Conserved Peptide with Therapeutic Potential to Overcome Nasopharyngeal Carcinoma

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is a squamous-cell carcinoma that arises in the upper lining epithelium of the nasopharynx. In this study, conserved peptide (Ulin-1) of Epstein-Barr virus constructed by Biomodelling and Biocomputation was tested for its ability to stimulate B cells to produce specific antibodies. Spleen cells were isolated and cultured with anti-CD3 and lipopolysaccharide (LPS), and treated or not treated with Ulin-1. Cell culture was harvested six days after incubation and analyzed by flow cytometry. Here, we demonstrated the ability of Ulin-1 to stimulate B cells to produce specific antibodies. The results of this study illustrate the importance of Ulin-1 engineered by Biomodelling and Biocomputation as both active and passive immunization agents against nasopharyngeal cancer.

KEYWORDS: Nasopharyngeal carcinoma, Ulin-1, gp 350/220, *in vitro*.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant tumor derived from the nasopharyngeal epithelium. It occurs in the nasopharynx area located behind the nose and above the back of the throat. It is commonly found in Southeast Asia, Southern China and Hong Kong.[1-4] Medium prevalence of nasopharyngeal carcinoma occurs to people in Africa, Mediterranean, Malays in Singapore, Indonesia, and Malaysia.[5-7] According to Devi *et al*. the risk of Bidayuh people in Sarawak State, Malaysia to suffer from NPC is higher than the highest recorded by any other population-based registries around the same period.[8] In Indonesia, nasopharyngeal carcinoma is most often found among malignant tumors in the field of ENT (Ear Nose and Throat). Its incidence is often found in patients over the age of 40 years, and its prevalence is quite high, namely 4.7 cases per year in 100.000 populations or in estimated 7000-8000 cases per year in Indonesia.[9] Nasopharyngeal carcinoma (NPC) is an Epstein-Barr virus (EBV) associated malignancy.[8,10-14] EBV is a DNA virus with icosahedral capsid structure.[15-18] Receptors considered to play a role in EBV entry into epithelial cells of nasopharyngeal is CD21/CR2.[18-19] Nasopharyngeal carcinoma prevention is still an issue, because the suitable and effective vaccine to inhibit EBV entry has not been found, as well as the hidden location of the nasopharynx. Nasopharyngeal carcinoma is generally asymptomatic until it reaches an advanced stage; thereby, it causes difficulty in a diagnosis and therapy. Therefore, it is necessary to prevent the disease through effective and non-toxic vaccination.[20-25] However, manufacturing EBV vaccine requires a good strategy by considering that EBV has many variants/strains.[26-28] Making a vaccine that works on all strains of EBV with conventional methods would require a long time and is expensive, so that a new strategy to make EBV vaccine to cover all the strains is very important thing to do.[24,29] Constructing EBV vaccine could be done carefully, quickly and accurately by Biocomputation and Biomodelling approach, and then tested by in vitro and in vivo systems, in order to obtain a high quality of vaccine.[24,26,29-31] Until now, radiotherapy remains an important option for therapy because a safer strategy has not been found.[33-39]

Based on the description above, a research to design a vaccine that works to inhibit the growth of nasopharyngeal carcinoma based on conserved regions of EBV that binds the host cell receptor is urgent and very important. Therefore, the aim of this study was to demonstrate Ulin-1 to stimulate EBV-specific B cells, and the outcome of this effort was to find a potential strategy for future therapeutic interventions in NPC.

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MATERIALS AND METHODS

Mice

Seven week-old female BALB/c mice were purchased from Gajah Mada University. They were maintained in pathogen-free condition in the Department of Molecular Biology at Brawijaya University, Indonesia. This study was approved by Ethics Committee of Brawijaya University, Indonesia.

Conserved Peptide (Ulin-1)

Conserved peptide was constructed by Biomodelling and Biocomputation. The conserved peptide used in this experiment consisted of 19-mer and was labeled Ulin-1.

Cell Culture and Antibody Induction *in vitro*

B lymphocytes were obtained from the spleen of seven week-old mice. Spleen cells were washed twice with PBS and resuspended with Iscove's medium of sodium bicarbonate containing 10% fetal calf serum (FCS), gentamycin, or RPMI-1640 medium added to gentamicin 0.01 mg/ml, two mM L-Gluta-mine, trypticase soy broth 0.03 mg/100ml, and 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, VA). Spleen cells (2 x 10^6/well) were cultured in 1.5 ml of RPMI-1640 medium containing 10% FCS, glutamin (30 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), 2-ME (5 x 10^{-5} M) in 24-well flat-bottom culture plates (Costar, Cambridge, MA) with anti-CD3 (2% culture supernatant), LPS (10 ng/ml, Sigma), IL-2 (10 µg/ml), and added to or without Ulin-1 (booked commercially) 1,000 ng/ml. Then the cells were incubated at 37°C in 5% CO_2 for 3 to 6 days.

