 leads to an important process in the maintenance of tolerance to self-molecules. A third signal is provided by cytokines and instructs the differentiation of T cells into TH1 or TH2 effector cells that can effectively deal with the inciting event, e.g. the infectious agents that induced the immune response. For example, the secretion of IL-12 directs the differentiation of CD4+ T cells to TH1 cells. Immature DCs localized in peripheral, non-lymphoid tissues are effective in the uptake of antigens through phagocytosis, receptor-mediated endocytosis, but do not effectively activate T cells because of the low expression of co-stimulatory molecules and cytokines. Maturation of DCs is characterized by increased expression of co-stimulatory molecules (signal 2) and secretion of cytokines (signal 3) resulting in mature DCs that efficiently activate antigen-specific naive T cells. Aluminium salts are common adjuvants in human and veterinary vaccines because of their excellent safety record, proven ability to enhance the immune response to a variety of antigens, and low cost. The two main types of aluminium containing adjuvants are aluminium hydroxide, and aluminium phosphate adjuvant. The mechanism by which aluminium-containing adjuvants enhance the immune response is poorly understood. A commonly held view is that aluminium-adsorbed vaccines form an antigen depot from which antigens are slowly released over time. Aluminium-containing adjuvants predominantly stimulate TH2 responses and this is independent of the cytokines IL-4, IL-6, and IL-13. The TH2 response was diminished in the absence of IL-18 (Sokolovska et al., 2007)

We postulated that aluminium-containing adjuvants stimulate the immune response via a direct effect on DCs. Previous studies did not demonstrate an effect of aluminium-containing adjuvants on mouse DCs in vitro, but the adjuvants did induce, indirectly or directly, the expression of co-stimulatory molecules and DC markers on human monocytes and macrophages. The experiments indicate that aluminium adjuvants directly enhance the presentation of antigens to T cells (signal 1), the expression of the co-stimulatory molecule CD86 (signal 2) and the secretion of IL-1 β and IL-18 (signal 3) by DCs. Neutralization of these cytokines and inhibition of their secretion demonstrated that they support TH2 cell differentiation (Sokolovska et al., 2007).

It forms a proton-selective ion channel, which is activated at acidic pH and is a specific target of the anti-influenza drugs amantadine and rimantadine. During virus entry, *via* receptor mediated endocytosis, M2 transport protons across the virus membrane reducing the pH of the virion interior (De Fillete et al., 2008).

The M2 also plays an important role in virus morphogenesis and assembly. The M2 protein has 96 amino acids, with three structural domains: an amino-terminal extracellular domain (comprising 23 residues), a trans membrane domain (TM) (19 residues), and a cytoplasmic domain (54 residues) (Betakova, 2007).

The extracellular domain of M2 is important for its incorporation into virions. (M2e), which remain nearly invariable since the 1918 Spanish flu (Reid et al., 2002).

MATERIALS AND METHODS

Mice; Female BALB/c mice (6–8 weeks of age) were obtained from an in house breeding colony at PT. Bio Farma, Bandung. Mice were maintained in a conventional barrier facility at 21 ± 2 °C and $50\pm 20\%$ relative humidity, and allowed free access to water and Lab Diet 5015 (Purina Mills Inc., Richmond, IN). Animal procedures were performed according to NIH guidelines for care and use of experimental animals and all experimental protocols involving mice were approved by Purdue Animal Care and Use Committee.

Immunization and bleeding protocols; Mice were grouped in three cages of 5 mice/cage, were inoculated twice on day 0 and 14 by subcutaneous route with 5, 10, and 20 nmol/dose peptides administered in alhydrogel compared to FCA/IFA adjuvant. Mice were bled through the retro-orbital plexus on day 10 to determine primary Ab response, and days 25 to determine secondary response.

Reagents; The epitope choice was M2e2-24 as this component conserved since 1933 (Fiers et al., 2008). The Peptide/epitope was successfully synthesized in in Biochemistry Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Padjadjaran University, using SPPHF moc/ btu method (Barlos and Gatos, 1999). The linear structure of epitope consist of B cell and T cell epitopes. Fitzmaurice et al., reported, branched structure of epitope has good immunogenicity and good antibody responses specific to M2e. We bought branched epitope from Genscript, USA. The linear structure has two epitopes, representing B cell from M2e2-16 and T cell epitope from P25 protein from canine distemper virus, (M2e 2-16-K-P25), and the branched structure has three target epitope representing B cell epitope from M2e-2-10, and two T cell epitopes from M2e 11-24 and P25, (M2e 2-10-K-(P25) M2e 11-24). The antigens then formulated with aluminium hydroxide gel compared to FCA/IFA adjuvant (Sigma-Aldrich. St. Louis, MO). Alhydrogeland FCA/IFA adjuvants was uses as an adjuvant for the experimental vaccines. Aluminium hydroxide adjuvant were diluted with 0.9% NaCl and adjusted to pH 7.4 with 0.1M HCl and autoclaved at 121°C for 20 min. Vaccines were tested their immunogenicity and the potency to mature the dendritic cells for stimulating either CD8+ T cell or antibody-mediated immune responses. The maturity of dendritic cells indicated by the cytokines concentration, (IFN- γ , TFN- α , IL2 and IL4) and antibody titre was measured by ELISA test.