Detection of Antibody Production by Flow Cytometry

Cell suspensions harvested from cell culture were incubated for 30 minutes at 4°C with FACS buffer (PBS, 1% bovine growth serum and 0.01% NaN_3). Samples were centrifuged and pellets were re-suspended in FACS buffer containing fluorochrome-conjugated antibody for 30 minutes at 4°C. The antibody used in this study were anti-B220 FITC (BD Biosciences, San Jose, CA, USA); PECy5-conjugated anti-IgG (BD Biosciences), PE-conjugated anti-IgE (BD Biosciences), PECy7-conjugated anti-IgD (Biolegand). Antibodies were purchased from eBioscience (San Diego, CA, USA) unless indicated. After washing, cells were re-suspended in FACS buffer and collected on a BD FACSCalibur flow cytometer. Data analysis was performed by using CellQuest. Intracellular antibody staining was performed in spleenic cell cultures according to the protocol provided by the manufacturer. At day 3 and 6, cells were collected, washed and subsequently permeabilized and stained for intracellular antibody, and the surface was stained for membrane antigens.

Statistical Analysis

Data were analyzed using ONE-way ANOVA at $p<0.05$ and Tukey HSD test using SPSS 16.0 for Windows.

RESULTS

Ulin-1 derived from proteins gp350/220 of EBV could stimulate B cells to produce a specific antibody. In addition to Ulin-1 1,000 ng/ml in cell culture, they profoundly increased antibody expression ($p<0.05$) compared to the control groups. In this experiment, it was known that in the first three days of post culture, antibodies had not yet been synthesized. In general, all immunoglobulin can be detected only after six days of post-culture. The addition of anti-CD3 in spleen cell cultures can support T cell survival, while the addition of LPS can support the APC survival (antigen presenting cells). Another interesting point of this study was that the Ulin-1 designed by Biomodelling and Biocomputation approaches was not an allergen. This result can therefore be an effective strategy for cancer immunotherapy.

DISCUSSIONS

B cells are one of the important components in the immune system which controls foreign antigens. These cells in turn synthesize antibodies when exposed to the appropriate antigen. Many studies describe the importance and function of B cells.[33-35] To perform the function as a component of immune system, B cells cooperate with other cells such as T cells, dendritic cells, and macrophages. Ulin-1 derived from gp350/220 proteins could stimulate B cells to produce antibodies (Fig. 1). To see the possibility of B cells which had synthesized antibodies in the early exposure of the given peptide, we analyzed the expression of antibodies at day 3 of post culture. The results of analysis at day 3 of post culture gave evidence that the antibodies had not yet been produced at the cellular level (Fig. 2). These results illustrated that B cells responding to antigen took time, and was different from the innate immune components which could respond to antigen spontaneously. There are at least two factors leading to the emergence of antibodies (IgG) which is relatively late. First, the dose of antigen is too small. Second, clones that match the given antigen are very little. The second possibility is more reasonable than the first one, since most peptides in the range of 1-10000 ng/ml is enough to stimulate B cells to produce antibodies.

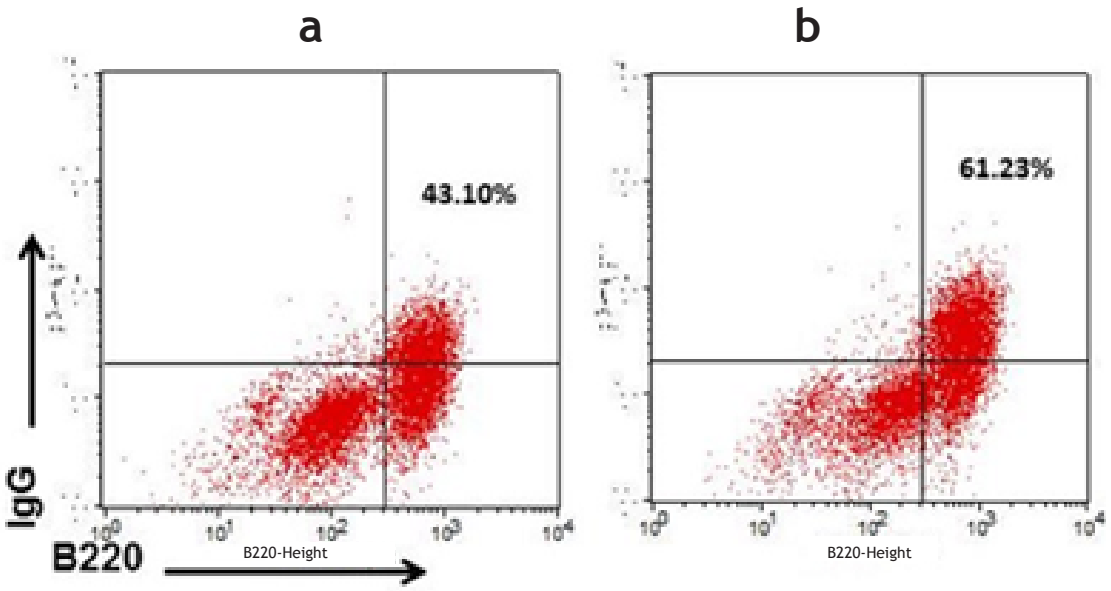

Figure 1. Ulin-1 derived from gp350/220 protein could stimulate B cells to produce specific antibodies. Spleen cells were cultured in RPMI medium for six days. a, Anti-CD3 and LPS were added to the culture. b, Anti-CD3, LPS, and Ulin-1 were added to the culture. On day 6, cell culture was harvested and analyzed by flow cytometry. Percentages of B220 cells positively stained by intracellular IgG were presented in the panels. Data were mean ± SD values of five mice in each group.

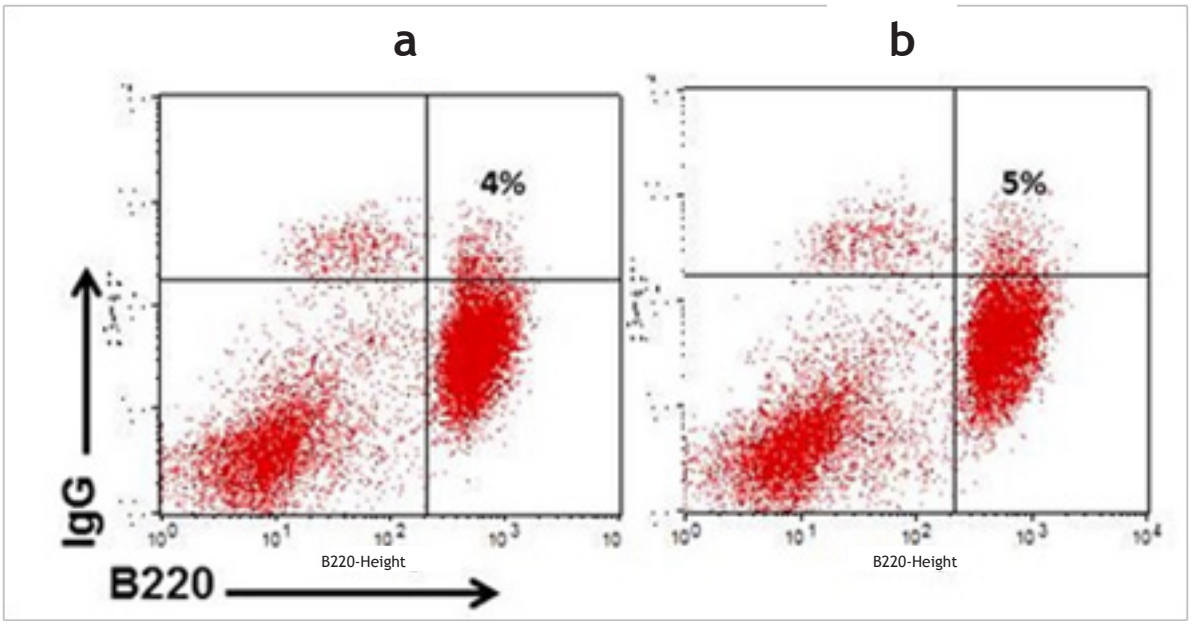

Figure 2. Stimulation of B cells with Ulin-1 for three days showed no increase in antibody synthesis. Spleen cells were cultured in RPMI medium for three days. a, Anti-CD3 and LPS were added to the culture. b, Anti-CD3, LPS, and Ulin-1 were added to the culture. On day 3, cell culture was harvested and analyzed by flow cytometry. Percentages of B220 cells positively stained by intracellular IgG were presented in the panels. Data were mean ± SD values of five mice in each group.