Determination of antibody titre; 96-well Immuno Platewere coated overnight at 4°C with 100 μ L/well of the M2e peptide diluted in PBS 1x. Plates were blocked with PBS/0.1% Tween-20/2% BSA (150 μ L/well) for 1h at 37°C, after washing three times with PBS/0.05% Tween-20, sera were added in 2-fold serial dilutions starting from 1:50. The plates were incubated for 90 min at 37 °C, washed four times and then incubated with 100 μ L of *goat peroxidase label anti mouse IgG* for 30 minutes at 37°C. After four washes, the presence of IgG was detected with 100 μ L of tetramethylbenzidine. The reaction was stopped by adding 100 μ L of 0.18M H₂SO₄. The OD was read at 450 nm. Results are expressed as an antibody endpoint titre.

Determination of cytokines (IL2, IL4 and IFN- γ) concentration; IL 4 was determined by ELISA (Affymetrix e-Bioscience). Corning Costar 9018 (or Nunc Maxisorp®) ELISA plate was coated with 100 μ L/well of capture antibody in 1X Coating Buffer (dilute as noted on C of A, which is included with the reagent set). Plate was sealed and incubated overnight at 4°C. Wells were aspirate and wash 3 times with 250 μ L/well Wash Buffer. Allowing time for soaking (~1 minute) during each wash step increases the effectiveness of the washes. Blot plate on absorbent paper to remove any residual buffer. Wells was block with 200 μ L/well of 1x ELISA/ELISPOT diluent. Incubate at room temperature for 1 hour. Optional: Aspirate and wash at least once with Wash Buffer. Using 1x ELISA/ELISPOT Diluent, dilute standards as noted on the C of A to prepare the top concentration of the standard. Add 100 μ L/well of top standard concentration to the appropriate wells. Perform 2-fold serial dilutions of the top standards to make the standard curve for a total of 8 points. Add 100 μ L/well of your samples to the appropriate wells. Seal the plate and incubate at room temperature for 2 hours (or overnight at 4°C) for maximal sensitivity. Wells was washed repeated for a total of 3-5 washes. Add 100 μ L/well of detection antibody diluted in 1x ELISA/ELISPOT diluent* (dilute as noted on C of A). Seal the plate and incubate at room temperature for 1 hour. Aspirate/wash as in step 2. Repeat for a total of 3-5 washes. Add 100 μ L/well of Avidin-HRP diluted in 1x ELISA/ELISPOT Diluent. Seal the plate and incubate at room temperature for 30 minutes. Aspirate and wash as in step 2. In this wash step, soak wells in Wash Buffer* for 1 to 2 minutes prior to aspiration. Repeat for a total of 5-7 washes. Add 100 μ L/well of 1x TMB Solution to each well. Incubate plate at room temperature for 15 minutes. Add 50 μ L of Stop Solution to each well. Read plate at 450 nm. If wavelength subtraction is available, subtract the values of 570 nm from those of 450 nm and analyse data. The procedures for IL-2 and IFN- μ determination is similar with IL 4. The difference only in primary antibody and standard antibody used.

RESULTS AND DISCUSSION

Antibody Titre

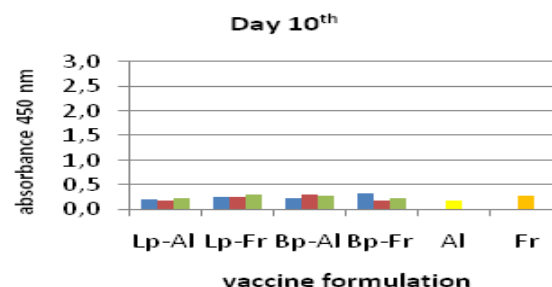


Figure 1. Antibody titre of BALB/C mice at day 10th after inoculation with formulated vaccines: inear peptide with alhydrogel (Lp-Al); linear peptide with CFA-and IFA (Lp-Fr); branched peptide with alhydrogel (Bp-Al); branched peptide with CFA-and IFA (Bp-Fr); Alhydrogel (control Al.); and CFA-IFA (control Fr.); peptide concentrations starting with (5 nmol/ dose.); (10 nmol/dose.); and (20 nmol/ dose.).

Antibody level of both linear and branched peptide are very low. This is the characteristic of a primary immune response occurring on an animal first exposure to an antigen. After some weeks, months or even years have elapsed, the animal immunized with the same antigen, it will usually produce a secondary immune response that differs from the primary response, as shown in the figure 2 below.