In this study, all of spleen mononuclear cells were cultured together without division by sorting. It was intended to allow cooperation between cells involved in the immune responses. In this culture, anti-CD3 was added to sustain the population of CD4 T cells in particular, to increase the survival. CD4 T cell populations were required to sustain B cell proliferation. LPS and peptide will be presented by MHC class II on macrophages, which in turn stimulate CD4 T cells to produce IL-2. IL-2 is required by B cells to survive and also to maintain proliferation. In addition, the added peptide may directly stimulate competent B cells, so that the cells will start to produce antibodies.[36-37] Macrophages, monocytes, and some lymphocytes have Fc receptors that will bind with the Fc region of IgG. IgG bindings in some cells mentioned above are very beneficial to the immune responses because they can promote the internalization of antigen entry. Complexes antigen antibody which have been internalized by the APC will be digested and processed in the cytoplasm and presented by MHC class II.[38-39] IgG is often referred as an opsonin regarding its functions to enhance phagocytosis. An association of IgG with Fc receptors on the cell will result in the activation and function of the cells in immune responses. In this experiment, we had evidence that the stimuli with the Ulin-1 did not cause IgE production. IgE was one of the antibodies closely associated with allergic reactions (Fig. 3). IgE bound very strongly with Fc receptors of basophils and mast cells, although no antigen interacted with that immunoglobulin. The consequence of that very strong bond was a very low concentration of IgE in serum, because most IgE was tied to the tissue. IgE contributes to the incidence of allergic reactions. The association of IgE with antigen causes the secretion of several molecular mediators affecting allergic symptoms. IgE plays an important role to eradicate the parasite, such as the worm larvae (helminth). In cases of illness caused by helminth, the concentration of IgE in serum increases so that it can be used for diagnostic test of this parasite type. Eosinophils have Fc receptors for IgE binding.[40] The association of eosinophils to IgE on the surface of helminth causes the death of this parasite. In this study, IgE was checked for the possibility of allergic reactions to peptide stimulation constructed by Biomodelling and Biocomputation.

To observe the ability of specific clone activation against the peptide, we analyzed the expression of IgD. IgD is an antibody located on the B cell-surface functioning as a receptor of B cells. The importance of IgD has been described in many different views.[34-35] While TCR (T cells receptor) functions as an activation tool for T cells, IgD is an activation tool for B cells. IgD increase on the B cell surface after peptide addition gives an evidence of the suitability of this peptide with B cell receptors, so that B cells turn into plasma and synthesize specific immunoglobulins against that peptide. The analysis of B cells in vitro after six days of post culture in the presence of peptide (Fig. 4) showed a significant increase in IgD production ($p < 0.05$).

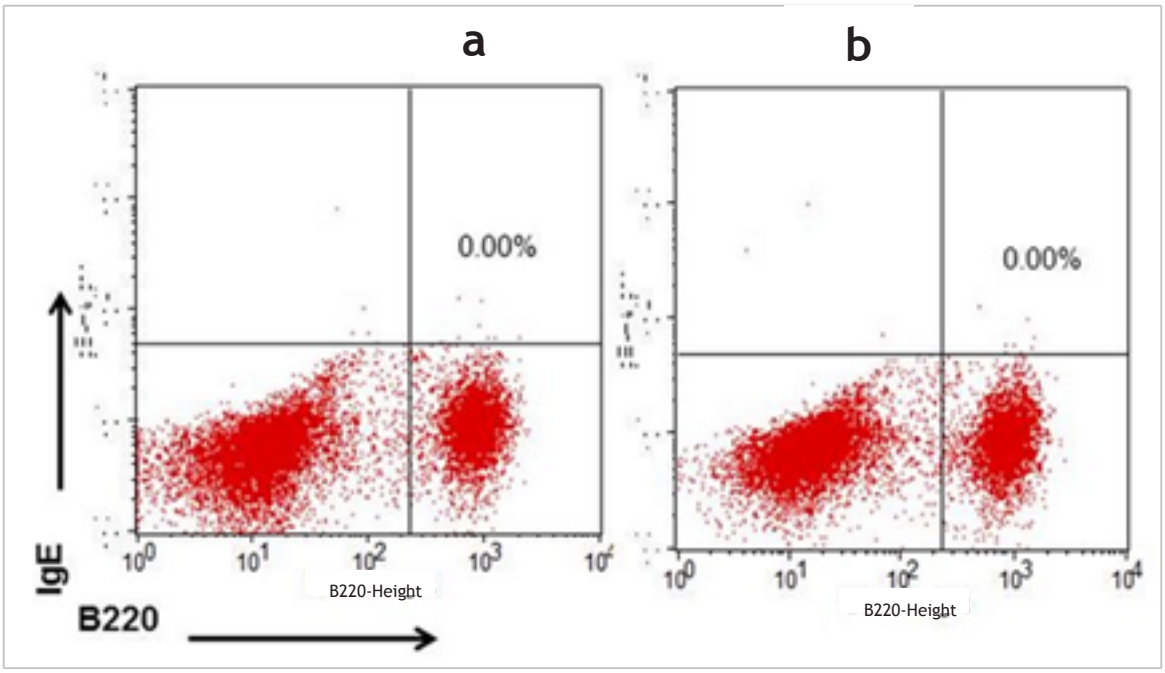

Figure 3. Peptide engineered by Biomodelling and Biocomputation (Ulin-1) did not have the nature of allergen. Spleen cells were cultured in RPMI-1640 medium for six days. a, Anti-CD3 and LPS were added to the culture. b, Anti-CD3, LPS, and Ulin-1 were added to the culture. On day 6, cell culture was harvested and analyzed by flow cytometry. Percentages of B220 cells positively stained by intracellular IgE were presented in the panels. Data were mean ± SD values of five mice in each group.

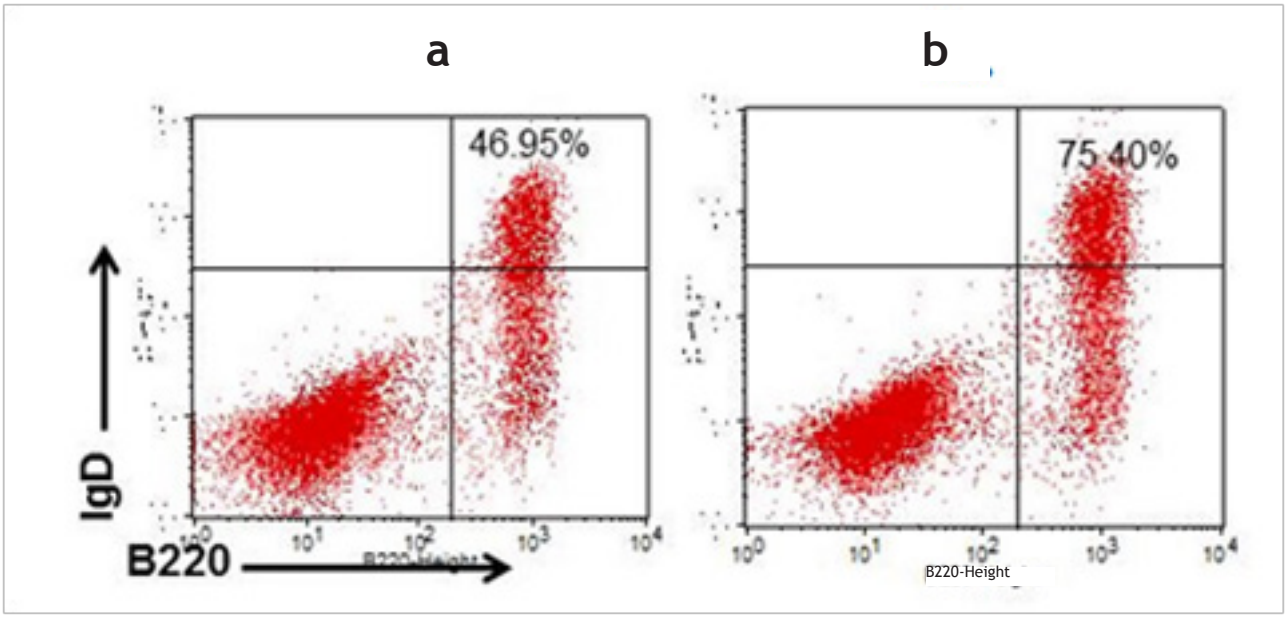

Figure 4. Stimulation of B cells by using Ulin-1 for six days showed the increase of IgD synthesis. Spleen cells were cultured in RPMI medium for six days. a, Anti-CD3 and LPS were added to the culture. b, Anti-CD3, LPS, and Ulin-1 were added to the culture. On day 6, cell culture was harvested and analyzed by flow cytometry. Percentages of B220 cells positively stained by intracellular IgD were presented in the panels. Data were mean ± SD values of five mice in each group

CONCLUSION

Epitope of gp 350/220 EBV, particularly Ulin-1 constructed by Biomodelling and Biocomputation can stimulate B cells to produce antibodies in vitro. The Ulin-1 is not an allergen and has a nature to change naïve B cells to plasma cells in vitro. It has a capacity to be a potent candidate for active and passive vaccines, which are important to overcome nasopharyngeal cancer.

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