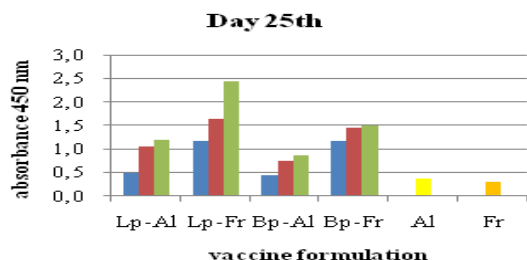


Figure 2. Antibody titre of BALB/C mice at day 25 after second inoculation with formulated vaccines: linear peptide with alhydrogel (Lp-Al); linear peptide with CFA-and IFA (Lp-Fr); branched peptide with alhydrogel (Bp-Al); branched peptide with CFA-and IFA (Bp-Fr); Alhydrogel (control Al); and CFA-IFA (control Fr.); peptide concentrations starting with (5 nmol/dose.); (10 nmol/dose.); and (20 nmol/ dose.).

The results show that antibody titer was increased significantly than shown in Figure 1. Linear peptide in FCA adjuvant is somewhat higher than linear peptide in alhydrogel. The same results also shown by branched peptide in FCA and alhydrogel, but the antibody level little bit lower than linear peptide.

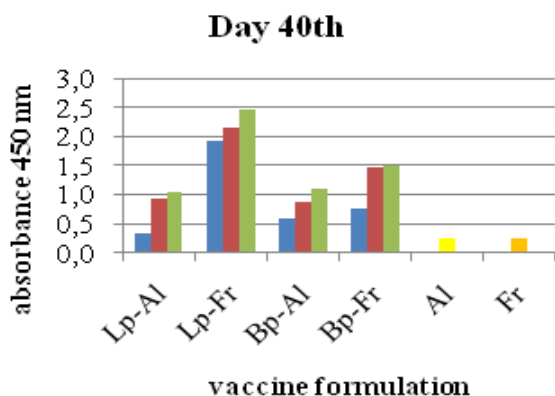


Figure 3. Antibody titre of BALB/C mice at day 40 after inoculation with formulated vaccines: linear peptide with alhydrogel (Lp-Al); linear peptide with CFA-and IFA (Lp-Fr); branched peptide with alhydrogel (Bp-Al); branched peptide with CFA-and IFA (Bp-Fr); Alhydrogel (control Al); and CFA-IFA (control Fr.); peptide concentrations starting with (5 nmol/dose.); (10 nmol/dose.); and (20 nmol/dose.).

In day off ourty we design to do the challenge test to determine the potency of vaccine by inoculation the immunized mice with Mem-71 virus strain (A/Memphis/1/71xA/Bellamy/142). Since there is no BSL-3 facility for doing this test, we decide to do the inhibition test using MDCK cell and determined by Elisa Reader. The results are not shown.

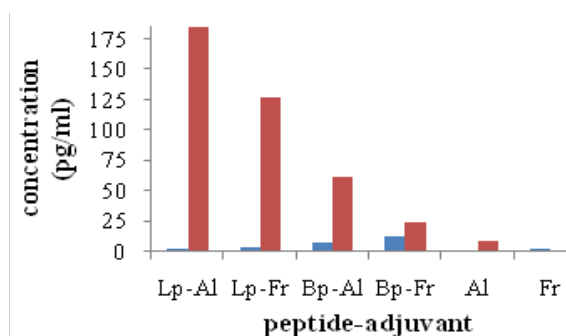


Figure 4. Concentration of cytokines, IL-4, and IL-2, in mice after inoculation subcutaneously with 20 nmol/dose.

Cytokines concentration

These results indicate that aluminium-containing adjuvants activate DCs and influence their ability to direct TH1 and TH2 responses through the secretion of IL-2 and IL-4. In this study, the effects of aluminium hydroxide and FC adjuvants on antigen presentation, expression of costimulatory molecules and cytokines by mouse dendritic cells (DCs) and the ability of DCs to induce T helper cell differentiation were investigated. Aluminium hydroxide adjuvant had a significantly stronger effect than FC adjuvant. Aluminium-containing adjuvants also stimulated the release of IL-2 and IL-4.

These results indicate that aluminium-containing adjuvants activate DCs and influence their ability to direct TH1 and TH2 responses through the secretion of IL-2 and IL-4. Aluminium-containing adjuvants enhanced antigen presentation by DCs as indicated by increased IL-2 and IL-4 secretion of naive T cells. This indicates that aluminium-containing adjuvants enhance the efficiency of T cell activation by DCs. In summary, aluminium-containing adjuvants have a direct effect on DCs and enhance their ability to activate antigen-specific T cells. Taken together, these experiments demonstrate that aluminium-containing adjuvants are not simple delivery vehicles for antigens, but directly activate DCs to effectively initiate immune responses and influence the ability of DCs to direct TH¹ and TH² responses.

CONCLUSIONS

From this study, demonstrating conserve epitope of Me2 2-16 when incorporated with P25 protein from canine distemper virus (linear structure) in alhydrogel adjuvant has greater potential to produce anti-M2e antibodies than in Freund adjuvant. Alhydrogeladjuvant had a stronger effect than Freund adjuvant. Alhydrogel also stimulate the release of IL-2 and IL-4.

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